Molecular and biological characterization of cell-free DNA using an *in vitro* cell culture model

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We may travel far through the realms of Evolution, but nowhere shall we find a more perfect co-operation or a more beautiful illustration of mutual help of one part for another, and of all parts for the whole, as well as of the whole for all its parts, than in the little insignificant cell, which seems to hold the very secret of the universe…

Jan Christiaan Smuts

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The expansion of mere aspirations to the current dimensions of this PhD thesis would not have been possible without the impact of many different people, and I regret that I am not able thank everyone in this space. My most grateful thanks are due to my family (and extended). You have supported me financially and emotionally through thick and thin, and not once have you reminded me of your efforts or asked for anything in return. I have very deep appreciation for that. Without you, this venture would have been cut short. Vida Ungerer, thank you for your unconditional love, profound understanding, and for constantly reminding me that I am just a human. You are my anchor. I also want to thank my close and colorful friends, F.J. Reynecke, Chris Badenhorst, Jaco Wentzel, and Angelique Lewies. You have influenced my thinking greatly, and I am certain that your friendship has made this process much less painful. To my colleagues, Leslie Peters and Janine Aucamp, the remaining members of our small cfDNA team, I extend a friendly salutation. Thank you for taking on this daunting task with me.

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Preface

The present thesis embodies a theoretical and empirical investigation of the origin, structure, fluctuation and function of cell-free DNA in human biology. This study is guided by the conviction that the true importance of cell-free DNA, along with the potential scale of its clinical utility, is undermined by a lack of understanding and appreciation of its biological properties and evolutionary history.

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This thesis is compiled in article-format according to the guidelines set by the North-West University, and consists of six published articles, two submitted manuscripts, four published book chapters, and two scientific posters. Papers in which I did not act as the lead author, but as co-author, is preceded by a statement of my contribution. Each article, chapter or manuscript was inserted in the thesis exactly as published or submitted and as such complies with the requirements set by the different journals or publishers. Documentation regarding permission from journals to use published articles in this thesis is provided in Appendix **VI**. Additional articles in which I participated as a co-author, that share points of contact with this study, but was not included in this thesis, is listed in Appendix **III**. Permission from authors to include all aforementioned articles in this thesis is provided in the following section. The structure and content of this thesis is summarized in Section 1.5

Author contributions and permission statements

I, Abel J. Bronkhorst, am the main researcher responsible for the proposal, planning and execution of this study, along with (i) extensive appraisal of the relevant literature, (ii) assessment, optimization and standardization of the experimental protocol or methods, (iii) collection, analysis, interpretation and presentation of data, (iv) design, planning and writing of research articles, (v) speaker and presenter of conference-related content, and (vi) writing of all sections of this thesis.

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Dr. Johannes F. Wentzel

Co-Supervisor and co-author responsible for guidance and expert advice on cell culturing, real-time PCR, and technical assistance on various flow cytometric assays.

Janine Aucamp & Dimetrie L. Peters

Colleagues that assisted with the writing and evaluation of article-related content.

Dr. Christoffel P.S. Badenhorst

Responsible for critical input and revision of the review articles presented in Chapter 2 of this thesis (Article **I** and Article **II**).

Prof. Lissinda H. du Plessis

Assistance with the analysis of flow cytometric data, the results of which are presented in Chapter 4 (Article **V**) and Appendix **I** (Article **XI**) of this thesis.

Dr. Etresia Van Dyk

Assistance with sequencing, electrophoresis and article-related content, the results of which are presented in Chapter 4 (Article **V**), Chapter 5 (Article **VII**) and Appendix **I** (Article **XI**) of this thesis.

Dr. Etienne de Villiers

Assistance with Next Generation Sequencing data analysis, the results of which are presented in Chapter 5 of this thesis (Article **VII**).

Prof. Francois H. Van Der Westhuizen & Hayley C. van Dyk

Assistance with the design, execution, data interpretation and article-related content of bioenergetics analyses performed on the Seahorse XFe96 Extracellular Flux analyzer, the results of which are presented in Chapter 4 of this thesis (Article **VI**).

Statement by the co-authors

I hereby confirm that I approved the publication of the aforementioned manuscript(s), and that my role related to the completion of this thesis, Molecular and biological characterization of cell-free DNA using an *in vitro* cell-culture model, is representative of my contribution. I also give my consent that the PhD student, Abel Jacobus Bronkhorst, may include the manuscript(s) as part of his thesis.

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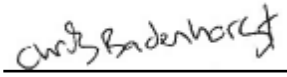

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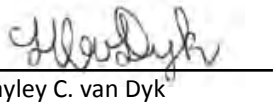

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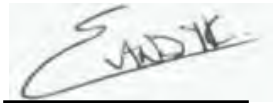

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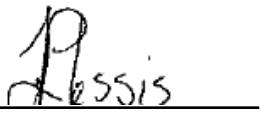

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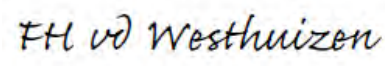

Prof Francois H. Van Der Westhuizen
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Abstract

Over the past 40 years, numerous proof-of-principle studies have intermittently demonstrated the translational potential of cfDNA as a non-invasive biomarker for the diagnostication, prognostication and therapy monitoring of a wide range of diseases, physiological conditions and other clinical scenarios. However, abstracting data in a research setting and applying it in medical practice proved to be more complicated than expected. It is commonly assumed that the development of comprehensive clinical cfDNA assays, along with the scope of the utility of cfDNA, is constrained mainly by a lack of an analytical consensus between research groups, and by the limits of current technologies. However, an increasing number of reports suggest that a lack of knowledge concerning the biological properties of cfDNA may be another substantial obstacle in the way of the rapid translation of research to medical practice.

Therefore, a major objective of this study was to develop a better understanding of the origin, structure, fluctuation and function of cfDNA in human biology. A review of the literature confirmed that the strain imposed on applied cfDNA research by methodological drawbacks, is indeed exacerbated by a poor understanding of the biological properties of cfDNA and by a lack of consideration thereof in clinical validation experiments. A multitude of intrinsic and extrinsic sources (e.g., apoptosis and necrosis) and causes (e.g., oxidative stress and bacterial turnover) can result in the presence of cfDNA in bio-fluids. Moreover, many of these sources and causes appear to be inextricably linked by a complex interplay of cellular and physiological interactions (e.g., endocrine signaling, metabolism, homeostasis), which are in turn influenced by a myriad of biological factors such as weight, fitness, health, diet, smoking, circadian oscillations and medicinal status, the nature of which can differ greatly between individuals of different age, gender and ethnicity, for example. The convergence of these factors result in a seemingly arbitrary presentation of both the quantitative and qualitative characteristics of cfDNA in the blood of an individual, and between individuals, at any instance, which severely complicates the characterization of cfDNA *in vivo*. In this regard, the development and utilization of alternative strategies for studying the biological properties of cfDNA is completely rationalized. Since two dimensional cell culture models are sequestered from many of the confounding elements inherent to the *in vivo* setting, it has the potential to overcome many of the obstacles associated with heterogeneous bio-fluid samples. However, despite its proven advantages in other domains of biological research, the application thereof in cfDNA research is largely lacking. Therefore, the most important aim of this study was to implement a cell culture model to investigate the biological properties of cfDNA.

Quantitative PCR and chip-based capillary electrophoresis in conjunction with flow cytometry revealed the presence of copious cfDNA fragments with a size of ~2000 bp in the growth media of 143B cells after 24 hours of incubation, which could not be correlated with apoptosis, necrosis or DNA replication. This indicated the involvement of some active release mechanism. Evaluation of different cancer and non-cancer cell lines suggested that this may be a common phenomenon. In an experiment intended for the optimization of cfDNA quantification and gene expression profiling, it was recognized that this ~2000 bp population is represented by different amounts of housekeeping genes, and that some housekeeping genes are absent in the cfDNA, despite being present in cellular mRNA. These studies suggested the intriguing possibility that there could be some intent and selectivity involved in the release of cfDNA.

Nucleotide sequencing of the actively released cfDNA revealed that the majority of this cfDNA consists of repetitive DNA (88 %), comprised largely of α-satellites and mini-satellites and the Alu, LINE1, ERV (K) class II, MaLR and TcMar-Tigger repetitive elements. A careful review of the literature indicated a strong correlation between the representation of these elements and their current transposition activity or their ability to become reactivated. Finally, local alignment analyses demonstrated that the majority of these sequences originate from the centromeres of chromosomes 1 and 16. Interestingly, it has been reported that the hypomethylation of DNA at the peri-centromeric regions of these two chromosomes leads to rearrangements, decondensation, and eventually chromosomal instability. Therefore, keeping in mind that hypomethylation is a hallmark of cancer cells, and that transposons can become reactivated by DNA demethylation, it was hypothesized that the demethylation of these regions in 143B cells leads to derepression and mobilization of transposons, followed by aberrant translocations and chromosomal instability. Based on the structural similarity between centromere protein B (CENP-B), a protein capable of inducing DNA breaks, and the transposase encoded by the Tigger DNA transposon, which are liable to activation by demethylation, both CENP-B and transposases may facilitate the excision of satellite DNA. Furthermore, considering the inextricably laced sequences of satellite DNA and transposons, it is likely that the presence of overrepresented transposons is a result of programmed DNA elimination.

Questions raised by these observations are whether satellite DNA and transposons are (i) deliberately released by cancer cells to perform specific functions in the extracellular environment, (ii) by-products of a normal cellular process and are incidentally biologically active, or (iii) biologically-inert byproducts. In this study it was not only demonstrated that certain repetitive element families are significantly overrepresented in the cfDNA released by 143B cells, but that specific members of each family are overrepresented, such as the L1P1 and HERVK9int subfamilies of LINE1 and ERV (K) class II, respectively.

The involvement of a single LINE1 element in the initiation of human colorectal cancer has recently been demonstrated, and the role of endogenous retroviruses in cancer is well documented. In keeping with this, numerous reports have described how cfDNA can be transported throughout the body, while other studies have demonstrated their capacity to enter target cells and alter their biology, with associated effects ranging from mutagenesis and oncogenesis to chemo-resistance and metastasis. Since the mechanisms involved in these phenomena are still unclear, satellite DNA and transposons may yet prove to be among the key effector molecules. Furthermore, a partial explanation for the phenomenon that cancer patients generally present with elevated levels of cfDNA can be derived from the observation made in this study that cultured cancer cells release notably more DNA than normal cells, and that this is related to their metabolism. This, together with the correlative relationship between the malignancy of cancer cells and rate of demethylation, suggests that the level of DNA release increases concomitantly with malignancy. Viewed in the light of the central theorem of the extended phenotype, in which the malignancy of cancer cells should maximize the survival of genetic instructions that promote malignant behavior, it stands to reason that cancer cells would up-regulate the mobilization and lateral transfer of transposons to neighboring cells with the purpose of transforming them. In line with this premise, it can be argued that the composition and function of the DNA released by normal cells will differ from cancer cells on a fundamental level, and it is also likely that the cfDNA from different cancer cells differ. Since it was demonstrated that normal cells also release DNA, these results not only implicate the active release of satellite DNA and transposons in detrimental effects, but also provide a potential mechanism for the transfer of satellite DNA and transposons between otherwise healthy somatic cells.

Taken together, the results and arguments presented in this thesis suggest that the commonly held assumption that apoptosis is the main origin, and most relevant fraction, of cfDNA in human blood may be incorrect, restrictive, and should be reconsidered. Further inquiry into the biological properties of actively released DNA will not only benefit applied research, but could also provide a new framework for a deeper understanding of molecular biology, pathology and the process of evolution. Furthermore, this study demonstrates the utility of *in vitro* cell culture models for studying the phenomenon of cfDNA, and as such also emphasizes the importance of consolidating basic and applied cfDNA research.

Keywords: cell-free DNA, cancer, satellite DNA, transposons, cell culture model, 143B cells, active release.

Uittreksel

In die afgelope 40 jaar het verskeie studies op die moontlikheid gedui dat selvrye-DNA (svDNA) in die mediese praktyk aangewend kan word as nie-indringende biomerkers vir die diagnose, prognose en terapie monitering van 'n verskeidenheid siektes, fisiologiese toestande en ander kliniese gevalle. Die ontginning van data in 'n navorsingsopstelling en die toepassing daarvan in die praktyk is egter meer uitdagend as wat aanvanklik verwag is. Daar word oor die algemeen aanvaar dat die ontwikkeling van omvattende kliniese svDNA toetse, tesame met die alledaagse bruikbaarheid daarvan, hoofsaaklik beperk word deur n gebrek aan ‘n konsensus tussen verskillende navorsingsgroepe ten opsigte van die analise van svDNA asook deur die beperkings van huidige tegnologie. Daar is egter 'n toename in studies wat daarop dui dat 'n algehele gebrek aan kennis met betrekking tot die biologiese eienskappe van svDNA 'n beduidende vertragende faktor mag wees in die spoedige benutting van basiese navorsing in die mediese praktyk.

Dus was een van die hoof doelwitte van hierdie tesis om 'n beter begrip te ontwikkel van die oorsprong, struktuur, fluktuering, en funksie van svDNA in menslike biologie. Na 'n deeglike ondersoek van die literatuur is dit bevestig dat die stremming wat op toegepaste svDNA navorsing geplaas word deur verskeie metodologiese tekortkomings inderdaad vererger word deur 'n gebrek aan kennis van die biologiese eienskappe van svDNA asook deur die feit dat dit grootliks geïgnoreer word in kliniese validasie eksperimente. 'n Groot aantal intrinsieke en ekstrinsieke bronne (bv., apoptose en nekrose) en oorsake (bv., oksidatiewe stres en bakteriële omset) kan lei tot die teenwoordigheid van svDNA in liggaamsvloeistowwe. Dit wil boonop voorkom asof baie van hierdie bronne en oorsake onskeidelik gekoppel is deur 'n komplekse raamwerk van verskillende sellulêre en fisiologiese interaksies (bv., endokrinologiese seine, metabolisme en homeostase), wat op hulle beurt weer beïnvloed word deur talle ander biologiese faktore, onder andere gewig, fiksheid, gesondheid, dieet, rook, sirkadiese ritmes en medisinale gebruik, wat uiteraard baie kan verskil tussen indiwidue van verskillende ouderdom, geslag en etnisiteit. Die kombinasie van hierdie faktore lei tot 'n skynbare arbitrêre verteenwoordiging van beide die kwantitatiewe en kwalitatiewe eienskappe van svDNA in die bloed van 'n indiwidu, en tussen indiwidue, op enige tydstip, wat as sulks die karaktarisering van svDNA *in vivo* tot 'n groot mate kompliseer. In hierdie verband, word die noodsaaklikheid van die ontwikkeling en gebruik van alternatiewe strategieë vir die bestudering van svDNA sterk beklemtoon. Omdat twee-dimensionele selkultuur modelle van meeste van die kompliserende elemente wat in *in vivo* sisteme aangetref word

geïsoleer is, kan dit baie van die struikelblokke wat met heterogene liggaamsvloeistowwe geassosieer is omseil. Ten spyte van bekende voordele van selkultuur modelle in ander domeine van biologiese navorsing, word dit nog nie algemeen toegepas in svDNA-navorsing nie. Die primêre doel van hierdie studie was dus om 'n selkultuur model aan te wend vir die bestudering van die biologiese eienskappe van svDNA.

Kwantitatiewe PKR en skyfie-gebasseerde kapillêre elektroforese tesame met vloeisitometrie het gewys dat daar 'n groot hoeveelheid DNA fragmente met 'n grootte van ~2000 bp in die groeimedium van 143B selle teenwoordig is na 24 ure se inkubering, en is skynbaar nie afkomstig van apoptose, nekrose of DNA replisering nie. Dit het gedui op die betrokkenheid van 'n aktiewe vrystellings meganismse. Bestudering van ander kanker en nie-kanker sellyne het aangetoon dat hierdie 'n algemene verskynsel mag wees. In 'n eksperiment waarin verskillende huishouding-gene gemeet is vir die optimisering van svDNA kwantifisering en geenuitdrukking analises, is dit waargeneem dat verskillende hoeveelhede huishouding gene in hierdie ~2000 bp voorkom, en dat van hulle afwesig is in die svDNA populasie ten spyte van hulle teenwoordigheid in sellulêre mRNA. Hierdie resultate dui op die aanloklike moontlikheid dat daar selektiwiteit en bedoeling betrokke mag wees by die vrystelling van DNA. Nukleotied-volgordebepaling van die aktief vrygestelde svDNA het aangetoon dat die meerderheid daarvan uit herhalende DNA-volgordes bestaan, wat hoofsaaklik saamgestel is uit α-satelliete, mini-satelliete en die Alu, LINE1, ERV (K) klass II, MaLR en TcMar-Tigger elemente. 'n Deeglike studie van die literatuur dui op 'n sterk korrelasie tussen die teenwoordigheid van hierdie elemente en hulle huidige transposisie aktiwiteit, of vermoë om geaktiveer te word. Laastens, dui analises daarop dat die meerderheid van hierdie fragmente van die sentromere van chromsome 1 en 16 afkomstig is. Dit is al voorheen waargeneem dat die hipometilering van DNA by die peri-sentromeriese gebiede van hierdie twee chromosome lei tot herrangskikings, dekondensering, en uiteindelik chromosomale onstabiliteit. Deur in gedagte te hou dat hipometilering 'n kenmerk van kankerselle is, en dat transposons geaktiveer kan word deur demetilering, was die hipotese voorgestel dat die demetilering van hierdie gebiede in 143B selle tot die derepressie en mobilisering van transposons lei, gevolg deur abnormale translokasies en chromosomale onstabiliteit. Op grond van die strukturele ooreenkomste tussen sentromeer protein B (CENP-B), 'n protein wat DNA breuke kan induseer, en die transposase wat gekodeer word deur die Tigger DNA transposon, wat albei dus deur demetilering geaktiveer kan word, is dit moontlik dat beide CENP-B en transposases satelliet-DNA uit die genoom kan sny. Verder, wanneer die strukturele ooreenkomste tussen satelliet-DNA en transposons in ag geneem word, blyk dit dat die oorverteenwoordigde transposons toegeskryf kan word aan geprogrammeerde DNA eliminering.

Vrae wat deur hierdie waarnemings na vore kom, is of die satelliet-DNA en transposons (i) opsetlik deur kanker selle vrygestel word om 'n spesifieke funksie te verrig in die ekstrasellulêre omgewing, (ii) byprodukte van 'n normale sellulêre proses is en terloops aktief is, of (iii) biologiese inerte byprodukte is. In hierdie studie is dit nie net aangetoon dat sekere herhalende element families in die vrygestelde svDNA oorverteenwoordig is nie, maar dat spesifieke lede van elke familie drasties oorverteenwoordig is, bv die L1P1 en HERVK9int sub-families van LINE1 en ERV (K) klass II, respektiewelik. Die betrokkenheid van 'n enkele LINE1 element in die inisiasie van menslike kolorektale kanker is onlangs aangetoon, terwyl die rol van retrovirusse in kanker al goed gedokumenteer is. Insgelyks, is daar 'n groot aantal studies wat beskryf hoe svDNA in die menslike liggaam vervoer kan word, terwyl ander studies gewys het hoe svDNA deur teikenselle opgeneem kan word en hulle biologie verander, met effekte wat strek van mutagenese en onkogenese na chemo-weerstandbiedendheid en metastase. Aangesien die meganismes wat by hierdie verskynsels betrokke is steeds nie verklaar is nie, is dit moontlik dat satelliet-DNA en transposons van die kern effektor molekules kan wees.

'n Gedeeldtelike verduideliking vir die verskynsel dat kanker pasiënte gewoonlik meer svDNA in hulle bloed het, kom uit die waarneming wat in hierdie studie gemaak is dat kanker selle beduidend meer DNA vrystel as normale selle, en dat dit toegeskryf kan word aan hulle unieke metabolisme. Hierdie, tesame met die korrelatiewe verhouding tussen die kwaadaardigheid van kanker selle en die vlak van DNA-demetilering, toon aan dat die mate van DNA vrystelling toeneem met kwaadaardigheid. Vanuit die perspektief van die sentrale stelling van die verlengde fenotipe, wat stel dat die kwaadaardigheid van kanker selle die oorlewing van genetiese instruksies wat kwaadaardigheid toelaat of bevorder, is dit denkbaar dat kanker selle die mobilisering en laterale oordrag van transposons na aangrensende selle, met die doel om hulle te transformeer, sal opreguleer. Op grond hiervan kan die argument gemaak word dat die samestelling en funksie van die DNA wat deur normale selle, en ook verskillende kanker selle, vrygestel word tot 'n groot mate sal verskil. Omdat dit ook aangetoon is dat normale selle ook DNA vrystel, word die aktiewe vrystelling van satelliet-DNA en transposons nie net in negatiewe effekte geimpliseer nie, maar verskaf ook 'n moontlike meganisme vir die oordrag van satelliet-DNA en transposons tussen normale selle.

Samevattend dui die resultate en argumente wat in hierdie tesis voorgelê is daarop dat die algemeen aanvaarde aanneming dat apoptose die hoof oorsprong, en mees relevante fraksie, van svDNA in menslike bloed is verkeerd en beperkend mag wees, en dat dit heroorweeg behoort te word. Verdere ondersoek van die biologiese eienskappe van svDNA sal nie net vir toegepaste svDNA-navorsing

voordelig wees nie, maar kan ook 'n nuwe raamwerk verskaf vir 'n dieper wetenskaplike insig oor molekulêre biologie, patologie en die proses van evolusie. Hierdie studie wys ook dat *in vitro* selkultuur-navorsing baie nuttig kan wees vir die bestudering van svDNA, en as sulks beklemtoon dit ook die belangrikheid daarvan om basiese en toegepaste navorsing te konsolideer.

Sleutelwoorde: selvrye-DNA, satelliet-DNA, transposons, selkultuur model, 143B selle, aktiewe vrystelling.

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Chapter 1: Introduction

1.1 Background and substantiation

Fragmented and unbound cell-free DNA (cfDNA) molecules were detected in human blood for the first time in 1948 (Mandel & Métais, 1948). This phenomenon seemed trivial at first, but 20-30 years later its potential clinical importance was realized when a number of studies indicated clear differences between both the quantitative and qualitative characteristics of cfDNA from healthy and diseased individuals.

Raised concentrations of cfDNA were first reported for patients with autoimmune disease and leukemia (Tan et al., 1966; Koffler et al., 1973). In the subsequent decades, numerous studies intermittently demonstrated that: (a) cancer patients generally have high levels of cfDNA compared to healthy subjects (Fleischhacker & Scmidt, 2007), (b) cfDNA levels can also be raised in a wide range of other physiological conditions and clinical scenarios, such as fatigue, smoking, aging, traumatic injuries, organ transplant rejection, diabetes and infections (van der Vaart & Pretorius, 2008), and (c) cfDNA levels often correlate with the severity, progression, treatment and recovery of patients in most of the aforementioned cases, especially cancer (Schwarzenbach et al., 2011).

In 1989, Stroun and colleagues recognized that a fraction of the cfDNA present in the plasma of cancer patients is derived from cancer cells (Stroun et al., 1989), and shortly thereafter another group detected *TP53* mutations in the DNA of urinary sediments collected from patients with invasive bladder cancer (Sidransky et al., 1991). Follow-up studies not only confirmed that both malignant and healthy cells do in fact release detectable amounts of cfDNA fragments into circulation and other body fluids, but also revealed that these fragments contain unique genetic and epigenetic alterations that can be traced directly back to the different cells from which they originate (reviewed in Wan et al., 2016).

These "proof-of-principle" studies demonstrated that kinetic analysis of cfDNA could serve as a tool for predicting the clinical outcome of conditions that cause endogenous tissue destruction. Moreover, it exemplified the translational potential of cfDNA as a multifaceted and highly specific non-invasive diagnostic, prognostic and theranostic marker for various pathologies. Thus, cfDNA analysis marks a new point of departure in the application of genomic and molecular techniques for comprehensive clinical tests based on personal non-invasive and precision medicine. The recent development of ultra-sensitive technology and concomitant improvements in most analytical techniques has opened up many new avenues for potential applications of cfDNA. An example of a very prominent milestone is the widespread establishment of several non-invasive prenatal testing (NIPT) facilities (Allyse et al., 2015). These facilities apply different genomic methods and approaches for the characterization of maternal plasma-derived cell-free fetal DNA (*first discovered by* Lo et al., 1997), which enables early and non-invasive sexing (Hyett et al., 2005), identification of multiple fetal genetic aberrations (Lo et al., 2010), and the detection of pregnancy complications (Bischoff et al., 2005).

Apart from the development and implementation of cfDNA analysis as a clinical tool, two other exciting research schemes are gradually emerging. First, cfDNA is being investigated as a possible mediator of intercellular communication. For example, several studies have implicated the lateral transfer of cancer cell-derived cfDNA as a causative agent in oncogenesis and the development of metastasis (Bendich et al., 1965; Garcia-Olmo et al., 2010; Trejo-Becerril et al., 2012). In addition, the lateral transfer of cfDNA has been implicated in the augmented resistance of cancer cells against radiation- and chemotherapy (Kostyuk et al., 2012; Glebova et al., 2015; Ermakov et al., 2011). Although the exact mechanisms are still unclear, it is suggested that the malignant phenotype of tumor cells are transferred to normal cells via the assimilation and transfection of genomic DNA contained in apoptotic bodies. Conversely, it has also been demonstrated that the lateral transfer of cfDNA derived from healthy cells can halt the proliferation of cultured cancer cells (Garcia-Olmo et al., 2014).

Second, the involvement of cfDNA in somatic genome variation and trans-generational inheritance is becoming increasingly clear. There is evidence not only showing that somatic cells are liable to genetic and epigenetic modifications via cfDNA, but that this information can also be transferred to sperm. For example, DNA has been detected in extracellular vesicles released by prostate cells that have been found to interact with sperm cells (Ronquist et al., 2011). More recently, it was shown that RNA from melanoma cells xenografted in mice is transported to spermatozoa via exosomes (Cosetti et al., 2014).

Taken together, it is clear that further inquiry into the biological properties of cfDNA will have a positive impact on clinical diagnositics, therapy development, and our general understanding of pathogenesis. Furthermore, if cfDNA is involved in adaptation and genome rearrangement, as the evidence suggests, further study of this phenomenon will improve our understanding of the process of evolution.

1.2 Problem statement

Despite more than 50 years of effort afforded to the development of cfDNA analysis as a screening tool, very few tests have been translated to clinical practice (Bronkhorst et al., 2015; van der Vaart, 2010). Excluding NIPT, only one other clinically validated and FDA approved application of cfDNA is currently available, namely the Cobas® EGFR Mutation Test v2, an assay designed to help clinicians identify lung cancer patients that are eligible for erlotinib or osimertinib treatment (Brown, 2016; Lowes et al., 2016). Furthermore, concerning the apparently wide-ranging messaging capabilities of cfDNA, we have a very limited understanding of the cellular circuits that mediate these effects, and the extent of its role in biology is still obscure. Lastly, although it is clear that cfDNA can be assimilated by all cell types *and* be incorporated into the genome (Mittra et al., 2015; Gahan & Stroun, 2010; Ronquist et al., 2011), the exact mechanisms involved remain unknown, and evaluation of its role in evolution is currently limited to theoretical deliberation (reviewed in Aucamp et al., 2016; Liu, 2008; Gahan, 2013).

It is commonly agreed that the advancement of cfDNA research in each of the aforementioned areas is constrained by three substantial drawbacks: (i) there is virtually no analytical consensus among different research groups, (ii) there is a lack of knowledge regarding the origin and function of cfDNA, and (iii) the molecular and structural characteristics of cfDNA under various conditions is insufficiently investigated. Many studies have attempted to overcome each of these issues, but all have been met with tenacious difficulties. This can be ascribed to the seemingly arbitrary fluctuation of the characteristics of cfDNA in bio-fluid samples. For example, in 2009 Beck and colleagues sequenced the cfDNA obtained from the serum of healthy individuals. Although a large number of sequences indicated an origin from apoptosis, they observed an uneven distribution of apoptotic and necrotic DNA across the genome. In addition, they showed that nonspecific DNA release is not the sole origin of cfDNA (Beck et al., 2009). As mentioned above, this heterogeneity is a result of the inherent complexity of the human body.

In an apparently healthy individual, for example, both the quantitative and qualitative characteristics of the blood-cfDNA fraction at any time are modulated by numerous internal processes (e.g., programmed cell death, inflammation and nuclease activity) which are in turn influenced by other biological and environmental factors, such as age, weight, gender, fitness, organ health, diet, circadian oscillations, and oxidative status (van der Vaart and Pretorius, 2008). Furthermore, it is generally understood that all cells are capable of, and are likely, continuously releasing cell-specific DNA into the extracellular environment (it has yet to be found absent in *in vitro* studies) (Gahan et al., 2008). A related issue of concern is the phenomenon of genetic mosaicism, a term used to describe the presence of two or more cell populations with different genotypes within one individual (Astolfi et al., 2010). Traditionally it is assumed that all of the somatic cells in a higher organism contain an exact replica of the entire genetic code, and that it is subject to change only by virtue of random mutations due to replication errors and inevitable damage to the genome (Aucamp et al., 2016).

However, there is accumulating evidence that the genome is continuously formatted by both intentional and incidental rearrangements, including duplications, deletions and insertions in both the germline and somatic cells, in both healthy and diseased states. This is possible because of compartmentalization, which creates a unique environmental niche for individual organs, tissues and cells, allowing adaptation and diversification according to localized conditions. Genetic diversification is achieved by mechanisms such as non-allelic homologous recombination (NAHR), non-homologous end-joining (NHEJ), and Fork Stalling and Template Switching (FoSTeS), which have been associated with transposon mobilization and insertion, drug-induced gene duplication, retroviral mutagenesis, and the action of minisatellites and small RNA molecules (for a concise review article, refer to Shapiro, 2013).

For these reasons, the aggregate cfDNA profile present in a single blood sample, for instance, comprises a muddled blend of both "wild-type" and genetically and epigenetically altered DNA fragments released by various cells from different tissues and organs by different mechanisms. This makes it very difficult to determine the biological properties and functions of cfDNA *in vivo*, and to make comparisons between different individuals. The magnitude of this issue is argued in a recent review article by Thierry et al., in which the heterogeneity of blood samples is illustrated by highlighting numerous putative sources and causes that result in the presence of cfDNA in the extracellular environment (Thierry et al., 2016).

1.3 Hypotheses investigated in this study

Two-dimensional or 2D cell culture models are insulated from most of the confounding elements that define a complete organism. Therefore, it has the potential to overcome many of the inherent obstacles associated with heterogeneous bio-fluid samples. Despite the fact that it has proven very useful, and perhaps more ethical, in many different domains of biological and translational research, the application of *in vitro* cell culture models in cfDNA research is largely lacking.

Therefore, in this study the utility of cell cultures in cfDNA research is explored by investigating four hypotheses, namely that *in vitro* cfDNA research can be used to:

I. Facilitate the optimization, standardization and development of robust preanalytical workflows for downstream cfDNA analyses.

II. Investigate the origin of cfDNA and the molecular mechanisms involved in its generation.

III. Elucidate the composition, function and role of cfDNA in normal biology and in molecular events that underlie the pathogenesis of cancer and other physiological conditions.

IV. Expedite the discovery and appraisal of cfDNA biomarkers.

1.4 Aims of this study

To explore the hypotheses stated in Section 1.3, the aims formulated for this study were:

I. To develop a better understanding of the various origins, structures and functions of cfDNA by conducting an extensive literature review (Article **I**, Article **II**, and Article **VIII**).

II. To establish both a robust cell culture model and reliable preanalytical workflow for the *in vitro* characterization of cfDNA (Article **III**, Article **IV**, Article **IX**, Article **X**, and Article **XII**).

III. To investigate the origin, fluctuation, structure and function of the cfDNA present in the growth medium of cultured cells (Article **V**, Article **VI**, Article **VII** , and Article **XI**).

1.5 Structure of this thesis

Chapter 2: Literature review

In this chapter the literature on cfDNA is appraised and consists of two review articles. The main focus of Article **I** is to provide a new perspective on the importance of (a) knowledge regarding the details of the historical events surrounding the discovery and conceptualization of cfDNA, and (b) elucidating both the evolutionary history and trajectory of cfDNA in human biology. It is argued that these two avenues of cfDNA research have been hugely neglected to date, and that an exploration thereof will improve our general understanding of the nature of cfDNA. Article **II** includes a systematic description of the various sources and causes, as well as their complex interplay, that result in the presence of cfDNA in human bodily fluids, especially blood. The purpose of this is mainly to demonstrate how the complexity of the *in vivo* setting translates to the heterogeneity of cfDNA in bio-fluid samples, and why "closed-circuit" *in vitro* cell culture models may prove to be useful in cfDNA research.

- **Article I**: J Aucamp*, AJ Bronkhorst*, CPS Badenhorst, PJ Pretorius. A historical and evolutionary perspective on the biological significance of circulating DNA and extracellular vesicles. *Cellular and Molecular Life Sciences* (2016), Volume 73, pp 4355-4381.

- **Article II**: J Aucamp, AJ Bronkhorst, CPS Badenhorst, PJ Pretorius. The diverse origins of circulating DNA in the human body: A Critical re-evaluation of the literature (*Manuscript submitted to Biological Reviews*).

* *Authors contributed equally to the drafting of this manuscript.*

Chapter 3: Method optimization and standardization.

This chapter consists of a brief summary of the cell lines used in this study, followed by a description of the preanalytical workflow developed and used for *in vitro* analysis of cfDNA in this study. This is based on the work reported in Article **III**, in which various methodological variables relating to cfDNA analysis are both theoretically and experimentally evaluated, and Article **IV**, a short communication focused on the optimization of cfDNA quantification.

- **Article III**: AJ Bronkhorst, J Aucamp, PJ Pretorius. Cell-free DNA: Preanalytical variables, *Clinica Chimica Acta* (2015), Volume 450, pp 243-253.

- **Article IV**: AJ Bronkhorst, J Aucamp, JF Wentzel, PJ Pretorius. Reference gene selection for in vitro cell-free DNA analysis and gene expression profiling, *Clinical biochemistry* (2016), Volume 49, pp 606-608.

Chapter 4: Molecular and biological characterization of cfDNA

This chapter consists of two research articles that describe the different origins, structures, fluctuation and other biological characteristics of the cfDNA present in the growth medium of both healthy and malignant cultured cells.

- **Article V**: AJ Bronkhorst, JF Wentzel, J Aucamp, E van Dyk, LH du Plessis, PJ Pretorius. Characterization of the cell-free DNA released by cultured cancer cells, *Biochimica et Biophysica Acta- Molecular Cell Research* (2015), Volume 1863, pp 157-165.

- **Article VI**: J Aucamp, AJ Bronkhorst, DL Peters, HC Van Dyk, FH Van der Westhuizen, PJ Pretorius. Kinetic analysis, size profiling and bioenergetic association of DNA released by selected cell lines *in vitro*, *Cellular and Molecular Life Sciences (Issue not yet assigned).*

Chapter 5: A provisional hypothesis for the origin and function of cfDNA in cancer

This chapter consists of an article describing the use of massively parallel semiconductor sequencing to investigate the composition of the cfDNA that is actively released by bone osteosarcoma cells *in vitro*. Based on the results, and an extensive review of the literature, a provisional hypothesis was formulated to explain the origin and function of actively released cfDNA in cancer.

- **Article VII**: AJ Bronkhorst, JF Wentzel, DL Peters, J Aucamp, E van Dyk, EP de Villiers, PJ Pretorius. Alpha-satellite DNA and active transposable elements are spontaneously released by bone osteosarcoma (143B) cells *in vitro* (*Manuscript submitted to BBA - Molecular Cell Research*).

Chapter 6: Concluding remarks and future prospects

In this chapter the major conclusions derived from the work in this study are discussed.

Appendix I: Conference outputs and published proceedings

Sections of the work from Article **I**, Article **III**, Article **IV**, and Article **V** were presented at the *Circulating Nucleic Acids in Plasma and Serum IX* congress, which was held in Berlin, Germany on 10-12 September 2015. The proceedings of this congress were peer-reviewed and included as separate chapters in a book published by Springer in the journal series *Advances in Experimental Medicine and Biology*. Moreover, sections of Article **V** and Article **VII** were presented as scientific posters at the 7th European Molecular Biology organization (EMBO) meeting, which was held in Mannheim, Germany on 10-13 September 2016. These book chapters and posters are derivative of their article-counterparts. Therefore, in order to avoid unnecessary repetition they were not included in the main text, but were appended at the end of the thesis. A list of the four book chapters and two posters are given below.

- **Article VIII:** J Aucamp, AJ Bronkhorst, PJ Pretorius. A Historical and Evolutionary Perspective on Circulating Nucleic Acids and Extracellular Vesicles: Circulating Nucleic Acids as Homeostatic Genetic Entities, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 91-95.

- **Article IX:** AJ Bronkhorst, J Aucamp, PJ Pretorius. Methodological Variables in the Analysis of Cell-Free DNA, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 157-163.

- **Article X:** J Aucamp, AJ Bronkhorst, JF Wentzel, PJ Pretorius. A Quantitative Assessment of Cell-Free DNA Utilizing Several Housekeeping Genes: Measurements from Four Different Cell Lines, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 101-103.

- **Article XI:** AJ Bronkhorst, JF Wentzel, J Aucamp, E van Dyk, LH du Plessis, PJ Pretorius. An Enquiry Concerning the Characteristics of Cell-Free DNA Released by Cultured Cancer Cells, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 19-24.

- **Poster 1:** AJ Bronkhorst, JF Wentzel, LH du Plessis, PJ Pretorius. Cell-free DNA is actively released by cultured cancer cells.

- **Poster 2:** AJ Bronkhorst, JF Wentzel, PJ Pretorius. Molecular characterization and profiling of the DNA released by cultured cancer cells using massively parallel semiconductor sequencing.

Appendix II: Data article

This article describes supplementary data obtained from the unpublished results reported in Article **III**.

- **Article XII:** AJ Bronkhorst, J Aucamp, PJ Pretorius. Adjustments to the preanalytical phase of quantitative cell-free DNA analysis, *Data in Brief* (2016), Volume 6, pp 326-329.

Appendices III to VI

Lists of publications, figures, tables, and copyright clearance documentation.

Bibliography

References made in each article are listed at the end of the article and are not included in the general bibliography. References made in Chapter 1 and Chapter 6 are included in the general bibliography.

1.6 Materials and methods used in this study

The different materials and methods that were used in this study are described in the relevant sections of each research paper, book chapter, submitted manuscript and poster, and are summarized in Table 1.

Table 1: List of methods used in this study

Method or technique	Used and described in Chapter(s)
Bioinformatics analysis of NGS data	5
Capillary electrophoresis using the bioanalyzer	4
cDNA synthesis	3
Cell cycle analysis using flow cytometry	4
Cellular protein isolation and quantification	3,4
Detection of apoptosis and necrosis using flow cytometry	4
Mammalian cell culturing	3,4,5
Next-generation sequencing using the ion torrent PGM and S5	5
Nucleic acid isolation and quantification	3,4,5
Polymerase chain reactions and real-time PCR	3,4

Chapter 2: Literature review

Article I

A historical and evolutionary perspective on the biological significance of circulating DNA and extracellular vesicles

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* *Authors contributed equally to the writing of this article*

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Cellular and Molecular Life Sciences

VISIONS AND REFLECTIONS

A historical and evolutionary perspective on the biological significance of circulating DNA and extracellular vesicles

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Abstract The discovery of quantitative and qualitative differences of the circulating DNA (cirDNA) between healthy and diseased individuals inclined researchers to investigate these molecules as potential biomarkers for non-invasive diagnosis and prognosis of various pathologies. However, except for some prenatal tests, cirDNA analyses have not been readily translated to clinical practice due to a lack of knowledge regarding its composition, function, and biological and evolutionary origins. We believe that, to fully grasp the nature of cirDNA and the extracellular vesicles (EVs) and protein complexes with which it is associated, it is necessary to probe the early and badly neglected work that contributed to the discovery and development of these concepts. Accordingly, this review consists of a schematic summary of the major events that developed and integrated the concepts of heredity, genetic information, cirDNA, EVs, and protein complexes. CirDNA enters target cells and provokes a myriad of gene regulatory effects associated with the messaging functions of various natures, disease progression, somatic genome variation, and transgenerational inheritance. This challenges the traditional views on each of the former topics. All of these discoveries can be traced directly back to the iconic works of Darwin, Lamarck, and their followers. The history of cirDNA that has been revisited here is rich in information that should be considered in clinical practice, when designing new experiments, and should be very useful for generating an empirically up-to-date view of cirDNA and EVs. Furthermore, we hope that it will invite many flights of speculation and stimulate further inquiry into its biological and evolutionary origins.

Keywords Inheritance of acquired characteristics · Pangenesis · Metabolic DNA · Genometastasis · Virtosomes · Lateral gene transfer

Introduction

In 1928, Fred Griffith [1] discovered that infectious bacteria, which had been heat-killed, could transfer a pathogenic property to a live non-pathogenic strain via an interchange of culture medium. Subsequent experiments by Avery et al. [2] indicated that such an acquisition of a pathogenic property can be ascribed to the transmission of nucleic material. Apart from paving the way to the discovery that DNA is the substance of inheritance, these observations were the first evidence for the existence of nucleic acids beyond the confinement of cells [3]. Shortly thereafter, another pivotal discovery was made when Mandel and Métais [4] demonstrated the presence of extracellular nucleic acids in human blood. In the two succeeding decades nucleic acids were found to be present in the extracellular environment of organisms from all kingdoms of life [5]. Although these nucleic acids were originally termed extracellular nucleic acids, the term "circulating nucleic acids in plasma and serum" (CNAPS) was adopted when numerous studies demonstrated the presence of tumor-derived nucleic acids in plasma and serum. This term was then gradually replaced by less awkward grammatical constructions, including circulating

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nucleic acids (cirNAs), cell-free nucleic acids (cfNAs), and circulating cell-free nucleic acids (ccfNAs). Although these terms are still used interchangeably, cirNAs is now the most commonly used. When referring only to the DNA fraction, the term circulating DNA (cirDNA) is used, although the term cell-free DNA (cfDNA) is used interchangeably. However, for the purposes of this review, the term cirDNA will be used to describe DNA present in biofluids, and when discussing in vitro studies, the term cfDNA will be used. Furthermore, the terms cirDNA and cfDNA describe any DNA existing in the extracellular environment, regardless of structure (association with a variety of protein complexes and extracellular vesicles).

Thus far, many endogenous and exogenous sources of cirDNA in humans have been identified, e.g., apoptosis, necrosis, bacterial, and viral DNA [6]. Although apoptosis and necrosis are considered to be the main processes for producing cirDNA, numerous studies have demonstrated that cirDNA can also be derived from active cellular release mechanisms [7–12]. Further examination showed that actively released DNA is often associated with RNA and a lipid-protein complex, can translocate to neighboring or remote parts of the body, enter target cells, and alter their biology [12–18]. In the following years, it became clear that the active release and uptake of nucleic acids are a characteristic shared by all organisms and cell types [19].

Pertaining to humans, the presence of cirDNA in human blood and the phenomenon of the lateral transfer of cirDNA between different cells within a body are implicated in a wide range of biological phenomena. First, the occurrence of elevated levels of cell-specific cirDNA in human blood has been associated with many benign and malignant diseases and other conditions. This is exemplified by many studies, in which the cirDNA derived from patients with wide-ranging conditions, such as cancer [3], preeclampsia [20], traumatic injuries [21], fatigue [22] and old-age [23], were shown to be represented by different sequences, sizes, quantities, and genetic and epigenetic alterations, as distinct from healthy individuals. These characteristics make cirDNA prime candidate biomarkers for the diagnosis and therapy monitoring of many diseases. Another exciting discovery worth mentioning here is that, in the form of cirDNA, the entire fetal genome circulates in maternal blood [24–26], enabling the non-invasive detection of several fetal genetic aberrations.

Second, several studies have demonstrated the capacity of cirDNA to act as an intercellular messenger of sorts. For example, the lateral transference of cirDNA between different cells has been implicated in oncogenesis, metastasis, the blocking of tumor growth [27–29], and the development of resistance against radiotherapy and chemotherapy [30–32]. Since our understanding of the *modus operandi* of intercellular communication is traditionally limited to cell–cell adhesion conduits and secreted hormones and neurotransmitters, cirDNA may provide a new perspective on how cells communicate and share potentially useful information with each other. In addition, it offers an entirely new paradigm that could potentially improve our understanding of various pathologies (discussed in "The role of circulating DNA in disease", "The bystander effect", and "Lateral transfer of circulating DNA from healthy tissues").

Finally, cirNAs may also play an active role in the shaping of genomes and ultimately the process of evolution. There is evidence not only showing that somatic cells are liable to genetic and epigenetic modification via cirNAs, but also that this information may be transferred to the germ cells. For example, DNA has been detected in EVs released by prostate cells that have been shown to interact with sperm cells [33]. More recently, Cosetti et al. [34] discovered that RNA of human melanoma cells xenografted in mice is transported via exosomes through the bloodstream to the spermatozoa, showing that somatic information can be transferred to and received by sperm cells (discussed in "Extracellular vesicles and mobile protein complexes").

However, despite showing great promise as both an investigative aid for a wide range of biological phenomena, and a potentially powerful tool for disease screening, cirDNA research already faces many challenges. In clinical diagnostics, qualitative analyses are not yet routinely applied to screen for diseases and as concerns quantitative analyses, no normal reference value has yet been correlated with any disease, and there is no cut-off value for diagnosis. Furthermore, regarding its role in intercellular communication, we currently have a very limited understanding of the cellular circuits that mediate its messaging functions, and regarding its role in somatic genome variation, transgenerational inheritance, and evolution, it still appears to be an improbable or inexplicable event for many scientists [35–37]. Except for a lack of an analytical consensus, these difficulties can be ascribed mainly to a severe insufficiency of knowledge of its biological properties and molecular origin.

Although cirDNA research is far from a fully fledged field, our capacity to probe these questions is not so much limited by technology as by our manner of thinking. We might thus very often be looking in the wrong place for answers. We contend that reviewing the early work associated with cirDNA and EVs, which has been badly neglected, is required to fully comprehend the nature of cirDNA. Concepts regarding horizontal gene transfer (HGT) and cirDNA were contemplated by many philosophers and scientists very long before (as far back as 500 years BC) its actual discovery. However, the difficulty, in that time, to discover or even conceive of such

mechanisms resulted, for those who believed in them, in many rhetorical enquiries that were nearly impossible to reciprocate. Consequentially, many ingenious ideas have been largely abandoned, and, to this day, still populate the garbage pail of the scientific literature. The progress that has been made in biology over the last couple of decades allowed us to perform a reconstruction of the historical path that led to the discovery and concepts of cirDNA and EVs and to provide an empirically up-to-date view (Fig. 1). This is essentially the task of this review: to rediscover parts of the forest which we have lost for the sight of the trees.

Earliest speculations on heredity

Ancient Greek philosophers

Anaxagoras (500–428 BC) believed that semen contained a miniature version of all human organs that, when planted in the uterus, grew and formed a well-developed fetus (reviewed in [38]). Hippocrates (460–370 BC) expanded this idea and proposed the Pangenetic theory of inheritance (reviewed in [39]). He hypothesized that semen contained all parts of a human body, and that it was secreted from the father's healthy and unhealthy organs to produce healthy or unhealthy parts in the child (reviewed in [40]). Aristotle (384–322 BC), however, argued against this idea [39]. He believed that semen was a secretion of blood that brought life to the coagulated menstrual blood in a woman's uterus [38, 41]. Only in the seventeenth century was this hypothesis refuted when William Harvey (1578–1657) demonstrated that the uteri of pregnant deer do not contain coagulated blood [41]. Hippocrates also believed in the inheritance of acquired characteristics, based on the observations which he made about the Macrocephali race. This was a primitive culture that associated nobility with the length of one's head, and so artificially elongated it. They would begin the process immediately after birth, first manipulating the child's head by hand, and then constraining it with bandages to force a lengthened shape. Over time, the practice was no longer required as the feature became inherited. Similar findings were reported in 1855 by Rivero and Von Tschudi [42]. Multiple collections of infant mummies and a 7-month-old fetus, still enclosed within the womb of a mummy of a pregnant woman, with cranial formations similar to the artificially manipulated heads of the adults were identified. Rivero and Tschudi further identified three different Peruvian races that share an interesting osteological anomaly, and the presence of an almost triangular-shaped interparietal bone in the crania of the infant mummies. However, one cannot rule out congenital disorders, e.g., Marfan syndrome (disorder of connective tissue), as a cause for the head morphology of these mummies.

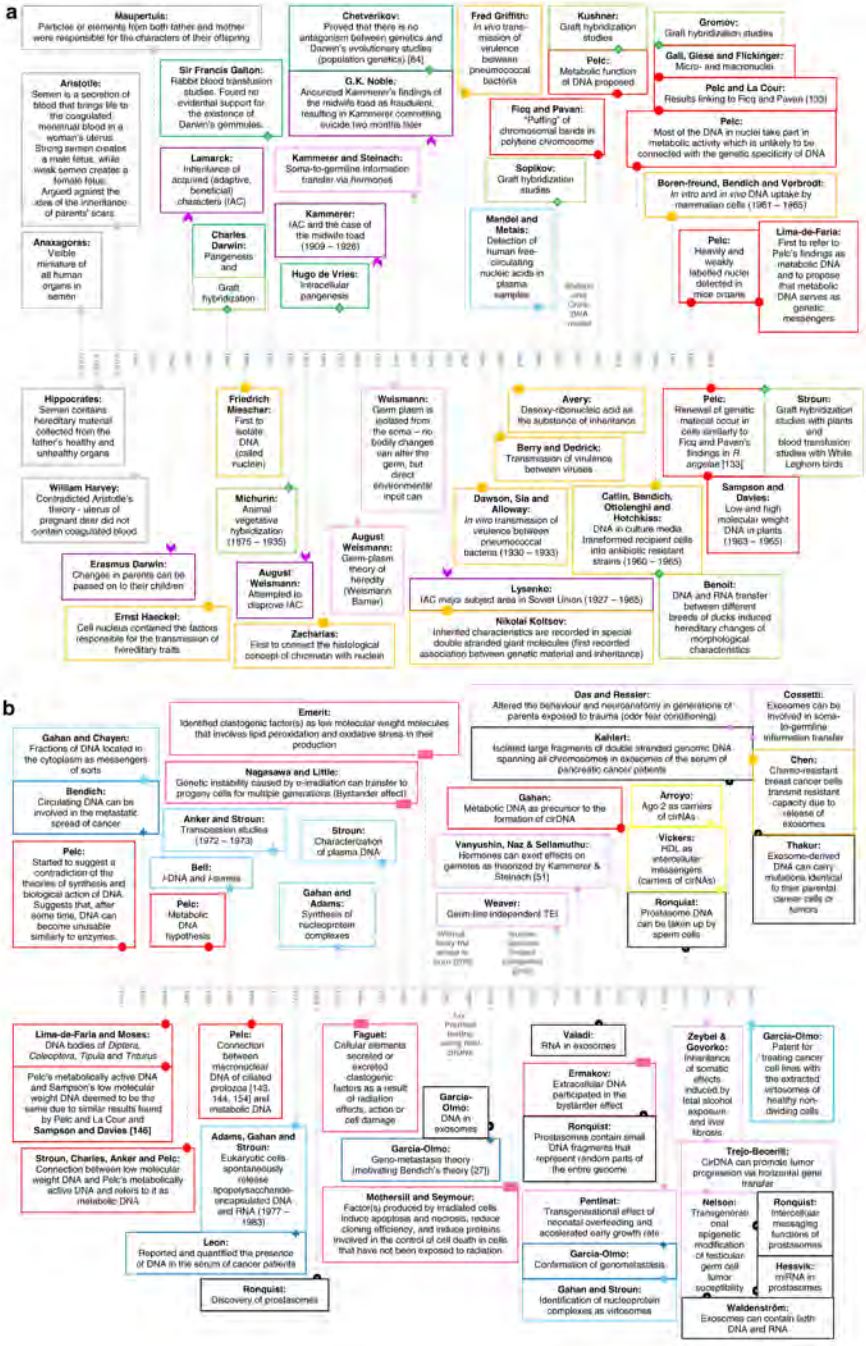
Predecessors of Charles Darwin and Jean-Baptiste Lamarck

Due to the empiricism of the seventeenth and early eighteenth century, the physical and experimental sciences were overemphasized, which restrained the deliberation of ideas on heredity. Indeed, natural history was studied primarily by ordained ministers, which led to a general belief that the world is perfectly designed. Three prominent figures held beliefs in the opposite direction. Based on Hippocrates' ideas, Pierre Louis Maupertuis (1698–1759) espoused a theory of Pangenesis, stating that particles or elements from both the father and mother were responsible for the characteristics of their offspring (reviewed in [43]). Similar to Maupertuis, Erasmus Darwin (1731–1802), the grandfather of Charles Darwin, rejected Aristotle's theory, but additionally suggested that changes in parental bodies, due to their activities, could be passed on to their children. Furthermore, he believed that one and the same kind of living filaments were and had been the cause of all organic life. These living filaments could acquire new parts in response to irritations, sensations, volitions, and associations, and could continue to improve its own inherent activity and be propagated from generation to generation [44]. He published these evolutionary speculations in *Zoonomia*, but never amplified them. Although the speculations of Erasmus Darwin are strikingly similar to Jean-Baptiste Lamarck's theory of the inheritance of acquired characteristics (IAC), it cannot be said with confidence that Erasmus influenced Lamarck, or vice versa [43].

The inheritance of acquired characteristics

Jean-Baptiste Lamarck: Heredity of adaptation

While devising his concept of evolution, Lamarck (1744–1829) identified two separate causes responsible for evolutionary change (reviewed in [39, 43]). The first cause involved the acquisition of greater complexity and perfection. Lamarck considered the power of acquiring progressively more complex organization to gain perfection an innate potential of animal life, a law of nature that did not, according to Lamarck, require special explanation. The second cause was the capacity of organisms to react to special conditions in the environment. According to Lamarck, the adaptations encountered in nature were due to the animals re-establishing harmony with their environment when this harmony was disturbed. This required the animal to either: (1) use certain parts more frequently


a
Maupertuis:
Particles or elements from both father and mother were responsible for the characters of their offspring
Aristotle:
Semen is a secretion of blood that brings life to the coagulated menstrual blood in a woman's uterus. Strong semen creates a male fetus, while weak semen creates a female fetus. Argued against the idea of the inheritance of parents' scars
Anaxagoras:
Visible miniature of all human organs in semen
Sir Francis Galton:
Rabbit blood transfusion studies. Found no evidential support for the existence of Darwin's gemmules.
Lamarck:
Inheritance of acquired (adaptive, beneficial) characters (IAC)
Charles Darwin:
Pangenesis and
Graft hybridization
Chetverikov:
Proved that there is no antagonism between genetics and Darwin's evolutionary studies (population genetics) [64]
G.K. Noble:
Accurated Kammerer's findings of the midwife toad as fraudulent, resulting in Kammerer committing suicide two months later
Kammerer and Steinach:
Soma-to-germline information transfer via hormones
Kammerer:
IAC and the case of the midwife toad (1909 – 1926)
Hugo de Vries:
Intracellular pangenesis
Fred Griffith:
In vivo transmission of virulence between pneumococcal bacteria
Kushner:
Graft hybridization studies
Pelc:
Metabolic function of DNA proposed
Ficq and Pavan:
"Puffing" of chromosomal bands in polytene chromosome
Sopikov:
Graft hybridization studies
Mandel and Metais:
Detection of human free-circulating nucleic acids in plasma samples
Gromiev:
Graft hybridization studies
Gall, Giese and Flickinger:
Micro- and macronuclei
Pelc and La Cour:
Results linking to Ficq and Pavan (103)
Pelc:
Most of the DNA in nuclei take part in metabolic activity which is unlikely to be connected with the genetic specificity of DNA
Boren-freund, Bendich and Verbrodt:
In vitro and in vivo DNA uptake by mammalian cells (1961 – 1965)
Pelc:
Heavily and weakly labelled nuclei detected in mice organs
Lima-de-Faria:
First to refer to Pelc's findings as metabolic DNA and to propose that metabolic DNA serves as genetic messengers
Hippocrates:
Semen contains hereditary material collected from the father's healthy and unhealthy organs
William Harvey:
Contradicted Aristotle's theory - uterus of pregnant deer did not contain coagulated blood
Erasmus Darwin:
Changes in parents can be passed on to their children
Ernst Haeckel:
Cell nucleus contained the factors responsible for the transmission of hereditary traits
Friedrich Miescher:
First to isolate DNA (called nuclein)
Michurin:
Animal vegetative hybridization (1875 – 1935)
August Weismann:
Attempted to disprove IAC
August Weismann:
Germ-plasm theory of heredity (Weismann Barrier)
Zacharias:
First to connect the histological concept of chromatin with nuclein
Weismann:
Germ plasm is isolated from the soma – no bodily changes can alter the germ, but direct environmental input can
Avery:
Desoxy-ribonucleic acid as the substance of inheritance
Berry and Dedrick:
Transmission of virulence between viruses
Dawson, Sia and Alloway:
In vitro transmission of virulence between pneumococcal bacteria (1930 – 1933)
Caitlin, Bendich, Ottolenghi and Hotchkiss:
DNA in culture media transformed recipient cells into antibiotic resistant strains (1960 – 1965)
Lysenko:
IAC major subject area in Soviet Union (1927 – 1965)
Nikolai Koltsov:
Inherited characteristics are recorded in special double stranded giant molecules (first recorded association between genetic material and inheritance)
Pelc:
Renewal of genetic material occur in cells similarly to Ficq and Pavan's findings in R. angulae [133]
Stroun:
Graft hybridization studies with plants and blood transfusion studies with White Leghorn birds
Sampson and Davies:
Low and high molecular weight DNA in plants (1963 – 1965)
Benoit:
DNA and RNA transfer between different breeds of ducks induced hereditary changes of morphological characteristics
b
Gahan and Chayen:
Fractions of DNA located in the cytoplasm as messengers of sorts
Bendich:
Circulating DNA can be involved in the metastatic spread of cancer
Pelc:
Started to suggest a contradiction of the theories of synthesis and biological action of DNA. Suggests that, after some time, DNA can become unusable similarly to enzymes.
Emerit:
Identified clastogenic factor(s) as low molecular weight molecules that involves lipid peroxidation and oxidative stress in their production
Nagasawa and Little:
Genetic instability caused by α-irradiation can transfer to progeny cells for multiple generations (Bystander effect)
Anker and Stroun:
Transmission studies (1972 – 1973)
Stroun:
Characterization of plasma DNA
Bell:
I-DNA and I-bases
Pelc:
Metabolic DNA hypothesis
Gahan and Adams:
Synthesis of nucleoprotein complexes
Das and Ressler:
Altered the behaviour and neuroanatomy in generations of parents exposed to trauma (odor fear conditioning)
Kahlert:
Isolated large fragments of double stranded genomic DNA spanning all chromosomes in exosomes of the serum of pancreatic cancer patients
Gahan:
Metabolic DNA as precursor to the formation of circDNA
Arroyo:
Ago 2 as carriers of circRNAs
Vanyushin, Naz & Sellamuthu:
Hormones can exert effects on gametes as theorized by Kammerer & Steinach [51]
Vickers:
HDL as intercellular messengers (carriers of circRNAs)
Weaver:
Germ-line independent TEI
Ronquist:
Proteasome DNA can be taken up by sperm cells
Cossetti:
Exosomes can be involved in soma-to-germline information transfer
Chen:
Chemo-resistant breast cancer cells transmit resistant capacity due to release of exosomes
Thakur:
Exosome-derived DNA can carry mutations identical to their parental cancer cells or tumors
Lima-de-Faria and Moses:
DNA bodies of Diptera, Coleoptera, Tipula and Triturus
Pelc's metabolically active DNA and Sampson's low molecular weight DNA deemed to be the same due to similar results found by Pelc and La Cour and Sampson and Davies [146]
Stroun, Charles, Anker and Pelc:
Connection between low molecular weight DNA and Pelc's metabolically active DNA and refers to it as metabolic DNA
Pelc:
Connection between macronuclear DNA of ciliated protozoa [143, 144, 154] and metabolic DNA
Adams, Gahan and Stroun:
Eukaryotic cells spontaneously release lipopolysaccharide-encapsulated DNA and RNA (1977 – 1983)
Leon:
Reported and quantified the presence of DNA in the sera of cancer patients
Ronquist:
Discovery of proteasomes
Fliguet:
Cellular elements secreted or excreted clastogenic factors as a result of radiation effects, action or cell damage
Garcia-Olmo:
DNA in exosomes
Garcia-Olmo:
Geno-metastasis theory (motivating Bendich's theory [27])
Mothersill and Seymour:
Factor(s) produced by irradiated cells induce apoptosis and necrosis, reduce cloning efficiency, and induce proteins involved in the control of cell death in cells that have not been exposed to radiation
Valadi:
RNA in exosomes
Ermakov:
Extracellular DNA participated in the bystander effect
Ronquist:
Proteasomes contain small DNA fragments that represent random parts of the entire genome
Pentinat:
Transgenerational effect of neonatal overfeeding and accelerated early growth rate
Garcia-Olmo:
Confirmation of genometastasis
Gahan and Stroun:
Identification of nucleoprotein complexes as virtosomes
Zeybel & Govorko:
Inheritance of somatic effects induced by fetal alcohol exposure and liver fibrosis
Trejo-Becerril:
CirDNA can promote tumor progression via horizontal gene transfer
Nelson:
Transgenerational epigenetic modification of testicular germ cell tumor susceptibility
Ronquist:
Intercellular messaging functions of prostasomes
Hessvik:
miRNA in prostasomes
Waldenström:
Exosomes can contain both DNA and RNA
Garcia-Olmo:
Patent for treating cancer cell lines with the extracted virtosomes of healthy non-dividing cells

◀**Fig. 1** Chronological summary of the key events that led to the discovery and characterization of cirNAs and EVs from 428 BC (**a**) to date (**b**). The research topics include inheritance theories (represented by *triangles*), Pangenesis and graft hybridization studies (*diamonds*), the inheritance of acquired characteristics (*chevron*), genetic material and inheritance (*squares*), soma-to-germline information transfer (*crosses*), metabolic DNA (*circles*), EVs (*half circles*), cirDNA (*stars*), the bystander effect (*rectangles*), mobile protein complexes (*arrow*), and cancer progression and/or metastasis (*plus*)

than before, considerably developing and enlarging these parts, or (2) use new parts that they have developed as a result of continuous stimulation. The idea that an organ could be strengthened by use and weakened by disuse was an ancient one. Lamarck simply provided a more rigorous physiological interpretation to this phenomenon. He considered it to be one of the cornerstones of his theory, referring to it as his "First Law" [43]. Lamarck's First Law posed the following question: How do these environmentally induced adaptations become inherited into a population? These forms of changes usually appear in a single individual or in a very small minority with respect to the population and though they tend to grow in number, there is still a risk of accidental extinction if they fail to spread among the population [45]. The accumulation of these micro-evolutionary changes, however, can result in an evolutionary change called genetic drift, a very slow process.

Lamarck's "Second Law" of evolutionary adaptation was the concept of IAC (reviewed in [43, 46]). He stated that all characteristics that individuals either acquire or lose due to environmental conditions that they were exposed to over a long period of time, were conveyed by the generation of new individuals descending therefrom, provided that the changes were present in both parents [43]. The phrase IAC was, however, quite imprecisely used by the majority of the public in that they did not emphasize the inheritance of adaptive (beneficial) traits, which Lamarck was very particular about [46]. The integration of various beliefs into Lamarck's theory of evolution made his paradigm highly persuasive, which continued to make some of his ideas widely accepted for almost a hundred years after Charles Darwin's publication of *The Origin of Species* in 1859 [43].

The germ-plasm theory of heredity vs soma-to-germline transfer

August Weismann: the argument against IAC through introducing the Weismann barrier

In the 1880s, August Weismann set out to disprove IAC. He hypothesized that cutting off the tails of experimental rats would not result in tailless pups or the shortening of the tail in successive generations. Indeed, long before Weismann, it was known that senseless mutilation had no effect on progeny as seen in cases of human circumcision, for instance [47]. Although Weismann's findings were irrelevant to Lamarck's original concept, it had grave ramifications on the public perception of IAC. Through the publication of multiple essays between 1883 and 1893, Weismann continued to discredit IAC and began introducing the germ-plasm theory of heredity.

Weismann's germ-plasm theory hypothesized that the nuclei of egg and sperm cells contained putative heredity particles which were transmitted from parent to child. However, contrary to IAC and Charles Darwin's Pangenesis theory (discussed in "The hypothesis of Pangenesis"), Weismann argued that these particles were immune to the lasting effects of environmental change, because he believed that germ cells were insulated from influences outside of the nucleus which allowed the preservation of parental types from significant environmental changes during transmission [48]. This theory of the immunity of germ-plasm against change was referred to as the Weismann barrier [49].

By the 1890s, the concept of "use inheritance" began to wane and Weismann's germ-plasm theory became increasingly popular, but IAC still remained favoured by many. Weismann then started suggesting that direct environmental effects on the germ-plasm were possible, but that functional changes of organs could not cause a corresponding change in the germ-plasm due to the germ being physically sequestered from not only somatic cytoplasm and idioplasm (nuclear contents), but also the germ-cell's cytoplasm [47, 50]. Furthermore, he believed that the idioplasm of the germ-cell was a substance of extreme stability, as it could absorb nourishment and grow enormously without changing its complex molecular structure. To support this, he referred to the persistence of unchanged species throughout thousands of years, particularly four thousand-year-old mummies of sacred Egyptian animals that remained identical to the animals existing then. Weismann believed that external effects could only affect germ-plasm growth rate and changes in entire species and that the only factor that could allow germ-plasm variation in individuals were changes that occur during sexual reproduction when germ-plasm is rearranged to produce offspring [47].

Demonstrating somatic induction in defence of IAC

Weismann's germ-plasm theory challenged the validity of IAC by implying that if the germ-plasm and soma were affected by environmental factors only, the changes in the germ-plasm would be transmitted to offspring and the somatic effects would have no effect on the germ-plasm

(direct or parallel induction) [50]. As mentioned in "August Weismann: the argument against IAC through introducing the Weismann barrier", Weismann believed that these environmental effects would, in any case, only influence germ-plasm growth rate or induce changes in an entire species, effectively refuting the idea that adaptive traits could be inherited by an individual. To prove that adaptive traits could be transferred from parent to offspring, one would have to demonstrate somatic induction, where the changes of only the soma would influence the germ cells to be transferred to offspring. Figure 2 briefly summarizes the progression of Hans Przibram, Paul Kammerer, and Eugen Steinach's research in support of IAC and the most prominent modern somatic induction studies that turned Weismann's theory on its head. Shortly after Steinach's discovery of hormones, the possibility of hormones-mediating somatic effects on the germline was questioned. Kammerer strongly believed that this was the case, developing a theory of somatic induction which he and Steinach tested in 1920 by exposing male rats to high temperatures, which resulted in morphological and physiological changes in their offspring and grandoffspring. It was theorized that the heat produced a change in hormone production in the interstitial cells and that the close proximity of the interstitial cells to the germ cells in the gonads facilitated hormonal interactions between them. The germline was, therefore, affected by the changes in hormone production and resulted in the inheritance of these changes by the offspring (reviewed in [51]).

Today, the concept of soma-to-germline information transfer through germline-independent transgenerational epigenetic inheritance is slowly growing. Vanyushin [52] has shown that methylation patterns in the rat genome are controlled by hydrocortisone dynamics, and a review by Naz and Sellamuthu [53] suggested that there are hormone receptors in mature sperm. Both of these studies strongly support Kammerer and Steinach's findings that hormones could exert their effects on gametes. Weaver et al. [54] showed that parenting behaviours and stress responses result in epigenetic alterations which can be transferred from the mother to her offspring. Dias and Ressler [55] showed altered behaviour and neuro-anatomy in the offspring of parents exposed to trauma in the form of odor fear conditioning. Mice were exposed to aromatic acetophenone to activate a known odorant receptor, *Olfr151*, and subsequently, conceived F1 and F2 generations were then

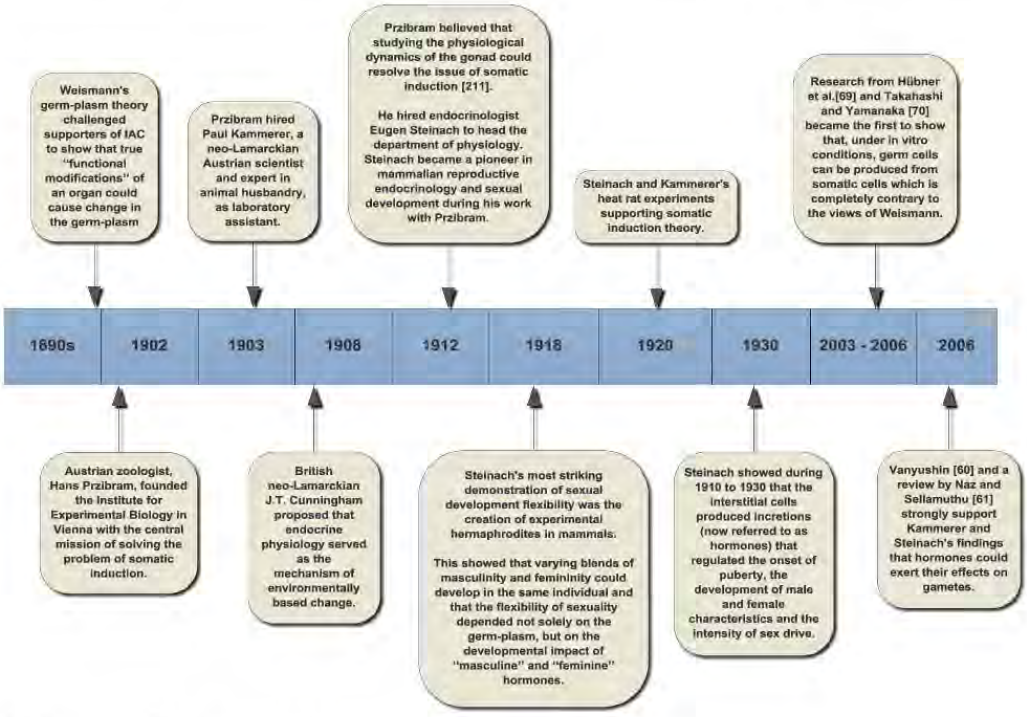


Fig. 2 Przibram and Steinach's somatic induction research [50]

screened for behavioural sensitivity to the acetophenone odor. The resulting behavioral sensitivity was found to be complemented by enhanced neuroanatomical representation of the *Olfr151* pathway and CpG hypomethylation was detected in the *Olfr151* gene of the F0 males and F1 generation. In vitro fertilization, F2 inheritance and cross-fostering showed that these transgenerational effects are inherited via paternal gametes. To ensure that the effects observed in the offspring were not due to direct exposure of the fetuses to the treatments, the parents were not yet pregnant when treated and cross-fostering was also applied. Nelson et al. [56] also discovered that genetically engineered Apobec1 deficiency could modify testicular germ-cell tumor susceptibility and embryonic viability in a transgenerational epigenetic manner. Other examples (reviewed in [57]) are the transgenetic inheritance of somatic effects induced by fetal alchohol exposure [58], neonatal overfeeding [59], and the induction of liver fibrosis [60].

In 2003, Hübner et al. [61] developed primordial germ cells or gametes from pluripotent stem cells of mice and humans. Three years later, Takahashi and Yamanaka [62] became the first to demonstrate that pluripotent stem cells can be derived from unipotent somatic mammalian cells. Researchers have, therefore, shown that, under in vitro conditions, germ cells can be produced from somatic cells which is completely contrary to the views of Weismann (that germ cells can produce both somatic and germ cells, whereas somatic cells produce only somatic cells [48]). The low probability of the fertilization of a transformed or untransformed germ cell and high turnover rate of sperm cells may limit the chances of a cirDNA-transformed sperm cell to fertilize an untransformed or transformed oocyte [63]. The successful transfer of genetic and epigenetic changes to following generations may, however, be dependent on the length and severity of exposure to the causing factor.

The case of the midwife toad

IAC became a major subject area in the 1930s to 1950s. Sigmund Freud was interested in IAC and refused to abandon this theory, even though it had become politically charged and regarded by the Nazis as Bolshevik and Jewish (discussed in [64]). This political prejudice is said to have started with Kammerer's experiment on the midwife toad (*Alytes obstetricans*), terrestrial amphibians that copulate, fertilize their eggs, and carry them on land during embryonic development [65]. Kammerer conditioned midwife toads to develop characteristics similar to closely related aquatic toad species. The few resulting generations showed a greater preference to copulate in water even under the normal environmental conditions and no longer carried their eggs (reviewed in [65, 66]). By generation F3, the male toads started to develop nuptial pads: rough, pigmented epidermal thickenings that male aquatic toads use to grasp the slippery females during copulation in water. However, William Bateson, a neo-Darwinist and Cambridge scientist, and G.K. Noble of the American Museum of Natural History announced that the toad was injected with India ink to create and represent nuptial pads (reviewed in [67]) and the controversial debate that followed pushed Kammerer to suicide (discussed in [65, 68, 69]). Many scientists believed that Kammerer's experiments were genuine, but his experiments were subsequently never repeated successfully. Arthur Koestler, a writer of scientific, political, and ethical issues, justified the reinvestigation of the Lamarckian experiments of Kammerer in 1971 [65, 67, 68], by identifying a natural occurrence of midwife toads with nuptial pads collected in the wild [65]. Harry Gershenowitz attempted the experiment in the 1980s using a similar method with *Bombina orientalis*, but could not continue due to financial constraints [67]. In 2009, Alexander O. Vargas stated that there was no proof that the nuptial pads of the experimental midwife toad were never present [65]. He believed that Kammerer stumbled upon non-Mendelian inheritance at a time when Mendelian genetics was becoming well accepted. Kammerer's experimental results were beyond the knowledge of biology of that time and were, therefore, deemed fraudulent [69]. Kammerer's midwife toad could, thus very well, be the earliest experimental evidence of epigenetic inheritance and, perhaps, even natural somatic induction. We are, therefore, in agreement with Koestler, Gershenowitz, and Vargas that these experiments should be reinvestigated.

The hypothesis of Pangenesis

Charles Darwin: variation and natural selection

Charles Darwin believed that two operative factors govern the organic world, the variation in the reproduction and inheritance of all living organisms, and natural selection. He devoted himself to describing the second factor and his name is principally associated with it. Variation, on the other hand, received considerably less attention. To Darwin, variation was a mysterious thing for natural selection to work on, and its spontaneous and uncontrolled character puzzled him (see [70] for a beautiful discussion). Nevertheless, inspired by his grandfather, Hippocrates, Maupertuis, and Lamarck, Darwin made an attempt to understand variation and formulated the theory of Pangenesis, which he presented in the chapter "Provisional hypothesis of Pangenesis" of his book "The variation of animals and plants under domestication", which was published in 1868 [71].

The fundamental building blocks of the Pangenesis theory were microscopic particles that Darwin called gemmules. These particles were constantly shed by all living cells at every developmental stage. They were self-replicating, could vary in response to the environment, and were capable of dormancy. They could then be released into the circulatory system, in which they were conveyed throughout the body, and could eventually reach the reproductive cells. Consequentially, information could be transferred to the next generation (reviewed in [19, 39, 72]). Darwin believed that gemmules could aggregate with one another or fuse into nascent cells to form new cells and connected the purpose of gemmules to not only heredity, but to the healing of wounds and regrowth of limbs (as seen in lizards). Darwin also hinted that gemmules show specificity regarding which cells they enter. He compared the affinity of gemmules for certain cells to the affinity of tissues for special organic substances, e.g., poisons only affecting certain organs or cancers and diseases affecting certain tissues or glands [71].

Darwin admitted that the sheer abundance of self-replicating gemmules from throughout the body at every developmental stage seemed inconceivable, but compared the vast numbers of gemmules with the numerous amounts of seeds and eggs that certain plant and animal species can produce and with the amount of contagions spread by disease to show that it was not impossible. He also believed that this vast abundance of gemmules could result in certain developmental oddities, e.g., the abnormal multiplication or transposition of organs and limbs (goldfish with supernumerary limbs or the development of a double tail in lizards whose tails were severed) [71]. In cases where mutilation or amputation did not result in the regrowth of the organ, Darwin believed that the remaining damaged and diseased tissue somehow resulted in the destruction of the gemmules released by that organ before it was removed, which prevents the body from rebuilding the organ.

Although Darwin's idea of free information-carrying particles in the circulation was a "gratuitous assumption" [71], Pangenesis served as a very efficient solution to both the 1865 Mendelian theory of inheritance and a central unsolved problem in Darwin's theory of natural selection, the so-called "blending hypothesis". The latter states that the repeated 'blending' of parental traits to form new generations would eventually remove all the diversity in a population, due to a lack of new sources of variations, and could eventually bring evolution to a grinding halt (reviewed in [39]). Pangenesis provided a ready source of heritable variation, as environmentally induced changes in the cells of organisms would be transmitted to offspring via modified gemmules, providing a wide range of diversity through the inheritance of acquired traits. However, despite showing great insight, Pangenesis was never adopted by his successors [70]. In fact, they actually held strong beliefs in quite the opposite direction. On top of that, Darwin's overemphasis of natural selection did unfortunately, in all innocence, directly, and quite powerfully reinforce the mechanistic conception of the universe. In other words, the true cause of organic change and progress was, by most, ascribed to natural selection. The difficult part of the theory, which had even Darwin perplexed, the internal factor of variation, was ignored. Be that as it may, Darwin went on and devised the concept of graft hybridization, a technique in which heritable changes could be transferred over graft junctions, to test his hypothesis.

For Darwin graft, hybridization referred to the production of individuals (graft hybrids) from the united cellular tissues of two different plants [73]. He collected large amounts of information about multiple graft hybrids, including that of the Bizzaria orange, jessamine, oleander, ash, hazel, grape, hyacinths, rose, and potatoes. The most famous example of graft hybridization is that of *Cytisus adami*, which bore its own hybrid flowers (dingy-red color) and that of its parent plants (large, bright yellow, and small, purple flowers) due to a graft of a *C. purpureus* (purple laburnum) bud into a *C. laburnum* (common laburnum) stock [71] (discussed in [73]). Darwin believed that graft hybridization proved that the elements required to produce a new being were not formed by sexual organs, but were present in cellular tissues where they could unite without the aid of sexual organs to form a new bud with characteristics of both the parental sources. Although he was successful and many other well-known researchers have created and demonstrated the existence of graft hybrids (reviewed in [73]), the acceptance of it as a reality was/is challenged by the perception that the phenomenon involves "simple" chimeras (discussed in [74]).

Francis Galton: argument against Pangenesis

Darwin's half first cousin, Sir Francis Galton, believed that mental and physical traits were inherited and that a superior race of men can be created, similar to the production of a superior breed of cattle or horse (reviewed in [39]). To prove this, Galton used Belgian mathematician Adolphe Quetelet's theory of a normal curve, where measurement variables would be distributed in the form of a bell curve. The frequency of the most common variables was proportional to the area under the apex of the bell and the frequency of the outlying variables was found near the tails. Galton believed that the size of the brain, the amount of grey matter or brain fibers, and, therefore, mental capacity of a population could be plotted with this method. The Pangenesis theory provided him with further justification that intelligence, or any other mental of physical

trait, would follow normal distribution. If a trait, in this case, intelligence was determined by two forms of a particular gemmule which exists in equal numbers in each of the parents, the result would be a bell-shaped distribution of intelligence among the offspring [39]. Pangenesis could, therefore, be used to predict the distribution of intelligence from one generation to the next. In the early 1870s, Galton set out to demonstrate the existence of gemmules by conducting blood transfusion experiments on rabbits [75]. The study was based on the theory that gemmules had to be present in the blood to be transported from one part of the body to distant reproductive cells, making it possible to transfer these gemmules from one animal to another via blood transfusion. Focussing on morphological changes alone, he attempted to "mongrelize" silver-grey rabbits with the blood of yellow, common grey, or black and white rabbits using three different blood transfusion techniques: (1) moderate transfusion of partially defibrinated blood; (2) large transfusions of wholly defibrinated blood; and (3) the establishment of a cross-circulation system between the carotid arteries of a silver-grey and common rabbit. None of these techniques produced any morphological changes which would suggest that gemmules existed [75]. For decades then, the concept of Pangenesis was excluded from the expanding knowledge of genetics.

Karl Pearson (an influential English mathematician, biometrician, protégé and biographer of Galton), however, discredited Galton's findings in 1900 by stating that Pangenesis was no more disproved by stating that gemmules have not been found in the blood than the atomic theory is disproved by the fact that atoms have not been found in the air [76]. Furthermore, Galton's unsuccessful experiments were later questioned due to the repeated successes of modified inheritance via graft hybridization. The most prominent graft hybridization research was performed by Russian geneticist, horticulturist, and a strong supporter of Darwin's views, Ivan Vladimirovich Michurin (1855–1935). His work resulted in the creation of more than 300 new fruit plant species (reviewed in [77]) and played a major role in (1) the works of Lysenko ("Trofim Denisovich Lysenko"), (2) the investigation of the effects of blood transfusions on hereditary traits by animal breeders (known as animal vegetative hybridization), in particular studies by P.M. Sopikov who transferred multiple physical traits between various bird species ("The connection between IAC and Pangenesis"), (3) the development of the circulating DNA theory by Stroun, Anker, Gahan, and Chayen using graft hybridization in plants ("The concept of circulating DNA"), and (4) more recent immunological tolerance and HGT studies. With regard to Galton's unsuccessful blood transfusion experiments, it was proposed that transfusion incompatibilities due to blood group differences, species differences, the transfusion method, frequency, and duration, and blood volume could be factors that affected the experiment (reviewed in [72]). Moreover, heritable changes may be easier to detect in poultry than in rabbits, and also, bird erythrocytes are nucleated and contain DNA, whereas mammalian erythrocytes do not, meaning that to observe heritable changes in mammals may require larger volumes of blood transfusion and more generations of progenies.

Trofim Denisovich Lysenko

The Soviet Union was under the leadership of Josef Stalin who welcomed the actions of the agronomist Trofim Lysenko who was a follower of Michurin's graft hybridization experiments and interested by the work of Kammerer. This made matters worse for Pangenesis and IAC, in the sense that on becoming President of the Lenin Academy of Agricultural sciences of the U.S.S.R. in 1948, Morgan-Medelian genetics were considered redundant and replaced by the "progressive, materialist, Michurin trend". Lysenko became notorious for forcing Soviet scientists to accept Michurin's teachings or be banned from doing research—or killed. Thus, as a keen supporter of IAC, he led large-scale experiments on graft hybridization and the conversion of winter and spring wheat from the 1930s to the early 1960s (reviewed in [77]). Repetition of these experiments by Western geneticists produced negative results, and Lysenko was declared a forger and a criminal (discussed in detail in [78]). Later studies, however, showed evidence that supported Lysenko's findings, showing that Lysenko was, perhaps, falsely accused (discussed in detail in [73, 79]), similar to Kammerer. On the other hand, Lysenko and his assistants, many of whom were not actual scientists, also transcribed Lamarck's ideas into bizarre claims, such as the "extraordinary adaptation" of cuckoos who constantly materialized *de novo* from eggs was (reviewed in [46, 80, 81]). The saga of Lysenko rendered Pangenesis and Lamarckian inheritance, an unacceptable and repulsive idea to the majority of biologists for a very long time [82].

De Vries: intracellular Pangenesis

In 1889, the Dutch botanist Hugo de Vries introduced intracellular Pangenesis, a modified version of Darwin's Pangenesis. He described discrete hereditary particles (called pangenes) coding for individual cells or smaller entities inside cells that corresponded to enzymes and the most basic components of the cell (reviewed in [39]). De Vries proposed that the nuclei of all cells contained an identical collection of pangenes that served as a repository of the totality of the hereditary information and that the distinctive nature of different cell types was determined by

the types and numbers of the pangenes transported from the nucleus to the cytoplasm. This theory has formed the crux of the modern understanding of the process of cell differentiation.

DNA, heredity, and the advent of extracellular DNA

The mid-nineteenth century marked the beginning of cytogenetics and the connections between genetic material and heredity. The events that led to the discovery of DNA, its connection to chromosomes, and to heredity are summarized in Fig. 3. In studying the nuclein of spermatozoa, Friedrich Miescher came very close to understanding the mechanisms controlling the development of an embryo and how characteristics and traits were passed on from one generation to another (reviewed in [83]). He stated that if one had to consider that a single substance is the specific cause of fertilization and heredity, then nuclein should be the first to be considered. However, Miescher did not believe that only a single substance could result in the diversity of different animal species. Although he believed that different types of nuclein could exist, he did not think that these differences would be enough to even warrant the slight differences between the individuals of the same species, because he could not imagine the combinatorial diversity possible with biological polymers. In fact, the majority of scientists believed that the more complex proteins served as the carriers of genetic information and not nuclein. It was assumed that nuclein was too small to store the vast amount of genetic information (discussed in [83]). In 1927, Nikolai Koltsov proposed that each chromatid of a chromosome consisted of one giant hereditary molecule, in which inherited characteristics were recorded (reviewed in [84]). This special giant double-stranded molecule consisted of two mirror strands that would replicate in a semi-conservative fashion using each strand as a template and each segment within this molecule would represent a gene. Koltsov's theory was confirmed 25 years later when James Watson and Francis Crick introduced their theoretical model of a double-stranded DNA helix in

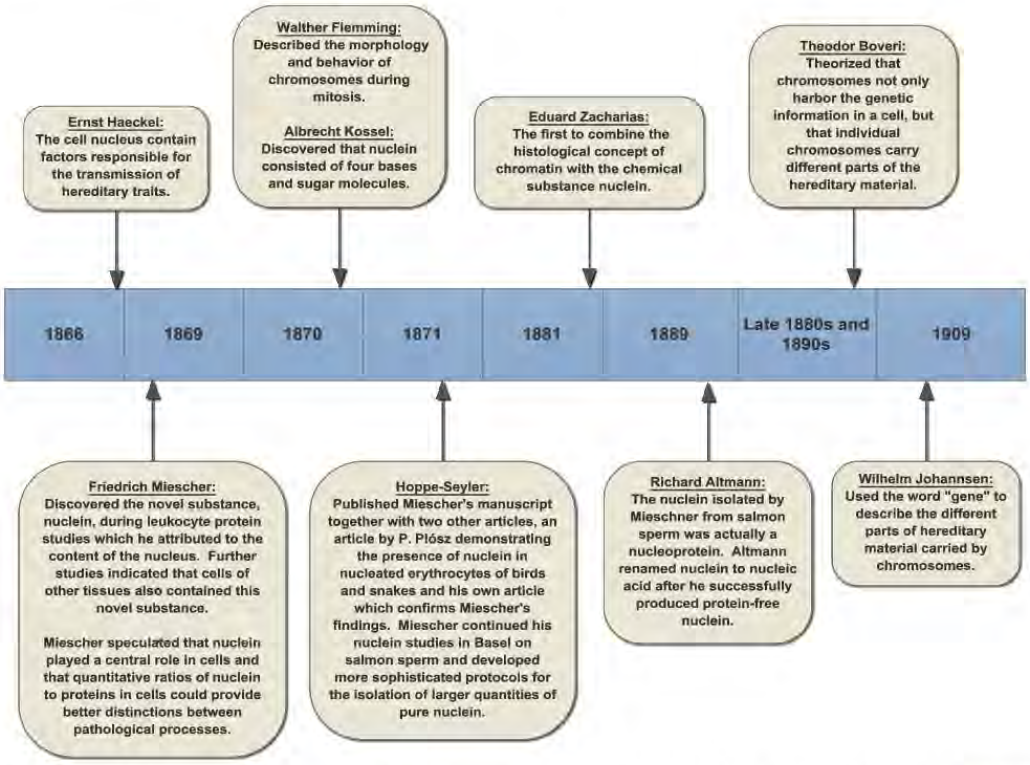


Fig. 3 Chronological summary of the events that led to the discovery and elucidation of nuclein as hereditary material in genes [83, 212, 213]

1953 [83, 84]. Koltsov's giant hereditary molecule was the first hint that it was possible for nucleic acids to serve as genetic information carriers.

In 1928, Fred Griffith [1] described the transmission of a virulent property from pneumococcal bacteria killed by heat to a living non-virulent attenuated strain by adding large doses of the killed cells' culture to the living cells. His findings were later confirmed by several studies [85–88] and were also found to occur in viruses [89]. Avery et al. [2] demonstrated the transfer of genetic material between different pneumococcal strains by managing to isolate a biologically active fraction in a highly purified form which, in exceedingly small concentrations, could transform cultured non-virulent pneumococcal cells into virulent cells. This active fraction was determined to contain a highly polymerized and viscous form of desoxyribonucleic acid [2], proving that DNA is the substance of inheritance (the transforming principle originally described by Griffith in 1928) (reviewed in [3]). It is also the first observation of the existence of DNA beyond the confinement of cells. Later studies indicated the existence of similar DNA fractions in both plant and animal kingdoms [3] and Mandel and Métais [4] were the first to report the detection of freely cirNAs in human plasma samples. In the 1960s, *Neisseria* and strains of *Diplococcus pneumoniae* (*Streptococcus pneumoniae*) were found to release biologically active DNA into culture media which resulted in the transformation of recipient cells [27, 90–92], similar to the virulence transmission studies of Griffith [1]. Transformation was obtained by treating a mixture of two pneumococci strains, one of which was penicillin-resistant and streptomycin-sensitive, and the other penicillin-sensitive and streptomycin-resistant, with penicillin. The treatment resulted in the lysis of the penicillin-sensitive strain, whose DNA transformed the penicillin-resistant strain to streptomycin-resistant [90, 91]. Similar to bacteria, mammalian cells can also take up DNA in vitro and in vivo [6, 27, 93, 94].

The connection between IAC and Pangenesis

In 1950, the Russian P.M. Sopikov became greatly inspired by Michurin's hybridization experiments. He found that the periodical transfusion of Black Australorp rooster blood to White Leghorn hens, which later mated with White Leghorn roosters, delivered offspring with noticeable modified inheritance. In exchanged donor and recipient roles, similar results were obtained. In comparison with purebred controls, changes in body mass, neck and body size, leg length, and leg pigmentation were noted. These characteristics became more pronounced in each successive generation of continually treated birds [95]. With Chuvash geese and Bronze turkey donors and White Leghorn recipients, similar changes were observed [96]. In response to Sopikov's unexpected results, a deluge of similar investigations by different researchers followed, which included donor/recipient pairs, such as the New Hampshire chicken/White Leghorn chicken [97] and the Bronze turkey and White Leghorn chicken [98]. Most obtained positive results. Further studies outside the Soviet Union also showed similar results, including the study by Stroun et al. [99], reporting that White Leghorn birds repeatedly injected with the blood from grey guinea fowl produced progeny with grey or black-flecked feathers in second and later generations [72]. All of these experiments have provided evidential support for the existence of Darwin's gemmules. However, what really actualized the credibility of Pangenesis is the association of gemmules with the discovery of extracellular DNA in human blood [4]. For the first time, then, DNA extracted from the Khaki Campbell was exclusively used to induce heritable changes in the Pekin duck. Supplementing these results, DNA and RNA transfer between different breeds of ducks induced hereditary changes of morphological characteristics [100].

The concept of circulating DNA

Owing to the fact that Western geneticists considered graft hybridization to be a dubious technique, no one outside Russia had for a long time attempted to repeat the experiments [101]. The first to do so successfully was Maurice Stroun and colleagues [102, 103]. They found that after three generations of grafting between two varieties of eggplant, hereditary modifications were obtained in the pupil plant. However, unlike the Russians who ascribed the hereditary modifications to acquired characters, because of a change of medium, Stroun suggested that it was due to DNA circulating between the mentor and pupil plants. It was also suggested that these DNA molecules could be transported to, and integrated with the genome of the reproductive cells of the pupil plant [102].

In concurrence with Stroun, Gahan and Chayen then suggested that a fraction of the DNA located in the cytoplasm had the ability to act as a messenger of sorts [104]. Stroun and Anker then went on and partly validated the hypothesis of cirDNA when they demonstrated that DNA could travel from the bottom of a tomato stem to the upper part of the plant, after dipping the stem in purified DNA [105]. Shortly afterwards, Eugene Bell discovered informational DNA (*I*-DNA), a non-mitochondrial cytoplasmic DNA found in the cytoplasm of a variety of embryonic cells that are packaged in particles called *I*-somes, which can be associated with rapidly labelled RNA and polyribosomes [106]. Bell theorized that *I*-DNA, rather than

mRNA, passes into the cytoplasm where it becomes associated with protein to form *I*-somes. This DNA then served as the templates for RNA synthesis, while transcription occurs after the DNA leaves the interior of the nucleus or while the DNA is associated with the outer nuclear membrane. Inhibitors of DNA synthesis do not affect *I*-DNA and nuclear DNA synthesis equally, indicating that *I*-DNA is not an artefact of tissue fractionation and DNA isolation [106, 107]. Bell's *I*-DNA and *I*-somes were hypothesized to represent copies of nuclear genes and to serve as information intermediates between the nucleus and the cytoplasm in eukaryotic cells, making them strikingly similar to De Vries' pangenes [39].

The concept of transcession

Similar to the viral and bacterial transformation studies of Griffith [1], Catlin [90, 91] and Ottolenghi and Hotchkiss [92], Anker and Stroun studied the transfer phenomenon (referred to as transcession) in the early 1970s, observing that it was sufficient to inject only the supernatant of a bacterial culture into animals to promote the transcription of bacterial DNA in animal cells [108, 109]. By placing frog auricles in Ringer's salt solution, Anker and Stroun discovered frog nucleoprotein complexes (containing DNA, RNA, lipids, and DNA and RNA polymerases) in the extracellular medium [7, 9–11, 101]. In 1982, Adams and Gahan determined that all the elements of the nucleoprotein complex are synthesized together in the cells, with the exception of the RNA, which is transcribed in the cytoplasm just before the complex exits the cells [101]. These nucleoprotein complexes, later named virtosomes [15], share striking similarities with Bell's *I*-somes. Both consist of DNA released into the cytoplasm, forming complexes with proteins, RNA and RNA transcription machinery that transcribe RNA in the cytoplasm before the complexes exit the cells. The studies by Stroun, Stephen Pelc (discussed in "The discovery of DNA with metabolic activity" and "Metabolic DNA"), Bell, Anker, and Gahan have suggested that DNA in the cytoplasm of cells act as messenger DNA [5, 110, 111], possibly explaining the cellular transformation occurrences observed by the multiple studies mentioned earlier [1, 2, 85–92], and providing one possible mechanism for the metastatic spread of cancer [27].

The role of circulating DNA in disease

Studies have indicated that diseases which cause endogenous tissue destruction, e.g., hepatitis, metastatic carcinoma, lupus erythematosus, and miliary tuberculosis, result in the increase of cirDNA levels [112–114]. Leon et al. [112] reported the presence of and quantified DNA in the serum of cancer patients. When compared with the healthy controls, 50 % of cancer patients presented elevated serum DNA levels and there was a correlation between the persistence of the elevated serum DNA levels and the weak response to therapy. As mentioned in "DNA, heredity and the advent of extracellular DNA", mammalian cells can also take up DNA in vitro and in vivo [27, 93, 94]. With this in mind, Bendich et al.'s hypothesized that cirDNA could be involved in the metastatic spread of cancer [27]. Tumorigenic DNA can be transported in a biologically active form via the circulatory or lymphatic systems, resulting in the penetration of various tissues. The disintegration of cells in necrotic areas of actively growing tumors in animals also results in the release of both normal and tumor-specific DNA fragments into plasma and serum. García-Olmo and colleagues hypothesized in 1999 and confirmed in 2010 that the horizontal transfer of tumor-derived cirDNA can transform NIH3T3 cells, producing cancerous cells [28, 115]. García-Olmo and colleagues referred to this hypothesis as the theory of genometastasis, the occurrence of metastasis via the transfection of susceptible cells, located in distant organs with dominant oncogenes that are derived from a primary tumor and are circulating in the plasma [28, 115, 116]. To date, there is no direct evidence that mutated cirDNA can initiate metastases. However, studies later confirmed that cirDNA (k-ras codon 12 mutated DNA) can promote tumor progression via lateral gene transfer (LGT) in rats that already have developed tumors, but does not itself initiate tumor formation in the host animal [117]. This concept of tumorigenic DNA being involved in cancer metastasis correlates so effectively with Darwin's gemmules that one cannot help but agree with Liu [118] that gemmules are analogues to cirNAs.

The bystander effect

The bystander effect refers to the effect of information transfer from targeted cells exposed to damaging agents of physical or chemical natures to adjacent, nonirradiated cells [119]. In 1984, Faguet et al. showed that cellular elements released clastogenic factors as a result of radiation effects, action, or cell damage, and Emerit et al. supported this finding in multiple studies in the 1980s and 1990s, identifying the factor(s) as low molecular weight (1000–10,000 Da) molecules that involved lipid peroxidation and oxidative stress in their production [120, 121]. It was also determined that patients with conditions, such as chronic inflammatory disorders or Fanconi's anemia, also had plasma with clastogenic activity [120]. Mothersill and Seymour also identified factors produced by irradiated cells and determined that these factors induced apoptosis and necrosis, reduced cloning efficiency, and induced proteins involved in the control of cell death in cells that were not

exposed to radiation [121, 122]. Mothersill and Seymour [121] theorized that the identified factors were proteins, but Ermakov and colleagues later indicated that cirDNA also participates in the bystander effect induced by exposure to X-ray irradiation in human lymphocytes [123] and endothelial cells [30, 32], providing the possibility that Mothersill and Seymour's factors may have been DNA rather than protein.

Lateral transfer of circulating DNA from healthy tissues

The bystander effect and genometastasis show that cancerous and damaging effects or messages can be transferred from cell to cell via LGT. However, healthy tissues are just as capable of transferring information between cells [124]. Garcia-Olmo et al. [29] demonstrated that the virtosomes of healthy non-dividing cells can modify the biology of recipient dividing tumor cells, resulting in the halting of DNA synthesis and tumor development, the possible reduction in tumor size and the prevention of metastases. It has been recently proposed that there is selectivity involved in cirDNA release, which supports the idea of cirDNA having an intended function rather than merely being the consequence of cellular death or damage. The screening of multiple housekeeping genes released by cell lines into cell culture [125] revealed the unequal representation of cfDNA sequences cell cultures. Similar results have been presented in human blood in 2009 [126].

The discovery of DNA with metabolic activity

In 1944, Ahlstrom, Euler, and Hevesy [127] discovered an increase in DNA content unproportional to the increase of organ weight during the growth of newborn rats, proposing that the formation of some of the labelled DNA molecules was not due to the synthesis of new cells, but due to an increase in the DNA content of cells already present [127, 128]. At first, it was assumed that DNA turnover occurred along with the cell formation, resulting in the difference between the calculated and radioactive data. However, this was deemed to not be possible, since the renewal of DNA only took place in very special cases. Fourteen years later, Stephen Pelc observed a similar incorporation of labelled adenine into the nuclei of non-dividing tissues. This was a significant finding, since DNA was considered to be stable in non-dividing tissues. He theorized that the synthesis or exchange of DNA took place in certain tissues as a metabolic function, unconnected with cell division, and that this metabolism was connected with the formation of certain proteins [129].

In 1959, Pelc questioned whether DNA might be broken down to a certain extent and reformed in cells and thought that this process should be detectable via the incorporation of DNA precursors in non-dividing cells or via the loss of DNA by processes other than pyknosis in dying cells. Pelc's autoradiographic studies of mouse seminal vesicles treated with colchicine indicated that the resulting incorporation of DNA precursors was considered at least 20 times above the requirement for cell division [130]. Mouse prostate cells showed a threefold excess of incorporation over that required for cell division. Feulgen-photometry of the cells during the study indicated that polyploidy was not responsible for the excessive DNA synthesis. Studies by Pelc and Gahan [131] showed similar results and it was hypothesized that all or most of the DNA contained in nuclei took part in metabolic activity that was unlikely to be connected with the genetic specificity of DNA.

In 1962, Pelc observed heavily and weakly labelled nuclei in the liver, smooth muscle surrounding the seminal vesicle, and interstitial cells of the testis of mice. It was assumed that the cells incorporated DNA precursors at different rates, producing the different levels of labelling [132]. There was no evidence of an immediate connection or direct connection between cells with weakly labelled nuclei and cells with heavily labelled nuclei. Pelc identified strong relationships between the presence of the weakly and heavily labelled nuclei to a similar occurrence observed by Ficq and Pavan [133], where considerable incorporation of tritiated thymidine into certain bands of polytene chromosomes of *Rhynchosciara angelae* occurred during the enlargement of the bands at certain steps of development. The "puffing" of the bands is due to a different activity of the genes at different times in the larval development and may occur similarly in the differentiated cells of mammalian organs. However, in differentiated cells, the same parts of chromosomes would be involved at all times, resulting in a lack of increase and decrease in the size of the chromosomal bands, as seen in *R. angelae* during different larval stages [134].

There were authors that could not identify any excessive DNA precursor incorporation or metabolic activity during their studies and some attributed Pelc's results to errors in methodology [135–137]. It was, however, possible that differences in the autoradiographic methods used (e.g., the use of the less reliable liquid emulsion technique versus the more reliable stripping film technique [138] and variations in the length of film exposure) contributed to these differences in results. In 1964, Pelc again determined that the incorporation of labelled thymidine in mouse seminal vesicles, heart muscle, and smooth muscle exceeded the requirements for cell division by a factor of eight to twelve [139] to refute results obtained by Gall and Johnson [136] who found that the incorporation of labelled thymidine was

due to premitotic DNA synthesis. He did, however, admit that premitotic and nonmitotic DNA synthesis had certain features in common. He also showed that diurnal rhythms could not be responsible for the excessive labelling of the cells, which Gall and Johnson suspected could have influenced Pelc's earlier results. Pelc believed that his studies started to suggest a contradiction of the theories of synthesis and biological action of DNA. It appeared possible for the strands of double helix DNA to separate and for one strand to be utilized for the synthesis of new molecules, while the second strand was discarded, thereby retaining a true copy of a 2C amount of DNA [139]. Parts, or all, of the DNA could, therefore, be renewed by self-reproduction, whereby DNA could still act as a template. It could, therefore, be assumed that after some time, DNA can become unusable, similar to enzymes [139], as they are subject to wear and tear while active and must be periodically renewed.

Metabolic DNA

Lima-de-Faria's DNA bodies and the origin of the term "metabolic DNA"

The presence of DNA bodies in oocytes was identified in *Dytiscus* as early as 1901, with similar bodies found in multiple *Tipula* species during the 1930s and 1950s [140]. In 1962, Lima-de-Faria [141] used tritiated thymidine incorporation to determine whether DNA synthesis occurred in the DNA bodies of *Tipula oleracea* larvae and to study their metabolic behavior. He determined that the DNA body of a *T. oleracea* oocyte consisted of approximately 59 % of the DNA of the whole nucleus. During diplotene, the DNA of the DNA body is suddenly released and becomes available to cellular components. Lima-de-Faria compared the occurrence and disintegration of DNA bodies to the metabolic behaviour of the "puffing" of chromosomal bands observed in *Rhynchosciara* [133] and also associated the occurrence and disintegration of DNA bodies to the occurrence of DNA with metabolic activity observed by Pelc [130, 141]. He was aware of the scepticism that Pelc received regarding the metabolic activity of DNA by Gall and Johnson [136], but believed that the occurrence of both the "puffing" chromosomal bands and the DNA bodies served as sufficient evidence to support Pelc and Gahan [131] 's hypothesis that all or most of the DNA contained in nuclei took part in metabolic activity that was unlikely to be connected with the primary function of DNA [141]. Gall and Johnson [136] were the first to refer to Pelc's DNA with metabolic activity as "metabolic" DNA, but their continuous use of scare quotes for this term illustrates their scepticism regarding its appropriateness. Lima-de-Faria was, in fact, the first to officially use the term metabolic DNA and the first to propose that metabolic DNA could serve as information carriers, as the released DNA of disintegrated DNA bodies could carry its own information to other cellular components [141].

The contents of DNA bodies

Lima-de-Faria and Moses studied DNA bodies in the females of the fly *Tipula oleracea* that formed in contact with the sex chromosomes in the oogonial interphases [142]. A DNA body synthesizes its DNA at a different time to that of the chromosomes, and its DNA forms complexes with histones, similar to the DNA of the chromosomes. At late diplotene, the DNA body disintegrates and its DNA is released into either the nucleus or the cytoplasm [142], similar to the destruction of macronuclei in ciliated protozoa (see "Macronuclei and metabolic DNA") after the micronuclei underwent division or meiosis [143–145]. The nucleoli of the *Tipula* oocytes are positioned within the DNA body and the presence of a band of RNA between the body and the chromosomes was detected, indicating that the DNA body has high RNA synthetic activity. Due to the positioning of nucleoli in the DNA body and the fact that the DNA of the body is complexed with histones, it was interpreted that the DNA body represents hundreds of copies of the operons of the nucleolar organizing region or neighboring regions.

DNA and RNA synthesis in DNA bodies during the different phases of meiosis

In 1968, Lima-de-Faria et al. [140] studied the DNA and RNA synthesis in the DNA bodies of *Acheta domesticus* oocytes (Fig. 4). Every female oocyte contains a DNA body, but no DNA bodies of comparable size or shape are present in the male meiotic prophase. Similar to *T. oleracea*, the DNA of the DNA bodies is synthesized at a different time to that of the chromosomal DNA and is complexed with histone, indicating that the bulk of DNA synthesis occurs in the DNA body.

The DNA body's DNA mass reaches its maximum at the beginning of the meiotic prophase and the chromosomes have been found to contract during this period. At pachytene and diplotene, the DNA body acquires the appearance of a puff, similar to the chromosomal puffs found in the polytene chromosomes of *R. angelae* [133] (Fig. 4). DNA synthesis within the DNA bodies ceases during the late pachytene and diplotene and the DNA body begins to disintegrate at the end of diplotene, breaking up the core into minor components and disintegrating the outer RNA shell. By late diplotene, the whole body is gone, releasing DNA, histone, and RNA into the nucleus with subsequent

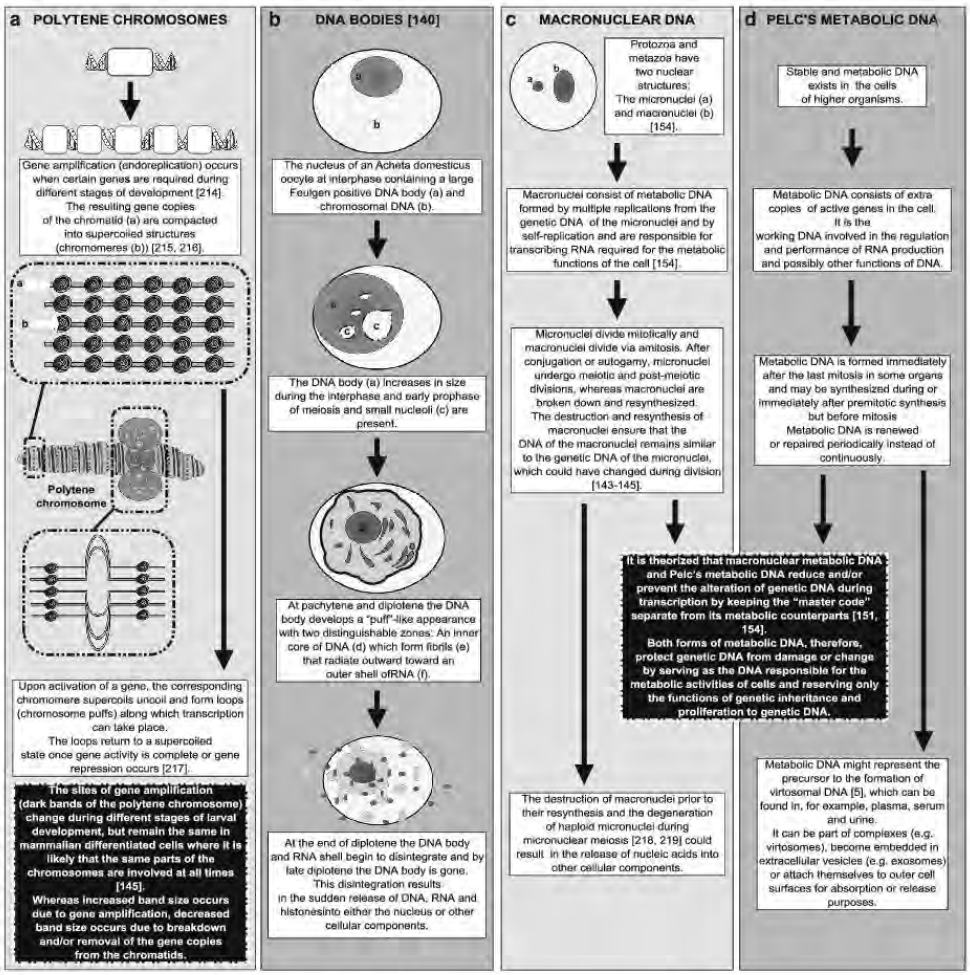


Fig. 4 Studies that contributed to the discovery and elucidation of Pelc's metabolic DNA. **a** Ficq and Pavan [133] 's discovery of DNA synthesis in the chromosomes of *R. angelae* larvae was the first indication that DNA played a very active part in the larval development. Polytene chromosomes are generated by the repeated replication of the chromosomes, followed by the alignment of the multiple copies of sister chromatids along their lengths, forming distinct banding patterns [214]. Pelc [132] later observed similar patterns of heavily and weakly labelled nuclei in mouse tissues and attributed the occurrence to the renewal of parts of genetic material that is highly active in the cells of an organ, similar to the puffing of chromosomal bands in *R. angelae*. **b** DNA metabolism in DNA bodies was also associated with Pelc's metabolically active DNA and Ficq and Pavan's chromosomal puffs by Lima-de-Faria [141]. Lima-de-Faria produced a detailed elucidation of DNA and RNA metabolism in the DNA bodies of *Acheta domesticus* oocytes in 1968 and proposed that the DNA released during the disintegration of the DNA body at the end of diplotene serve as information carriers [140, 141], theoretically making Lima-de-Faria not only the first to officially use the term metabolic DNA, but also the first to connect metabolic DNA to genetic messaging and cirNAs. **c** Pelc connected metabolic DNA with the macronuclear DNA of ciliated protozoa in 1970, giving reason to believe that metabolic DNA served a similar protective purpose towards genetic DNA as macronuclear metabolic DNA would protect micronuclear genetic DNA from alterations during transcription [156–161]. **d** Schematic summary of key characteristics of Pelc's metabolic DNA which share striking similarities with macronuclear metabolic DNA. The breakdown or removal of copied genes from polytene chromosomes during the larval development, the release of DNA and RNA from DNA bodies during disintegration, and the regular degeneration of old macronuclei and synthesis of new macronuclei during meiosis result in the release of nucleic acids into the nucleus, cell, and circulation, indicating a connection between Pelc's metabolic DNA and cirNAs as suggested by Lima-de-Faria [141] and later by Gahan et al. [5]

disintegration of the nuclear envelope at the end of prophase in meiosis. The DNA body forms two distinctive zones, an inner core of DNA, and an outer shell of RNA. The inner core forms Feulgen positive fibrils that radiate into the outer RNA shell, producing the appearance of the puff. RNA synthesis occurs in the outer RNA shell and in the chromosomes. Similar to the DNA bodies of *T. oleracea*, Lima-de-Faria et al. [140] have determined that the DNA body represents hundreds of copies of the genes of the nucleolar organizing region.

Pelc's metabolic DNA

Michael James Sampson identified two forms of DNA in *Vicia faba*, wheat, corn, and barley, namely high and low molecular weight DNA [146–148]. There was no evidence for turnover of the high molecular weight DNA, and this DNA also showed the same composition, irrespective of the tissue of origin, indicating that high molecular weight DNA in higher plants behaved typically as genetic material [147]. The metabolically labile, low molecular weight DNA of nuclear origin, however, was considered to have a physiological role rather than genetic due to its rapid formation in embryos induced to germinate [146, 147]. DNA synthesis detected during the period, in which $^{32}P_i$ was administered to barley roots, was that of the low molecular weight DNA [148]. The specific activity of high molecular weight DNA in barley roots labelled with $^{32}P_i$ was similar in both dividing cells and cells in which mitosis was inhibited. Metabolically, labile newly synthesized DNA was detected in the non-dividing cells that resembled the low molecular weight DNA of the dividing cells. It appeared that part of the newly synthesized low molecular weight DNA existed hybridized with the high molecular weight DNA [148]. In 1966, Sampson and Davies studied the synthesis of metabolically labile DNA in *V. faba* root cells. Autoradiography of labelled cells showed that the greater part of all thymidine incorporated into DNA was located in the nucleus. The region of cell elongation was also observed to contain both more nuclei and more heavily labelled nuclei than the mitotic zone, which corresponded with studies conducted by Pelc and La Cour on newly differentiated nuclei of *V. faba* roots in 1959, where intense labelling was observed in approximately 25 % of cells at a distance of 3–5 mm behind the 1.5–2 mm of meristem [146]. The similarities between Sampson's low molecular weight DNA and Pelc's DNA with metabolic activity suggested that they could be the same.

In 1967, Stroun et al. published an article, where the high and low molecular weight DNA fractions of mouse heart, skeletal muscle, and intestine were investigated. A low molecular weight fraction (between 5×10^5 and 4×10^6) and high molecular weight fraction (above 4×10^6) were obtained [149]. The specific activity of the low molecular weight fractions was significantly higher than that of the high molecular weight fractions in the three organs. Stroun et al. [149] theorized that the high specific activity of the low molecular weight fraction, especially in non-dividing muscle tissue, was possibly due to this DNA existing as a separate fraction in mammalian cells, and connected this DNA to the DNA responsible for the metabolic activity observed in the previous studies by Pelc, and referred to it as metabolic DNA. Pelc summarized the characteristics of metabolic DNA in 1968: It has been determined that (a) stable and metabolic DNA existed in cells of higher organisms; (b) metabolic DNA is formed immediately after the last mitosis in some organs and may be synthesized during or immediately after premitotic synthesis but before mitosis; (c) metabolic DNA is renewed or repaired periodically instead of continuously; (d) metabolic DNA is closely connected with the functional activity of differentiated cells; and (e) changes in DNA content, incorporation of precursors, or loss of labelled DNA correlates with a definite stage in development or with the stimulation of activity, indicating a definite correlation of metabolic DNA with function [150, 151]. The following hypothesis on metabolic DNA has been suggested by Pelc [151]: the metabolic DNA of a given type of differentiated cell consists of extra copies of active genes in the cell; metabolic DNA is the working DNA involved in the regulation and performance of RNA production, and possibly, other functions of DNA; metabolic DNA molecules are subject to wear and tear while active and are periodically renewed; DNA can, therefore, be labelled during premitotic synthesis, metabolic DNA formation, and metabolic DNA renewal or repair [150, 151]. Figure 5 summarizes the series of above-mentioned experiments that led to the hypothesis of Pelc's metabolic DNA.

The plant studies of Pelc and Sampson were mainly limited to root tissues behind the meristem, tissue which have different metabolic properties, and might not represent physiological homogeneous tissue [152]. Studies by Hurst et al. [152] and Hurst and Gahan [153] demonstrated via colchicine treatment that the use of shoot tissues, particularly collenchyma, could offer distinguishable, functionally differentiated and non-dividing tissues to efficiently study the presence of metabolic DNA. ^{3}H-thymidine (3H-Tdr) treatment and autoradiography of *Lycopersicon esculentum* shoots showed that the label was freely and equally available throughout the cells and that the tissues were seemingly heterogeneous, due to the presence of three different groups of collenchyma nuclei (no, low, and high levels of 3H-Tdr incorporation) after the isotope pulse. Hurst et al.'s [152] experiments proposed the following: (1) lack of change in mitotic activity in collenchyma tissue indicated that premitotic DNA synthesis

did not occur during the experiments and was not responsible for the incorporation of label into genetic DNA; (2) gradual loss of label in the collenchyma indicated that polyploidy was also not involved unless genetic DNA degradation occurred after the tissues became senescent (the loss of label would, however, not be gradual if that was the case); (3) if the observed labelling occurred due to the repair of a portion or portions of the DNA, it would indicate that the "repaired proportions" of the DNA were degraded again; and (4) if there was no indication of total DNA variation or increased strand breakage, metabolic DNA synthesis (and gradual turnover after acting as a transcriptional unit within these differentiated tissues) would be strongly indicated. The biological half-life of any metabolic DNA fractions in the shoot tissue was determined to be approximately 6 weeks, which is considerably longer than that of *V. faba* roots (24 h) [146]. These differences were proposed due to species differences or differences in the functions and metabolic requirements of these tissues.

Macronuclei and metabolic DNA

Studies regarding the nuclear behaviour of various protozoa and metazoa occurred since the late 1800s. These organisms were found to have two different nuclear structures, micronuclei and macronuclei [154]. The micronuclei consist of the genetic DNA of the organism involved in inheritance and cell division, and remain otherwise inactive. Macronuclei consist of metabolic DNA formed by multiple replications of the genetic DNA and by self-replication, and are responsible for transcribing RNA required for the metabolic functions of the cell [154]. In 1970, Pelc described connections between the macronuclear DNA of ciliated protozoa [143, 144, 154] and metabolic DNA [145] (refer to Fig. 4): (1) both macronuclear and metabolic DNA are observed as separate forms of DNA from their nuclear or genetic counterparts (the genetic DNA from micronuclei and stable, high molecular weight DNA, respectively); (2) both have been described to consist of multiple copies of either the genetic DNA or parts thereof; (3) both have been theorized to be involved in RNA production required for the metabolic functions of cells; (4) for micronuclei and macronuclei, DNA synthesis occurs at two different time intervals before vegetative DNA division can occur. The micronuclei divide mitotically and the macronuclei divide via amitosis [143–145, 155]. Metabolic DNA is formed before/immediately after the last mitosis or during/immediately after premitotic synthesis, depending on the organ; (5) when micronuclei undergo meiotic and post-meiotic divisions, the macronuclei are broken down and resynthesized. The destruction and resynthesis of the macronuclei result in the DNA of the macronuclei being similar to the genetic DNA of the micronuclei, which could have changed during division. A similar form of periodic renewal has been proposed for metabolic DNA; and (6) both have the potential to result in the release of DNA into the circulation. The destruction of macronuclei prior to their resynthesis could result in the release of nucleic acids into the extracellular space, and metabolic DNA has been proposed to represent the precursor to the formation of virtosomal DNA, which is released by cells into the circulation.

The similarities suggested that metabolic DNA could serve similar functions in higher organisms than that of the macronuclear DNA of protozoa and metazoa. The multiple copies of active genes of macronuclear and metabolic DNA may serve as a form of gene amplification, resulting in a higher rate of RNA production during the periods of high demand [151]. It has also been theorized that protozoa and metazoa have two separate forms of nuclei to reduce and/or prevent the alteration of DNA during transcription by keeping the master code separate from its metabolic counterparts. DNA damage, for example, can lead to the misreading or the presence of unsuitable molecules during transcription. Permanent cell damage may, therefore, occur if the gene copies of genetic DNA were directly involved in transcription. Multiple replaceable gene copies, on the other hand, are substituted through resynthesis if damaged. Thus, reducing the transcriptional involvement of the genetic DNA, by providing separate gene copies for these tasks in the form of macronuclear DNA, reduces the risk for accumulating genetic DNA damage or changes. To date, it has become evident that there are extremely high levels of regulation involved during transcription to prevent errors from occurring and whether metabolic DNA forms part of these regulation mechanisms is uncertain. This protection theory of macronuclei, however, does not account for the involvement of environmental factors in DNA damage, as the extra copies of DNA in separate nuclei would not be able to protect "dormant" genetic DNA from external factors.

The characterization of circulating DNA

Stroun et al. [156] were the first to characterize plasma DNA. CirNAs are detectable in various other fluids, including in serum, liquor, sputum, ascites, gastric juices, urine, bone marrow aspirates, stool samples, milk, lymphatic fluids, peritoneal fluids, cerebrospinal fluid, bronchial lavage, prostatic fluid, and biliary juices [3, 157, 158]. The generation of cirDNA, its accumulation in tissues, and elimination from the organism influences the concentration of DNA in circulating blood. Factors that

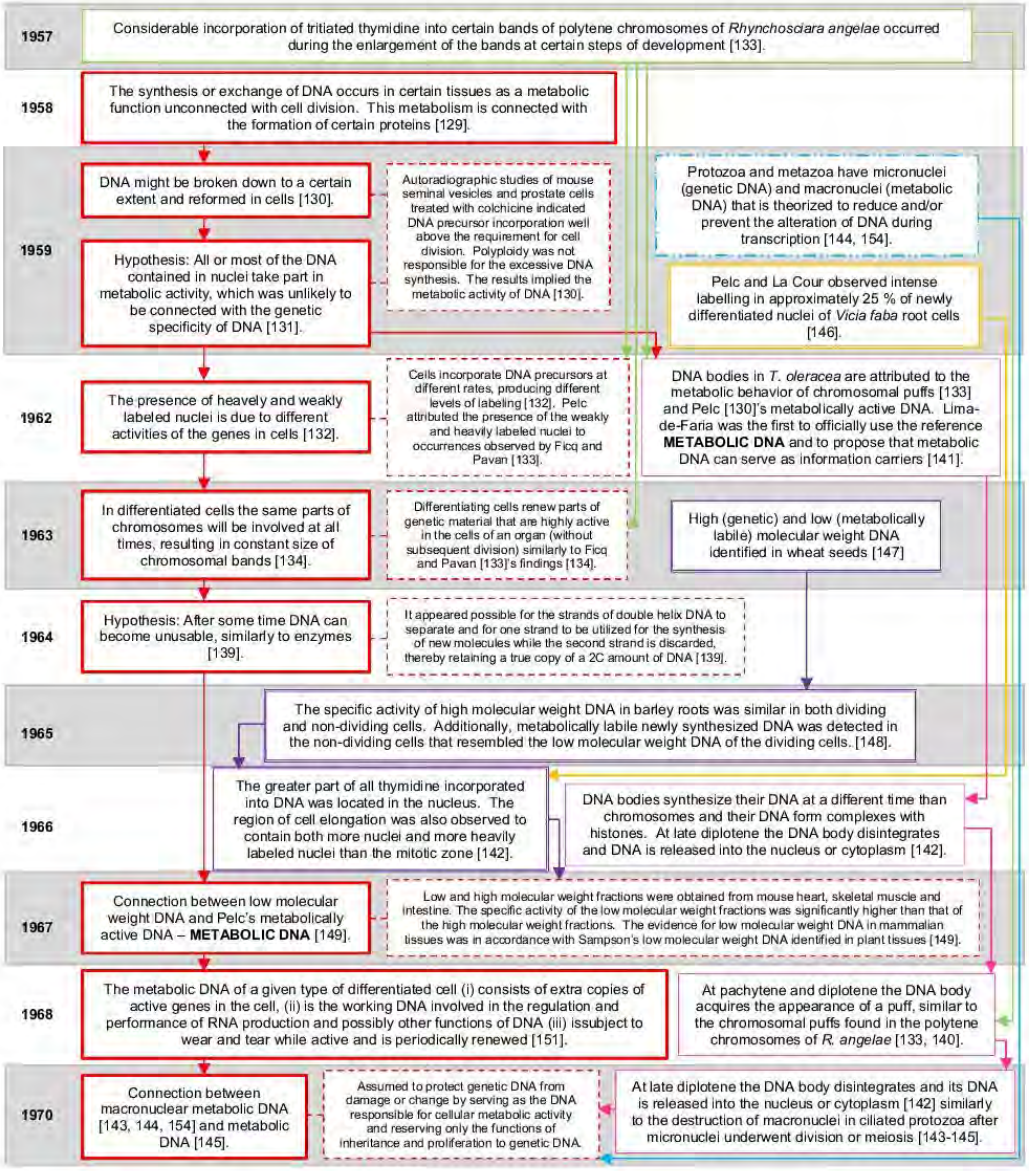


Fig. 5 Series of studies that influenced Pelc's research on metabolically active DNA and the development of Pelc's hypothesis on metabolic DNA

affect the circulation of cirDNA in the blood can include the activity of DNA-hydrolyzing enzymes in blood and tissues, the DNA's structure, and DNA–protein interactions in the blood [159]. CirDNA can be double stranded and single stranded [6, 7, 112, 156, 160, 161], is of low molecular weight, and is resistant to RNase and proteinase K [156], but can be hydrolyzed by DNase I, and is cleared predominantly by the liver and, to a lesser extent, the

kidneys [162, 163]. CirDNA has also been found to have a high background of overall methylation in healthy people, which can increase the stability of cirDNA, allowing it to circulate in the blood stream for a longer time [159]. CirDNA can be released by both dividing and non-dividing, differentiated cell populations [5]. Their fragment sizes typically range from 100 bp to 21 kbp [156, 164, 165], where the short fragments can be related to the histone octamer structure and apoptosis, while the longer fractions originate from necrotic cells [165]. A high proportion of the cirDNA originating from tumors, however, are more fragmented than that of healthy tissues, releasing DNA fragments smaller than 100 bp [6, 166]. Recently, we have reported a size of 2000 bp for the cfDNA of osteosarcoma cell cultures [167]. CfDNA samples-containing only these fragments showed no flow cytometric detection of apoptosis and necrosis, indicating that the 2000 bp cfDNA may be a product of active release.

Extracellular vesicles and mobile protein complexes

CirDNA is predominantly conveyed throughout the body in extracellular vesicles (EVs). These vesicles are generally categorized as exosomes, microvesicles (MVs), and apoptotic bodies (ABs). All of the formers have not yet been rigorously characterized, and, therefore, still remain poorly understood in terms of function [168–171]. EVs are categorized mainly on the grounds of their size and origin. Exosomes are classified as being 40–100 nm in diameter and are released due to fusion of multivesicular endosomes with the plasma membrane [172]. MVs, on the other hand, range between 100 and 1000 nm, whereas ABs can be as big as 4000–5000 nm. MVs and ABs, unlike exosomes, are shed from the plasma membrane [173]. There is, however, some confusion in the literature regarding nomenclature. This stems from three independent factors, namely: (1) most purification regimes cannot completely differentiate between the different EVs; (2) cells release a heterogeneous population of EVs [174–176], and (3) there is an overlap in the category of size. For instance, prostasomes (also classified as exosomes) have been documented to range between 40 and 490 nm [177], and some microvesicles have the same sizes as exosomes [178]. In the previous work, we have demonstrated that centrifugation time and speed influence the yield of cfDNA isolated from cell culture media [179]. These factors collectively contribute to the difficulty to reach a consensus on structural definitions. Except for EVs, CirDNA can be part of protein complexes or attach themselves to binding proteins or phospholipids on outer cell surfaces for absorption or release purposes [15, 167, 180–182]. CirDNA can also be in the form of mitochondrial DNA and nucleosomes as a result of apoptotic processes [182, 183]. In this paper, we will only focus on exosomes and protein complexes.

Exosomes

Exosomes are intraluminal vesicles secreted by cells consequential to the intracellular fusion of multivesicular bodies with the plasma membrane [158]. Although exosomes were discovered more than three decades ago, they were not given much attention due to a preconception that they were mere cellular waste disposal systems [184]. This view has changed once proteomic analysis showed that exosomes contain several proteins that are implicated in intracellular membrane transport, cell signalling, and apoptosis [185]. In the pursuing years, it has become apparent that exosomes act as haulers that convey an assemblage of cell-specific proteins and genetic material to other cells [184]. They have been shown to contain only RNA [186], only DNA [187], or sometimes both [188]. Interestingly, cancer tissue-derived exosomes have been shown to contain genomic DNA spanning all chromosomes. This information can be used to determine genomic DNA mutations that will be of diagnostic, prognostic, and theranostic value [189–192]. In any case, the selective packaging of specific nucleic acids into tailor made vesicles indicates a specialized function. Indeed, many publications have associated them with the messenger functions of various natures, such as the induction of tolerance against antigens, eradication of established tumors in mice, promotion of differentiation into T-regulatory cells, and adaptive recruitment against oxidative stress [25]. Furthermore, the composition and function of exosomes depend on the characteristics of the cells from which they originate and the conditions under which they are produced (toxic, oxidative stress, hyperglycaemia, for example) [24].

Ronquist et al. [177] discovered prostasomes in human prostatic fluid and seminal plasma. These vesicles were equivalent to exosomes from other cell types in that they were produced and secreted in the same manner [193]. The name "prostasome" exemplifies the cell type from which it originates (namely prostate cells), whereas "exosome" denotes the family name [194]. Further studies have shown that prostasomes can interact with spermatozoa [35]. This finding was met with greater enthusiasm when Ronquist et al. [36] discovered that prostasomes contain small fragments of DNA that seem to represent random parts of the entire genome. Two years later, Ronquist et al. [33] went on to demonstrate that these fragments of DNA could be taken up by sperm cells. Although, in the same year, Ronquist synthesized many examples of the intercellular messaging functions of prostasomes [195], he previously

stated his uncertainty regarding the extent of its biological functions [36]. In 2011, Ronquist stated that, at that point in time, there were no reports that indicated the presence of RNA in prostasomes [195]. Later that year, however, Hessvik et al. [196] demonstrated that exosomes derived from both non-cancerous (RWPE-1) and cancerous (PC-3) prostate cell lines did, in fact, contain miRNAs. Very interestingly, they found that, although the miRNA profiles of exosomes from both cell lines were very similar to that of their corresponding parental cells, there was a moderate amount of sorting involved. For instance, the PC-3-derived exosomes contained much less low number miRNAs than their parental cells. Moreover, although also with very similar profiles, some miRNAs were differentialy expressed between the two different cell lines. This selective sorting of miRNAs is surely a salient feature, but we will not elaborate on it in this review. However, here, we can speculate that prostasomes may have the ability to convey adaptive genetic and epigenetic changes, which the soma has undergone, to the sperm cells. The packaging of nucleic acids in EVs likely promotes its uptake into cells, and protects it from nuclease and nucleic acid-binding proteins present in the seminal vesicles, which is believed to facilitate the direct uptake of nucleic acids by sperm cells [197].

Recent findings of Cosetti et al. [34] confirm that exosomes can be involved in soma-to-germline information transfer, showing that human melanoma cells xenografted into mice released RNA which was transported through the bloodstream via exosomes and eventually into spermatozoa, implicating exosomes, carriers of cirDNA, in the heredity of acquired changes. This possibility is also demonstrated by another landmark study, in which it was demonstrated that exosomes can traverse physical barriers and affect the biology of another cell [197, 198].

Mobile protein complexes

So far, the predominant view is that cirDNA is only transported throughout the body via EVs. There is, however, substantial evidence that protein complexes can do the same. Indeed, it has been reported that 90 % of circulating miRNAs are not associated with EVs. Here, we will briefly discuss three types of protein complexes that have been described in the literature, namely virtosomes, high-density lipoprotein (HDL), and argonaute2 (Ago2). Virtosomes were the first protein nucleocomplexes to be described ("The concept of transcession"). Virtosomes have been demonstrated to be composed of both DNA and RNA. They have also been shown to be actively released by all living cells studied to date, but not dead or dying cells, and have the ability to enter recipient cells, followed by incorporation of the DNA into the genome. The DNA content has been demonstrated to hybridize with nuclear DNA, and thus bears great similarity to cellular DNA. The type of RNA present has not yet been determined, but it has similar effects on recipient cells than that of siRNA or miRNA [15]. Liposomes, containing apolipoprotein A-I, have many times been used as an artificial vehicle to deliver small interfering RNAs to target animal cells. Interestingly, apoA-I is the main constituent of HDL. Based on this fact, and, keeping in mind, the knowledge that HDL interacts with nucleic acids, Vickers et al. [199] hypothesized that HDL could be associated with endogenous miRNAs. Not only did they demonstrate this, but they have also shown that HDL can deliver miRNAs to target cells with gene regulatory consequences. HDL could evidently play a role in intercellular messaging. Ago2 is commonly known as the key effector protein of gene silencing mediated by miRNA. Arroyo et al. [200] then discovered Ago2 in human plasma, but have also demonstrated that it is associated with miRNA. They have also found that miRNA is much more stable when associated with Ago2. After demonstrating that the liver-specific miRNA miR-122 was released only in the form of lipoprotein complexes, Arroyo et al. hypothesized that Ago2-miRNA complexes reflected the biology of the cells from which they originated. This suggests that cells purposely secrete functional miRNA gene silencing complexes into circulation, possibly to convey intercellular messages.

Discussion and conclusion

Natural selection and IAC are the two principle schemes devised to explain evolution. After decades of scrutiny, the majority of scientists subscribe to the idea that evolutionary change occurs through the guidance of selective processes, and not IAC. However, Darwin himself believed in IAC and formulated the hypothesis of Pangenesis to account for it. This does not mean that he disregarded random variations, indeed, he considered IAC to be an auxiliary mechanism to natural selection [201]. In Darwin's era, however, there was a lack of interest to elucidate the mechanisms implied by Pangenesis and IAC, which guaranteed the position of IAC and Pangenesis in the garbage pail of history. However, in this review, we present ample evidence that acquired characteristics can, indeed, be inherited, and also show that this process seems to be governed by rules that undermine the basic assumption that variation is random [202]. Thus, it is has become clear that, although their ideas seem naive today, Darwin and Lamarck were not entirely wrong. In the present-day genetics terms, IAC and Pangenesis imply that an environmental factor can induce genetic and epigenetic changes

in targeted sites, which, in turn, allows adaptation to the original factor of invocation, and that this new information can be transferred to neighboring cells, germ cells, and the next generation of offspring. Contemporary molecular genetics shows us that this happens quite often, and many different mechanisms are involved.

First, it has generally been assumed that all the somatic cells of a higher organism contain an exact replica of the entire genetic code, and that it is subject to change only by virtue of random mutations due to replication errors and inevitable damage to the genome. In contrast, there is a great deal of evidence, indicating that the genome is continuously formatted by directed rearrangements and inscriptions (for a comprehensive review, refer to [203]). This is corroborated by Astolfi et al. [204] who presented substantial experimental and theoretical evidence that the cells of higher organisms are a mosaic of genetic variants. This is owed to the fact that individual organs, tissues, and cells are vulnerable to environmental stress under the localized conditions, allowing them to generate genetic diversity. One of the best examples we could elaborate on is gene duplication, which is well known to be a major source of genetic variation for normal non-cancerous cells. In 1978, Biedler and Spengler found that cultured mammalian cells implemented selective gene duplication as a mechanism for acquiring resistance to methotrexate (MTX) [205, 206]. Subsequent studies then established that gene amplification could be utilized as a mechanism for resistance to other toxic chemicals as well. Since then, gene duplication has been found to be a rather common phenomenon under selective pressure [207]. Interestingly, quite rapidly after gene duplication, the copy starts to gradually accumulate mutations, causing the parent and duplicate genes to diverge. This divergence may result in (1) non-functionalization, wherein either one of the copies are silenced; (2) neofunctionalization, wherein one copy develops a novel function, but the other remains normal, and (3) subfunctionalization where both copies obtain complementary functions that, together, perform the original function. It appears to be a general rule that gene expression is modified, and relatively quickly, after gene duplication. Indeed, results presented by Aldana et al. [208], indicate that duplication and diversification of a single gene could result in the emergence of novel phenotypes and, subsequently, the reconfiguration of gene expression pathways (refer to [208] for a more detailed description).

The benefit of genetic variation is exemplified by numerous studies showing that prokaryotes borrow genetic ideas from one another, allowing adaptation to toxic substances. Similarly, the implication of somatic genome variation in higher organisms is that, although it may, in some cases, compromise normal cellular functions (as seen in cancer metastasis, for example), it creates new coding and regulatory sequences. This may offer many new capabilities to pre-existing frameworks. In a comprehensive synthesis, Koonin and Wolf [46] showed that HGT is a major component of Lamarckian evolution. Ever since comparative genomics has revealed the ubiquity of HGT in prokaryotes and unicellular eukaryotes, the phenomenon has been avidly documented. However, the evidence indicating that lateral gene transfer occurs between different cells within one organism, including humans, has not been given adequate attention and is still very poorly characterized. Similar to prokaryotes, is it conceivable that the genetic variants created in specific somatic cells in higher organisms are receptive to intraorganismal Darwinian selection? It is likely that this is a common phenomenon, but has been occurring mostly unnoticed. This could be due to the difficulty to trace adaptive responses in non-pathogenic events. We propose that these genetic variants are shared between cells by the transference and transfection-like uptake of cirDNA in the form of EVs and protein complexes. However, the elucidation of this phenomenon is thus far complicated primarily by a lack of understanding, regarding the origin and biological functions of cirDNA. We argued that many, and, perhaps, some crucial, answers to these questions are well hidden in the literature of the past. For this reason, we reconstructed the historical path that led to the discovery and concepts of cirDNA and EVs. From this, we learned that the discovery of cirDNA and EVs can be traced backed to the ancient Greek philosophers, and it is clear that it is tightly interwoven with some of the most prominent discoveries in heredity, genetics, and evolution. Today, there is a lot of evidence that cirDNA deliberately, genetically, and epigenetically alters the biology of recipient cells and that this is strongly implicated in the progression of several diseases, immunomodulation, cell differentiation, adaptation, and transgenerational genetic and epigenetic inheritance.

We also presented evidence indicating that metabolic DNA may be the precursor to the bulk of cirDNA. With this in mind, the following three characteristics of metabolic DNA can provide important clues that can help elucidate the origin of cirDNA: (1) there are two types of DNA existing in the cells of higher organisms, namely the stable high molecular weight DNA and the low molecular weight fraction of DNA, which are renewed periodically rather than continuously (indicating exposure to wear and tear); (2) metabolic DNA synthesis occurs at different times than the stable genetic DNA (before or after mitosis); and (3) the content of metabolic DNA represents multiple copies of genes that correlate with a definite stage in development or with the stimulation of an activity, indicating a definite correlation of metabolic DNA with cellular function. Metabolic DNA is, therefore, considered

as specially synthesized DNA that serve as the working DNA involved in the regulation and performance of RNA production and other DNA functions. In other words, the occurrence of cirDNA is not just the result of cell lysis and apoptosis that can affect neighboring cells, as many tend to believe, but is newly synthesized and complexed with lipoproteins or packaged in vesicles specifically to serve as messengers. Furthermore, the tailor made EVs and protein complexes that facilitate the transfer of cirDNA between cells are also capable of transporting cirDNA to sperm cells. This is very similar to the idea that Darwin had in mind when he suggested the existence of gemmules. CirDNA may thus have a special role in adaptation, transgenerational inheritance, and by implication, evolution.

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The diverse origins of circulating DNA in the human body: Critical re-evaluation of the literature

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The diverse origins of circulating DNA in the human body: Critical re-evaluation of the literature

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Abstract

Since the detection of circulating DNA (cirDNA) in human plasma in 1948, the use of this DNA as a new non-invasive screening tool has been studied for many diseases, such as solid tumours and fetal genetic abnormalities and/or traits. However, to date our lack of knowledge regarding the source and purpose of cirDNA in a physiological environment has limited its use to more obvious diagnostics, neglecting the potential utility of cirDNA in the identification of predispositions to diseases and the earlier detection of cancers and epigenetic changes due to lifestyles. The concept or mechanism of cirDNA can also have potential therapeutic uses such as immuno- or gene therapy. This review provides a comprehensive compilation of putative origins of cirDNA and then contrasts the contributions of cellular breakdown processes and active mechanisms to the release of cirDNA into the extracellular environment. The involvement of cirDNA derived from both cellular breakdown and active release in lateral information transfer is also discussed. With this we hope to encourage researchers to adopt a more holistic view of cirDNA research, taking into consideration all of the biological pathways in which cirDNA is involved, and also consider the integration of *in vitro* and *in vivo* research. We also wish to encourage researchers to no longer limit their focus to the apoptotic or necrotic fraction of cirDNA, but to take advantage of the intercellular messaging capabilities of the actively released fraction of cirDNA to investigate role of cirDNA in, for example, pathogenesis.

Keywords: active DNA release; cellular breakdown mechanisms; circulating mitochondrial DNA; extracellular vesicles; lateral information transfer

1. Introduction

The presence of cell-free circulating nucleic acids (cirNAs) in human blood was first reported by Mandel and Métais (1948). The number of studies regarding cirNAs increased since this discovery, which led to the detection of correlations between circulating DNA (cirDNA) levels and pathological conditions, including cancer and diseases that cause endogenous tissue destruction (Leon *et al.*, 1977; Tan *et al.*, 1966). To date the use of cirDNA in the diagnosis of cancer and other diseases are studied extensively, but the origin of cirDNA remains under debate due to our lack of knowledge regarding the purpose of cirDNA in cellular and systemic functions. There are various forms of biological features that can not only directly contribute to the final cirDNA concentration, but can also interact with one another to form a further cascade of effects that result in cirDNA release. These features have been discussed and reviewed in several publications (Bryzgunova & Laktionov, 2014; Gahan, 2012; Lichtenstein *et al.*, 2001; Stroun *et al.*, 2001; Thierry *et al.*, 2016; Ulivi & Silvestrini, 2013), many with different conclusions regarding which feature serves as the main origin of cirDNA. Two controversial contenders for main origins are cellular breakdown mechanisms (consisting of necrosis, apoptosis, pyroptosis, autophagy and mitotic catastrophe) and active DNA release mechanisms (which also includes the vesicular transport of nucleic acids). This continuous "controversial" argument was originally thought to be due to their generalised involvement in the release of DNA into circulation from most, if not all, of the biological features (e.g. cancer, exercise, aging, inflammatory and immune reactions). However, a seemingly unintended lack of consensus regarding the very definition of the term "main origin" became a more likely explanation, as the brunt of the "controversy" is based on whether the main origin refers to the fraction of cirDNA that is the most abundant or the most likely to have biological function. In this review a comprehensive compilation of putative biological features that can contribute to cirDNA release is provided. Furthermore, a categorisation of these putative features as (1) from living or dead cells and (2) as either sources, causes or a combination thereof is introduced and a schematic illustration of the interactions between these features is provided to not only demonstrate the cascade of effects induced once these features interact with one another, but to also illustrate the generalised involvement of cellular breakdown mechanisms and active DNA release in the release of DNA into the extracellular environment.

With this we hope to achieve two main goals, the first of which is to clarify the lack of consensus regarding the meaning of the term "main origin of cirDNA" and using the resulting argument to propose a more specific means of classifying cirDNA fractions, namely most abundant versus most functional. With this classification, along with our more in-depth evaluation of the source and cause of cirDNA release, we argue in favour of utilising the most functional fraction of cirDNA for further cirDNA research endeavours. Secondly, we wish to emphasise the introduction of in vitro models in cirDNA research. By using this review's presentation of the myriad of relationships and interactions that can occur between the different putative sources and causes of cirDNA, we wish to disseminate the idea of utilising the "closed-circuit" models that in vitro methods can produce to restrict the potential sources and causes of cirDNA release to only that of the site, tissue or physiological system in question. By using these models in conjunction with *in vivo* sampling, future research can strongly contribute not only to the discovery of novel biomarkers for diagnostics and prognostics, but to the discovery of other novel therapeutic uses and the elucidation of the true physiological purpose of cirDNA.

2. The categorisation of circDNA origins: Sources versus causes, and living versus dead cells

Putative biological features that can produce or result in the release of cirDNA can be divided into three categories, namely (1) sources of cirDNA, (2) causes of cirDNA release and (3) a combination of both source and cause. Fig. 1 and Table 1 provides a comprehensive summary of putative biological features and the various mechanisms involved in the release of cirDNA from each feature. By separately categorising the different mechanisms involved in each biological feature, it becomes easier to see where the true origin of each feature's cirDNA lies and how these features can change, when interacting with one another, from sources of cirDNA to causes of cirDNA release and *vice versa*. Fig. 2 schematically illustrates this interaction between the putative sources and causes of cirDNA that further contribute to cirDNA release, emphasising the complexity of cirDNA contents that so clearly complicates the elucidation of the tissue origins and biological function of cirDNA and the discovery of novel, disease- or physiology-specific biomarkers. It also becomes clear from Table 1 and Fig. 2 that most, if not all, of these features have one thing in common, they require cellular breakdown and/or active DNA release mechanisms in order to release DNA. Table 1 also indicates whether cells may be alive or dead when contributing to cirDNA release, an important factor to keep in mind as this is the one key difference between cirDNA released from cellular breakdown mechanisms (damaged and/or dying cells) and active DNA release (only from living cells). For the purpose of this paper the term cellular breakdown mechanisms is used as a collective term for necrosis, apoptosis, pyroptosis, autophagy and mitotic catastrophe. These processes share common mechanisms to release cirDNA from damaged or dead cells, including the use and avoidance of phagocytosis and lysosomal degradation. Fig. 3 schematically illustrates the process of cirDNA release for each process and their relationships. The following sections discusses the different putative sources of cirDNA, causes of cirDNA release and the combinations thereof as listed in Table 1, Fig. 1 and Fig. 2.

3. Sources of circulating DNA

(1) Exogenous sources

Foreign nucleic acids contained in the exogenous sources that enter the body may be released during immune defences or metabolic digestion and enter the bloodstream. Various water sources, the soil in which fruit and vegetables are grown, the sediment and water of rivers and oceans to which aquatic life are exposed contain various sources of DNA from fungi, viruses, bacteria and decomposed organisms (Nielsen *et al.*, 2007). Exposure of the body to inhaled DNA (e.g. pollen in the air) and ingested DNA (daily intake of raw, unprocessed and processed food) can also serve as sources of cirDNA (Spisák *et al.*, 2013).

Studies regarding the ingestion of genetically modified organisms by pigs, goats, mice and rainbow trout, have demonstrated that small fragments of nucleic acids may pass to the bloodstream and reach various tissues (reviewed in (Rizzi *et al.*, 2012; Spisák *et al.*, 2013). Through screening all publically available cirDNA sequencing data of over 1 000 human subjects at the time, Spisák *et al.* (2013) also determined that, in humans, meal-derived DNA fragments large enough to be able to carry complete genes can avoid complete degradation and pass from the digestive tract to the circulation. Only 71.1 % of the sequence reads could be mapped to the human reference genome. Of the remaining 28.9

%, over 25 000 sequence reads aligned to plant chloroplasts, particularly potato (*Solanum tuberosum*) and/or the closely related tomato (*Solanum lycopersicum*). Further hints of the presence of DNA from other food related species were detected, but the samples sizes were not large enough for convincing results. Interestingly, plant chloroplast reads were not detected in umbilical cord blood, despite being present in the maternal blood.

Bacterial and viral DNA could contribute to cirDNA levels of patients with certain diseases or infection-induced cancers (Van der Vaart & Pretorius, 2008a), including nasopharyngeal carcinoma and Hodgkin's disease due to the Epstein-Barr virus (Tong & Lo, 2006), cervical cancer or head and neck squamous cell carcinoma due to the human papilloma virus (Shimada *et al.*, 2010; Tong & Lo, 2006) and hepatocellular carcinoma due to the hepatitis B virus (Shimada *et al.*, 2010). Infection-induced pyroptosis and the autophagic clearance of these exogenous organisms can cause the release of exogenous DNA into circulation, which in turn can result in even further damage. Bacterial and viral DNA can serve as an endogenous molecule or danger-associated molecular pattern (DAMP) that can trigger innate immunity when released. The DNA contains inflammatogenic unmethylated CpG motifs and can bind to Toll-like receptor TLR9 once released from dead or dying cells, resulting in a potent inflammatory reaction (Bliksøen *et al.*, 2016; McIlroy *et al.*, 2015; Nasi *et al.*, 2016) (discussed in Section 5(3)(a)). In parasitic diseases, there is a large level of turnover of parasites involving replication, maturation and death of organisms and multicellular parasites, e.g. the flatworm *Schistosoma*, can contain DNA copies in stoichiometrical excess over parasite count (Wichmann *et al.*, 2009). DNA of parasites can, therefore, also be present at high levels in the circulation of patients, e.g. with bilharzia. Circulating DNA fragments of endoparasites (e.g. schistosomes and trypanosomes) and parasitic bacteria (e.g. *Wolbachia*) can transfer genetic information to the host via horizontal gene transfer (HGT) (Schaack, Gilbert & Feschotte, 2010) and even become heritable traits (Hotopp *et al.*, 2007). Nearly the entire *Wolbachia* genome was transferred to the fly nuclear genome and several other insect species have been shown to contain *Wolbachia*-related genes in their genomes (Hotopp *et al.*, 2007). Hou *et al.* (2014) demonstrated HGT of a gene from *Wolbachia* in the C6/36 cell line derived from *Aedes albopictus* (mosquito). This gene (WP0273 (C6/36)) was found to display high transcription levels and the results proposed that the gene is functional and has been expressed and translated into protein in the host cells. Similar findings were also reported by Klasson *et al.* (2009), who discovered the transfer of the genes AAEL004181/8 from *Wolbachia* to *Aedes aegypti* and *mascarensis.*

Organ transplantations and blood transfusions are also examples of exogenous sources of cirDNA and are commonly used to monitor complications or graft rejection (Lo *et al.*, 1998b; Lui *et al.*, 2002; Tong & Lo, 2006). In cases of complications or rejection the transplanted tissues, which normally serve as sources of exogenous DNA, causes cellular degradation that results in the release of DNA into circulation. However, donor-specific DNA can also be found in the bloodstream of transplant patients in the absence of any transplant-related complications and multiple studies have taken advantage of this concept to elucidate the origins of cirDNA under normal physiological conditions (refer to Section 5(2)) and during exercise (refer to Section 4(8)). Burnham *et al.* (2016) also discovered the presence of donor-derived mitochondrial DNA (mtDNA) in the plasma of lung transplant patients and it is theorised that the release of mtDNA accompanying graft injury promotes harmful immunologic responses observed in solid-organ transplantation (discussed in Section 5(3)(a)).

(2) Necrosis

Necrosis (also known as oncosis) occurs as accidental cell death (Bryzgunova & Laktionov, 2014; Viorritto, Nikoloy & Siegel, 2007) during which the cellular potential for energy production and regeneration is lost, leading to the breakdown of ATP-dependent functions (Holdenrieder & Stieber, 2009). This results in osmotic water influx, cellular and organelle swelling, blebbing, increased plasma membrane permeability and disintegration due to the disruption of the ionic pumps of the plasma membrane, liberation of cellular content, nuclear chromatin clumping and non-specific chromatin digestion (Bryzgunova & Laktionov, 2014; Holdenrieder & Stieber, 2009; Majno & Joris, 1995; Weerasinghe & Buja, 2012) (Fig. 3). The rapid loss of membrane integrity during necrosis can also stimulate a host inflammatory response. Necrosis occurs more rapidly than apoptosis, removal of necrotic cells is slower and the resulting DNA fragments released from necrotic cells (~10 000 bp) are larger than most of the apoptotic DNA ladder fragments (with multiples of ~140 – 200 bp) (Bortner, Oldenburg, & Cidlowski, 1995; Suzuki *et al.*, 2008; Van der Vaart & Pretorius, 2007; Viorritto *et al.*, 2007). *In vitro* studies by Choi, Reich and Pisetsky. (2005) have shown that necrosis does not spontaneously release DNA. Clearance of necrotic cells via macrophages is required to release DNA into culture medium and the co-culture of macrophages with necrotic cells leads to the release of DNA into growth medium in a dose-dependent manner.

Necrosis can occur with apoptosis and dominates the end stages of irreversible injury. Incidences of necrosis have been detected in cell death via radiation exposure, chemotherapy, environmental toxins, atherosclerotic plaques, ischemic heart disease, stroke and killing of macrophages by viral and bacterial infections (Weerasinghe & Buja, 2012). The presence of large DNA fragments has been demonstrated in plasma samples during multiple cancer studies, hinting towards necrosis as an origin for cirDNA (Bronkhorst *et al.*, 2016b; Deligezer *et al.*, 2008). However, the concept of necrosis as a primary source for cirDNA was strongly contradicted in radiation therapy studies where cirDNA levels decreased in 66 – 90 % of lymphoma, lung, ovary, uterus and cervical tumour patients and in 16 – 33 % of glioma, breast, colon and rectal tumour patients (Leon *et al.*, 1977), instead of a surge in cirDNA release that one would expect from dead and dying irradiated cells. This decrease in necrotic DNA, on the other hand, may be due to radiation therapy causing the inhibition of tumour cell proliferation rather than killing the cells (Deligezer *et al.*, 2008). Though necrosis is normally regarded as accidental cell death, there are several forms of regulated and programmed necrosis, e.g. necroptosis and pyronecrosis. Necroptosis refers to programmed and regulated necrosis mediated through a receptor-interacting protein kinase (RIP)1-RIP3 complex-dependent pathway (Degterev *et al.*, 2005; Yang *et al.*, 2015). It can be induced by a class of death receptors that includes tumour necrosis factor TNFR and Fas. Necroptosis can also induce inflammation (discussed in (Yang *et al.*, 2015)). The cell death mechanism of necroptosis is unclear, but shares similar subcellular events with necrosis, including oxidative burst, mitochondrial membrane hyperpolarisation, lysosomal membrane and plasma permeabilisation. These subcellular events may, however, not occur via the same mechanisms as that of necrosis. The involvement of necroptosis has been identified in several pathological or cell death conditions, including ischemic brain injury, myocardial infarction and chemotherapy-induced cell death (Hitomi *et al.*, 2008). The immune and nervous system may be particularly susceptible to necroptosis as these areas have enriched expression of necroptosis-related genes. Pyronecrosis is also a necrosis-like cell death process independent of caspase 1 and caspase 11, but dependent on ASC and lysosomal protein cathepsin B and results in the secretion of proinflammatory mediator

HMGB1. It is induced by pathogens (e.g. *Neisseria gonorrhoeae*, *Toxoplasma gondii*, *Bacillus antrhacis lethal toxin* and *Staphyolococcus aureus*) (Yang *et al.*, 2015).

(3) Apoptosis

Studies have proposed that apoptosis serves as the source of cirDNA in both normal and diseased tissues (Delgado *et al.*, 2013; Jahr *et al.*, 2001; Sai *et al.*, 2007). A fundamental apoptotic process possibly involved in the release of cirDNA is internucleosomal chromatin cleavage via caspase-activated DNase (CAD). CAD is found in proliferating cells as part of a complex with ICAD, an inhibitor of CAD that serves as a chaperone during CAD synthesis (Nagata, 2000). The induction of apoptosis results in the dissociation of the CAD-ICAD complex by caspase (particularly caspase 3), allowing CAD to cleave chromosomal DNA into large fragments, followed by further cleavage into multiples of 160 – 180 bp nucleosomal fragments (Beck *et al.*, 2009; Holdenrieder & Stieber, 2009; Jahr *et al.*, 2001; Nagata, 2000; Stroun *et al.*, 2001; Suzuki *et al.*, 2008; Van der Vaart & Pretorius, 2008a) (Fig. 3). These nucleosomal fragments are packaged with other cell fragments into vesicles (apoptotic bodies (ABs)) to accommodate the efficient clearance of cell debris via the engulfment of ABs by phagocytes and other nearby cells, which in turn may generate moieties that incite or amplify immune reactions (Aarthy *et al.*, 2015; Savill, 1997; Ziegler, Zangemeister-Wittke & Stahel, 2002).

Internucleosomal chromatin cleavage via apoptosis forms a ladder pattern similar to the pattern visible after electrophoresis and sequencing reactions of cirDNA, indicating to many researchers that apoptosis may be the main source of cirDNA (Anker *et al.*, 1999; Beck *et al.*, 2009; Bicknell & Cohen, 1995; Fournié *et al.*, 1995; González-Masiá, García-Olmo & García-Olmo, 2013; Jahr *et al.*, 2001; Pinzani *et al.*, 2010; Stroun *et al.*, 2001; Van der Vaart & Pretorius, 2008b; Van der Vaart *et al.*, 2009). However, this characteristic ladder pattern is also found in the case of actively released DNA (Stroun *et al.*, 2001; Van der Vaart & Pretorius, 2007). Cells and whole organs in culture also spontaneously release nucleoprotein complexes in a homeostatic environment and newly synthesised DNA is preferentially released, indicating the presence of an active DNA release mechanism and arguing against apoptosis (Anker *et al.*, 1999; Anker, Stroun & Maurice, 1975; Stroun & Anker, 1972; Stroun *et al.*, 2001). Additionally, the presence of apoptotic cells is short-lived, even in tissues with high cellular turnover, due to highly efficient clearance mechanisms (Gahan, 2012; Hochreiter-Hufford & Ravichandran, 2013; Ravishankar & McGaha, 2013).

(a) How escaping phagocytosis may promote high circulating DNA levels

Phagocytosis of apoptotic cells is considered as a non-immunogenic and non-inflammatory process with minimal neutrophil recruitment (Bicknell & Cohen, 1995; Elliott & Ravichandran, 2010; Hochreiter-Hufford & Ravichandran, 2013; Savill, 1997; Viorritto *et al.*, 2007). Proper recognition, clearance and degradation of apoptotic cell material are needed to maintain a protective environment against uncontrolled inflammation and eventual autoimmunity. Apoptotic cells not removed via phagocytosis (due to impaired uptake by phagocytes or excessive cell death) leak cell material over time, resulting in inflammation, exposure to self-antigens and a break in tolerance (Hochreiter-Hufford & Ravichandran, 2013; Viorritto *et al.*, 2007).

If apoptosis is the main source of cirDNA, inflammation and autoimmune reactions would, therefore, be persistent symptoms in cancer and any other conditions with increased cirDNA levels (Van der Vaart & Pretorius, 2007; Viorritto *et al.*, 2007). Inflammation and autoimmune reactions are, however not persistent or frequently present symptoms in pregnant mothers (refer to Section 5(4)) or in subjects performing regular exercise (refer to Section 4(8)), indicating the unlikelihood of apoptosis being the main contributor of cirDNA. Moreover, researchers have reported that apoptosis and necrosis are not necessarily responsible for the occurrence of cirDNA due to the fact that the treatment of tumours do not increase cirDNA levels regardless of treatment-induced cell death (Anker *et al.*, 1999; Leon *et al.*, 1977; Pisetsky, 2012; Van der Vaart & Pretorius, 2007). Certain cancer cells can also resist apoptosis and continue to proliferate with a concomitant increase in cirDNA (Anker *et al.*, 1999; Anker *et al.*, 1975; Stroun *et al.*, 2001; Van der Vaart & Pretorius, 2008b). However, there are cases where chromatin degradation products escape further degradation into acid-soluble products and appear in the bloodstream and urine without eliciting inflammation and/or autoimmune reactions normally seen in phagocytic impairment (Beck *et al.*, 2009; Botezatu *et al.*, 2000). Defects in apoptotic cell clearance and enhanced cell death are also believed to play a role in many human pathologies where increased cirDNA levels are detectable, including autoimmune diseases (systemic lupus erythematosus and rheumatoid arthritis), pulmonary diseases (chronic obstructive pulmonary disease, cystic fibrosis and asthma where inflammation occurs possibly due to impaired phagocytic functions), cardiovascular diseases, atherosclerosis, sepsis and septic shock, neurological conditions (Alzheimer's disease, Huntington's disease and Parkinson's disease) and cancers (Hochreiter-Hufford & Ravichandran, 2013; Ravishankar & McGaha, 2013; Saukkonen *et al.*, 2008; Viorritto *et al.*, 2007), indicating that apoptosis may be a more likely source of cirDNA in certain diseases, depending on the disease pathophysiology.

(4) Pyroptosis

Pyroptosis refers to an inherently proinflammatory, caspase 1-dependent programmed cell death (Bergsbaken, Fink & Cookson, 2009) that results in the activation of inflammatory cytokines, interleukins IL-1β and IL-18, and rapid cell death characterised by plasma-membrane rupture and the release of proinflammatory intracellular contents (Fink & Cookson, 2005). The loss of mitochondrial integrity and release of cytochrome c, which activates apoptotic caspases, does not occur (reviewed in (Bergsbaken *et al.*, 2009)). This indicates that pyroptosis does not directly result in the release of mtDNA into circulation and that caspase 1 does not degrade ICAD to CAD to cleave DNA between nucleosomes as observed in apoptosis. Instead pyroptosis-mediated cell death will likely cause autophagic clearance of mitochondria and release of mtDNA (discussed in Section 4(2)) and DNA cleavage will result from the activity of a caspase 1-activated nuclease that does not produce oligonucleosomal DNA fragmentation patterns characteristic of apoptosis. Nuclear integrity is maintained and marked nuclear condensation does occur. Membrane rupture involves pore formation, resulting in water influx and an increase in cell size, but no swelling and lysis as it is prevented by cytoprotective glycine, which non-specifically blocks ion fluxes in damaged cells. Pyroptosis is stimulated primarily by a range of microbial infections (e.g. *Salmonella*, *Shigella*, *Francisella* and *Legionella*) (Bergsbaken *et al.*, 2009; Fink & Cookson, 2005). Caspase 1 is also involved in sepsis, organ failure and the pathogenesis of multiple diseases, including myocardial infarction, cerebral ischaemia, inflammatory bowel disease and neurodegenerative diseases. Pyroptosis is

also implicated in the cell death mechanisms of infections, ATP treatments, diseases and disorders (including gout and asbestosis) and ultraviolet radiation therapies (Bergsbaken *et al.*, 2009).

(5) Actively released DNA: Metabolic DNA and protein-DNA complexes

Ahlstrom, Euler and Hevesy (1944) and Stephen Pelc (1958) were the first to discover labelled DNA molecule formation not due to the synthesis of new cells, but due to the increase in DNA content of cells already present (reviewed in (Aucamp *et al.*, 2016)). In 1968 Pelc determined that these low molecular weight DNA molecules, dubbed metabolic DNA, existed along with stable, high molecular weight DNA in the cells of higher organisms and that metabolic DNA synthesis (1) occurs in both dividing and non-dividing, differentiated cells, (2) is independent of DNA synthesis and repair, (3) is renewed or repaired periodically instead of continuously and (4) is closely connected with the functional activity of differentiated cells (Appleton, Pelc & Tarbit, 1969; Pelc, 1968). Changes in metabolic DNA content, incorporation of precursors, or loss of labelled DNA also correlated with a definite stage in development or with the stimulation of activity, providing a definite correlation of metabolic DNA with function.

Later studies by Anker *et al.* (1975) and Rogers *et al.* (1972) reported a similar form of low molecular weight DNA. Human blood lymphocytes released double-stranded DNA *in vitro* with and without any stimulation. The released DNA was smaller than cellular DNA (with a molecular weight that ranged from 3.5×10^6 to 3.7×10^6 Daltons (5.384 – 5.692 kbp)) and did not seem to be due to dead or dying cells (Anker *et al.*, 1975; Gahan, Anker & Stroun, 2008). Later *in vitro* studies also showed that actively growing normal and malignant cells can shed nucleic acids into the culture medium, while dead cells cannot (Choi, Reich & Pisetsky, 2004; Fleischhacker & Schmidt, 2007; Rogers *et al.*, 1972). It may take longer than an hour to reach the maximum concentration of DNA released by cells into growth medium *in vitro* (termed cell-free DNA (cfDNA)) and this maximum concentration cannot be exceeded, regardless of incubation period and mechanical stress (e.g. centrifugation), suggesting an active regulatory mechanism independent of mechanical stress (Anker *et al.*, 1975). The cells that released DNA retained their functional integrity, fully maintaining their capacity to increase DNA synthesis after stimulation, and cell death rates did not affect the amount of cfDNA. The specific activity of the released DNA was different from that of the cellular DNA, depending on the time of labelling (lymphocyte cultures were subjected to two successive incubations of 16 and 4 hours, [^{3}H]TdR label was added to the growth medium during either the first or second incubation and the amount and specific activity of cellular and cfDNA were determined after the second incubation). Anker *et al.* (1975) also observed that the released DNA in the medium could not be destroyed by several DNases but were, however, sensitive to DNase degradation once purified. This indicated that the DNA could have been released as a complex (e.g. as a mononucleosome (Ziegler *et al.*, 2002)). Rogers *et al.* (1972) confirmed that released DNA from stimulated lymphocytes could be complexed with proteins or lipids and later studies demonstrated that oligonucleotides are partially protected from nucleases when they form complexes with proteins, such as serum albumin and IgG proteins (Laktionov *et al.*, 1999; Tamkovich, Vlassov & Laktionov, 2008). Proteins, such as high density lipoprotein (HDL) and argonaute2 (Ago2), can also facilitate the transport of nucleic acids throughout the body, similar to extracellular vesicles (EVs) (Arroyo *et al.*, 2011; Aucamp *et al.*, 2016; Vickers *et al.*, 2011).

Gahan and Stroun (2010) have reported that actively released DNA can also be complexed with glycolipoproteins and associating RNA, and these specific complexes were referred to as virtosomes. The DNA, RNA, protein and lipid components have also been determined to be newly synthesised. The DNA fraction of the virtosome is synthesised in the nuclei of living, dividing and differentiated cells and not dead or dying cells, where it then passes into the cytosol accompanied by newly synthesised lipid and protein (including DNA-dependent DNA and RNA polymerases) (Gahan & Stroun, 2010). Due to both dividing and differentiating cells being able to spontaneously release DNA, the DNA-complex is not specifically related to mitotic DNA synthesis and could be synthesised during either the G0 or G1 phases of the cell cycle. The RNA is synthesised after the completion of the synthesis of the DNA-lipoprotein complex and prior to leaving the cell, possibly through the action of the DNA-dependent RNA polymerase. Inhibition of the respiratory activity of living cells leads to the inhibition of the release of virtosomes, indicating that an active energy-dependent secretory mechanism is involved. Additionally, equilibrium appears to be reached when culture medium concentrations of the virtosomes reach a certain level and this level cannot be exceeded. This equilibrium is maintained either by a negative feedback mechanism or by the establishment of equilibrium between the release and uptake of virtosomes by cells.

There are striking similarities between the synthesis of the virtosomal DNA and metabolic DNA (reviewed in (Aucamp *et al.*, 2016; Gahan *et al.*, 2008; Pelc, 1968)). Both are synthesised in actively dividing and non-dividing, differentiated cell populations, both have similar molecular weights that are lower than that of the stable DNA fraction and the synthesis of both DNA forms in differentiated cells is paralleled by different metabolic processes (e.g. cardiac muscle function or metabolic functions in hepatocytes) (Gahan & Stroun, 2010). It was therefore suggested that the metabolic DNA could be the origin of the released DNA. Virtosomes are also strikingly similar to Bell's *I*-somes, packaged particles containing non-mitochondrial cytoplasmic DNA (*I*-DNA), found in the cytoplasm of various embryonic cells, that can be associated with rapidly labelled RNA and polyribosomes (Aucamp *et al.*, 2016; Bell, 1969; Bell, 1971). Both the virtosome and *I*-some's DNA fractions are released from the nucleus, where they associate with proteins in the cytoplasm, followed by the synthesis of RNA that associates with the DNA-protein complexes to form the virtosomes and *I*-somes. It is, therefore, very possible that virtosomes and *I*-somes are one and the same.

(a) Cell-surface-bound DNA versus cell-free circulating DNA

CirDNA can be complexed to the cell-surfaces of cells and can circulate freely in the extracellular space complexed with proteins or as virtosomes. It has been speculated that cirDNA actively released by cells initially becomes attached to cell membranes (Breitbach, Tug & Simon, 2012) by binding with cell-surface DNA-binding proteins (Bennett, Gabor & Merritt, 1985) and/or with the phospholipids of the cellular membrane through bivalent ions (Skvortsova *et al.*, 2006). This surface-bound-DNA can be detached with trypsinisation, confirming the involvement of cell surface proteins (Bryzgunova *et al.*, 2015). Direct correlations between the concentration of cell-free and cell-surface-bound cirDNA and the concentrations of weakly bound DNA and cell-free cirDNA have been demonstrated, indicating the occurrence of partial exchange between cell-surface-bound and cell-free DNA. In theory, as the DNA-binding capacity of the cells become saturated the cirDNA detaches from cells and enters the bloodstream (Breitbach *et al.*, 2012).

An equilibrium, therefore, forms between intracellular DNA, extracellular surface-bound-DNA and cell-free cirDNA that is likely in order to: (1) facilitate the assimilation of cirDNA at cell surface (DNA-binding receptors (including cytokeratins), linker and core histones (including histone1x-like proteins) and serum proteins (such as albumin) can promote the internalisation of DNA in blood cells (Rykova *et al.*, 2012)); (2) protect cirDNA from degradation via nucleases in the extracellular environment (Bryzgunova *et al.* (2015) confirmed the potential protective properties of binding to cell surfaces as they have demonstrated that there are no correlations between both cell-surface-bound DNA fragmentation and dissociation and DNase activity in blood plasm); (3) regulate cirDNA levels in circulation via the management of the equilibrium; (4) promote the transport of cirDNA (cirDNA bound to monocytes can migrate into the tissues from the blood vessels, thereby playing the role of ―carriers" of surface-bound DNA to cells (Skvortsova *et al.*, 2006)).

Cases regarding changes in the ratios of cell-surface-bound and cell-free DNA levels have been reported for cancers (Bryzgunova *et al.*, 2015; Skvortsova *et al.*, 2006) and proposed for exercise (Breitbach *et al.*, 2012). It is believed that changes in the cytoplasmic membrane structures of blood cells may be involved as changes in lipid ratios in blood cell membranes have been associated with oncologic disease development that can result in disorganisation of protein composition and cell surface architectonics and membrane cation transport dysfunction (Bryzgunova *et al.*, 2015). The combination of this change in cell-free and cell-surface-bound DNA ratio and decreased DNase activity in cancers (discussed in Section 4(4)) may be responsible for the increased levels of cirDNA measured in cancer patients compared to that of healthy subjects.

(b) Mechanisms of active DNA release

Other than cirDNA release from cell membranes, Morozkin *et al.* (2008) proposed that the formation of nucleoprotein complexes could mediate the active release of DNA by cells and, correspondingly, that the inhibition of secretory mechanisms responsible for active protein, proteoglycan and proteolipid secretion should block cirDNA release. It was determined that endoplasmic reticulum/Golgi-dependent secretory pathways and ABC-1 family of membrane transporters, involved in energy-dependent membrane translocation of various proteins via a Golgi-independent pathway, is involved in DNA traffic, indicating the existence of DNA-binding proteins that can be excreted outside the cells in the same manner. The inhibition of these pathways in human umbilical vein endothelial cells (HUVECs) only affected cell-surface-bound DNA concentrations, indicating that the actively released DNA initially remains bound to the cell membrane before being released. Increasing the pH of endosomes, Golgi vesicles and lysosomes via chloroquine treatment, however, inhibits the extra- and intracellular transport of molecules and results in the induction of apoptosis in actively proliferating cells, implying that increased DNA levels in the growth medium of HUVECs treated with chloroquine is due to apoptosis rather than active release. When using HeLa cells, however, only the inhibition of Golgi apparatus function decreased the cell-surface-bound DNA of HeLa cells. The inhibition of the ABC1 family of membrane transporters resulted in an increase in DNA concentrations by 50 % instead of a decrease, and chloroquine treatment did not affect *in vitro* cfDNA levels, thereby not inducing apoptosis in HeLa cells. Morozkin *et al.* (2008)'s, therefore, indicated that the active DNA release mechanisms in primary and cancer cells can be different.

(c) The functional aspects of actively released DNA

(1) Intercellular messengers. Apart from the metabolic activity of Pelc's metabolic DNA, actively released DNA is said to function as intercellular messengers. The concept of cirDNA serving as intercellular messengers originated from the research of Avery, MacLeod and McCarty (1944), who discovered that DNA was the biologically active fraction responsible for the transformation of non-virulent pneumococci to virulent strains when exposed to the growth medium of dead virulent pneumococci. Nearly two decades later, Maurice Stroun and colleagues demonstrated that graft hybridisation of two varieties of eggplant can result in hereditary modification in the pupil plant (Aucamp *et al.*, 2016; Stroun, Mathon & Stroun, 1963a; Stroun *et al.*, 1963b). It was theorised that the DNA circulating between the mentor and pupil plants were responsible for the transfer of hereditary information and that this circulating DNA can be transported to, and integrated with, the genome of the recipient cells. With this research in mind, Gahan and Chayen (1965) proposed that it was the DNA located in the cytoplasm that had the ability to act as a messenger of sorts. Eugene Bell discovered *I*-somes shortly after this, theorising that this form of DNA represented copies of nuclear genes that serve as information intermediates between the nucleus and cytoplasm (Bell, 1969; Bell, 1971). Indeed, the molecular weight of cirDNA is believed to be high enough for part of it to serve as a carrier of information of several genes (Anker *et al.*, 1975).

(2) Synchronising cell differentiation. Bell (1969) postulated that the *I*-somes serve as mediators of cell differentiation (messengers between the nucleus and cytoplasm for the synthesis of proteins). On the other hand, Gahan and Stroun (2010) identified virtosomes as messengers between cells. If these forms of DNA are indeed one and the same, it is possible that the DNA found in the circulation associated with protein and lipids are tools for cell differentiation that are actively released into the circulation to serve as messengers to neighbouring cells, perhaps to Synchronise the activities of the cells of a tissue or organ. Virtosomes have been found to readily enter other cells *in vitro* and *in vivo* and modify the biology of the recipient cells (Adams, Diaz & Gahan, 1997; Garcia-Arranz *et al.*, 2016; Garcia-Olmo *et al.*, 2015; García-Olmo *et al.*, 2010; García-Olmo, Ruiz-Piqueras & García-Olmo, 2004).

(3) Genometastasis. The cirDNA in the plasma of cancer patients have also been found to transfer oncogenic information to susceptible cells (García-Olmo *et al.*, 2010). In 1965 Bendich, Wilczok and Borenfreund hypothesised that cirDNA could be involved in the metastatic spread of cancer. It was theorised that tumorigenic DNA can be transported in a biologically active form via the circulatory or lymphatic systems, resulting in the penetration of tissues and organs (Aucamp *et al.*, 2016; Bendich *et al.*, 1965). Later research by García-Olmo *et al.* (1999) resulted in the genometastasis theory, the occurrence of metastasis through the transfection of susceptible cells in distant organs with dominant oncogenes, from a primary tumour, that circulates in the plasma (García-Olmo *et al.*, 1999; Garcia-Olmo *et al.*, 2000; García-Olmo *et al.*, 2010).

García-Olmo *et al.* (2010) examined the effects of plasma from healthy subjects and from patients with *K-ras*-mutated colorectal tumours on cultured NIH-3T3 and human adipose-derived stem cells (hASC). hASCs treated with plasma from the cancer patients were resistant to transformation and showed no indication of mutated *K-ras* sequences by real-time PCR. Most of the NIH-3T3 cell cultures, however, showed mutated human sequences soon after the start of

incubation that persisted up to three weeks after the removal of human plasma from the growth medium. The tumour cultures also showed low concentrations of DNA in plasma relative to the tumour cells after the simple addition of plasma without prior DNA extraction, indicating considerable efficiency of the transfection phenomenon when DNA is added and originates in plasma. This transfection efficiency, interestingly, declines when the DNA extracted from the plasma is administered instead of plasma. In addition to *K-ras* sequences, human *p53* and human β-globin-encoding sequences were also found to be transferred to the NIH-3T3 cells from the plasma, but not transferred to hASC cells. DNA transfer was, therefore, not restricted to oncogenic DNA sequences. Further investigation showed that the treated NIH-3T3 cell DNA could not recombine with the plasma DNA to form a similar mutation in the DNA of the host. However, once injected into NOD-SCID mice these treated NIH-3T3 cells can generate undifferentiated carcinomas and mutated human *K-ras* sequences could be detected in distant parenchyma (liver and lungs) and the plasma of the mice. The NIH-3T3 cells, therefore, acquired the potential for oncogenic activity *in vivo*, confirming both Bendich *et al.* (1965)'s theory and the genometastasis theory.

(4) Anticancer treatment. Where treating susceptible healthy cells with tumorigenic cirDNA can promote metastasis, treating tumour cells with cirDNA derived from healthy cells can potentially be used to prevent metastasis. Adams *et al.* (1997) revealed that mouse spleen lymphocytes can rapidly and efficiently import DNA from tumour cell medium or growth medium containing tumour cell cytosol, resulting in a three to four-fold increase in [^{3}H]-thymidine incorporation. Mouse tumour cell lines, J477 and P497, incubated in growth medium containing either non-dividing lymphocyte or hepatocyte cytosol, on the other hand, showed a reduced rate of [^{3}H]-thymidine incorporation and it was proposed that this was an indication of the inhibition of tumour growth in response to the DNA of the non-dividing cells' cytosol. This growth inhibiting effect on tumour cells have been confirmed to occur both *in vitro* and *in vivo* by more recent studies (Garcia-Arranz *et al.*, 2016; Garcia-Olmo *et al.*, 2015). Garcia-Olmo *et al.* (2015) patented the *in vitro* and *in vivo* utilisation of the virtosomes isolated from non-dividing cells to reduce or block tumour growth and metastasis. Virtosomes can be extracted and purified from any non-dividing cells or tissues either in its newly synthesised form in the cytosol or after its spontaneous release from cells. To determine whether the purified virtosomes retained their biological activity, the virtosomes are tested against a culture of mouse tumour cell line J774 with similarly prepared J774- or P497-derived virtosomes serving as a control. The control tumour-derived virtosomes will have no effect on the tumour cells and little effect on normal dividing 3T3 fibroblasts. The virtosomes from the non-dividing cells or tissues, however, will result in a dose-dependent 60 – 70 % reduction of J774 DNA synthesis and cell replication. Similar experiments using non-dividing hepatocyte virtosomes and tumour cell lines SW480 and fibrosarcoma (HT1080) showed a reduction in cell replication of more than 80 % and 90 %, respectively, after 24 hours of treatment. However, once the treatment ceases, the remaining cells are able to continue with cell replication processes and escape the effects of the virtosomes. *In vitro* studies of Garcia-Arranz *et al.* (2016) has shown that the continuous presence of rat liver virtosomal concentrations, varying between 2 and 100 % of the growth medium added to the cells, can result in almost complete cell replication inhibition, in the absence of cell death, of both tumour (human Duke's type B colorectal adenocarcinoma and human connective tissue fibrosarcoma) and normal (smooth muscle, human umbilical vein endothelial, human fibroblasts and mouse embryonic fibroblasts) cell lines.

In vivo studies by Garcia-Olmo *et al.* (2015) involved the intravenous injection of virtosomes from non-dividing cell populations into tumour-bearing BDIX strain rats. As observed in their *in vitro* results, the virtosomes had no effect on healthy BDIX rats. Ten days of consecutive daily injections into BDIX male rats, previously inoculated with DHD/K12-PROb cells to form a tumour similar to colon adenocarcinoma, resulted in the partial or complete reduction in tumour size for the following 14 days. Using the same rat models, Garcia-Arranz *et al.* (2016) confirmed that virtosomes have no effects on healthy controls when treated with ten consecutive daily doses or with an additional ten consecutive daily doses three weeks later. Rats with induced tumours receiving ten consecutive daily doses of virtosomal injections showed a reduction in tumour size up to two months later with no liver and colon metastases. Rats that received a second series of doses four weeks after the first series showed little response toward the treatment. Due to the presence of proteins in virtosomes, histological analysis of a range of tissues were performed and confirmed that there were no indications of immune reactions resulting from the injection of proteins. These histological analysis results and the syngenic animals used in the experiment confirms that virtosomal proteins are not responsible for the observed inhibition in tumour growth during treatment and subsequent release from inhibition when virtosomes are no longer administered.

(6) Vesicular transport of nucleic acids

All human cells are capable of releasing/producing extracellular vesicles (EVs), which are generally categorised as exosomes, apoptotic bodies (ABs) and microvesicles (MVs), which are sometimes referred to as microparticles or shedding vesicles (Raposo & Stoorvogel, 2013). However, the functions of the different EVs are still poorly understood, and exosomes are the most thoroughly characterised (Barteneva *et al.*, 2013; Distler *et al.*, 2005; Flaumenhaft, 2006; Shet, 2008). In the past, EVs ranging from 40-1000 nm have been referred to as exosomes. However, after their origin was elucidated the nomenclature was adopted for a size of 30 – 100 nm and restricted to vesicles released during reticulocyte differentiation due to the fusion of multivesicular endosomes with the plasma membrane. Conversely, MVs and ABs are shed from the plasma membrane (Raposo & Stoorvogel, 2013). Microvesicles generally range between 100 and 1000 nm, whereas ABs can be up to 4000 nm (Mause & Weber, 2010). Confusion regarding nomenclature stems from the inability of most purification regimes to completely fractionate the different EVs. This is further complicated by the phenomenon that many cells release a heterogeneous population of EVs (Deregibus *et al.*, 2007; Heijnen *et al.*, 1999; Muralidharan-Chari *et al.*, 2009). Additionally, there is an overlap in the category of size. For example, prostasomes, also of endosomal origin, have been shown to range from 40 to 490 nm (Ronquist *et al.*, 1978). Moreover, some plasma membrane derived vesicles have the same sizes as exosomes, and are often wrongfully categorised (Booth *et al.*, 2006).

Exosomes are released into various body fluids where they are broken down or can perform functional roles strongly associable with intercellular communication (Peters & Pretorius, 2011; Urbanova *et al.*, 2010). Exosomes contain various forms of RNA relative to the RNA profiles of the originating cells (Hood, San & Wickline, 2011; Peters & Pretorius, 2011; Raposo & Stoorvogel, 2013; Urbanova *et al.*, 2010). However, Raposo and Stoorvogel (2013) emphasised that many studies involving RNA in exosomes failed to demonstrate whether the identified extracellular RNAs were truly associated with the exosomes or rather with RNA-protein complexes that may have been co-isolated

with the exosomes. Whether exosomes can carry DNA is controversial, due to many studies both confirming and denying the presence of DNA in these microvesicles. However, there are reports of microvesicles and exosomes of different sources containing transposable elements, single stranded DNA, genomic DNA in rats (Serrano-Heras, García-Olmo & García-Olmo, 2011) and mitochondrial DNA. Kahlert *et al.* (2014) recently reported identifying large fragments (>10 kb) of double-stranded genomic DNA spanning all chromosomes in human exosomes isolated in the serum of pancreatic cancer patients. They further determined that exosome-depleted serum did not produce any PCR products, suggesting that the majority of serum cirDNA may come from inside the exosomes and are not present as free floating cirDNA. This suggests that exosomes may be a new source of cirDNA (Kahlert *et al.*, 2014). Thakur *et al.* (2014) reported similar findings in tumour-derived exosomes. Waldenström *et al.* (2012) have demonstrated that cardiomyocytes can release DNA and RNA containing microvesicles or exosomes in vitro, termed cardiosomes, and proposed that microvesicles and cardiosomes may be involved in metabolic events in the microenvironment of the heart to facilitate cellular processes through the transfer of nucleic acids to target cell nuclei.

Microvesicle biogenesis occurs during many processes, including cancer, cellular differentiation, stress, activation, senescence, stimulation with cytokines, stimulation by shear force, exposure to ATP, apoptotic cell death, changes in the microenvironment, hypoxia and malignant transformation (Hood *et al.*, 2011; Rak, 2010). Studies have shown that tumour cells and cells in the tumour microenvironment can secrete exosomes, which could contribute to tumour progression via angiogenesis and metastasis promotion (Rak, 2010; Raposo & Stoorvogel, 2013), as well as the increased circulating tumour DNA levels in cancer patients. Kahlert *et al.* (2014) and Thakur *et al.* (2014) demonstrated that exosome-derived DNA can carry mutations identical to their parental cancer cells or tumours. Interestingly, it was also shown that healthy and cancerous prostate cells release exosomes with very different nucleic acid contents (Hessvik *et al.*, 2012).

By carefully examining the literature cited above, it becomes clear that every cell-type in the human body has the capacity to release and assimilate extracellular vesicles (or at least the phenomenon has yet to be found absent). Moreover, there are many different functional classes of EVs and not all cell-types release all EV types and there seems to be a degree of specificity with regard to uptake. The secretion of EVs also appears to be an energy dependent and regulated process, which could be both constitutive or a reaction upon a stimulus, wherein the structure (shape and size), content (relative amounts of DNA, RNA and proteins), and function of EVs depend on the characteristics of the cell from which they originate and are modulated by the conditions under which they are produced. It is, therefore, very important for researchers to keep in mind both the morphology and functions of EVs when planning their cirDNA experiments. In other words, the different shapes and sizes of EVs can result in different profiles of cirDNA when different blood-processing protocols are used. Similarly, the exosome-derived cirDNA profile can also be influenced by the physiological status of the individual at the time of blood-withdrawal.

(7) Erythroblast enucleation

Mature erythrocytes do not contain nuclei in order to optimise oxygen transport. As they mature, chromatin becomes condensed and transcription is generally suppressed via the involvement of a network of chromatin factors and histone modifying proteins (Keerthivasan, Wickrema & Crispino, 2011). After exiting their final cell cycle, the nuclei of the now orthochromatic erythroblasts become polarised to one side of the cell. These cells then enucleate to form reticulocytes and pyrenocytes, the extruded nucleus with a thin cytoplasmic rim and surrounded by a plasma membrane. The mechanism of enucleation as reviewed by Ji, Murata-Hori & Lodish (2011) and Keerthivasan *et al.* (2011) may include: (1) apoptosis, (2) asymmetric cytokinesis (nuclear extrusion in the form of cell division, where the nucleus is separated from the cytoplasm via a form of abscission or vesicle trafficking), (3) differential protein sorting to the pyrenocyte and reticulocyte during enucleation (requires vesicle trafficking that may couple protein sorting with enucleation), (4) macrophages (an important *in vivo* enucleation and erythroid homeostasis process where pyrenocytes are engulfed by macrophages and digested in lysosomes via DNase II), (5) autophagy of nuclei (and mitochondria) of erythroblasts, (6) the involvement of actin (to maintain cell shape, maintenance of the polarity of the nucleus, assist in the formation and movement of endocytic vesicles) and (7) the involvement of microRNAs (e.g. miR-144/451 cluster is required for erythropoiesis, whereas miR-191 overexpression can block chromatin condensation and enucleation).

As observed in the previous sections, apoptosis (discussed in Section 3(3)), macrophages (phagocytosis) (discussed in Section 3(3)(a) and Section 4(1)) and autophagy (discussed in Section 4(2)) serve as putative sources of cirDNA and/or causes of cirDNA release into circulation (Fig. 3), indicating that pyrenocytes can release DNA fragments from their nuclear contents when either undergoing, or perhaps even escaping, any or all of these processes. It is also possible that the vesicular form of the pyrenocytes can promote engulfment by nearby cells other than that of macrophages to promote phagocytosis, which may result in lateral information transfer between the pyrenocyte contents and the recipient cell. Categorising erythroblast enucleation as a source or cause of cirDNA is significantly different from other biological features. Biological features that actively involve cellular breakdown mechanisms, e.g. inflammation and sepsis, in the same manner as erythroblast enucleation to release DNA into circulation are categorised as causes of cirDNA release, because they induce or cause cellular degradation that in turn releases DNA from the cells. However, erythroblast enucleation does not cause apoptosis and autophagy, it is rather these processes that result in the enucleation process. Moreover, there is no resulting cell death as the enucleated cells become mature oxygen-transporting erythrocytes, making erythroblast enucleation the only biological feature that involves cellular breakdown mechanisms in the release of DNA from living cells. Erythroblast enucleation, therefore, serves primarily as a source of cirDNA.

This process, however, does become a cause for DNA release once pyrenocytes become targeted by phagocytic cells for degradation. The membranes of pyrenocytes express phosphatidyl serine on their surface to deliberately promote phagocytosis in order to maintain effective enucleation processes and erythroid (Keerthivasan *et al.*, 2011). Should pyrenocytes not be degraded or the DNase II activity of macrophages be inhibited or overwhelmed by the amount of pyrenocytes for any reason, the accumulation of pyrenocytes will trigger anaemia under *in vivo* conditions, resulting in cell damage and death, due to the disruption of enucleation and homeostasis.

(8) NETosis

NETosis is limited to certain hematopoietic cell types. It is a rapid, almost explosive process consisting of nuclear disintegration and cell death, leading to the extrusion of NETs and the snaring and accumulation of both defence-related substances and the invading microorganisms (Lögters *et al.*, 2009; Mesa & Vasquez, 2013). The resulting high molecular weight cirDNA fragments of NETosis are similar to that of necrosis, whereas apoptosis produces low molecular weight DNA species (Pisetsky, 2012). NETs are delicate in structure and their functions depend on their scaffold DNA and histones (Mesa & Vasquez, 2013). They are able to trap almost all types of pathogens (including pathogens considered too large for phagocytosis) to prevent the spread of microorganisms throughout the body and to facilitate antimicrobial processes by concentrating antimicrobial factors at the infection site (Zawrotniak & Rapala-Kozik, 2013). Correlations between cirDNA levels and NETs have been found in cases of preeclampsia, sepsis, cancer and thrombosis (Chowdhury *et al.*, 2014; Fuchs, Brill & Wagner, 2012). Ineffective clearance or excessive formation of NETs have several pathological effects (Almyroudis *et al.*, 2013; Zawrotniak & Rapala-Kozik, 2013). In autoimmune diseases, NET formation is induced (e.g. in small-vessel vasculitis) and NET degradation reduced (e.g. in lupus nephritis and systemic lupus erythematosus) which could have cytotoxic effects (Hawes, Wen & Elquza, 2015; Lögters *et al.*, 2009; Mesa & Vasquez, 2013; Zawrotniak & Rapala-Kozik, 2013). The storage of non-leukoreduced blood-transfusion products has also been found to produce cirDNA associated with histones and myeloperoxidase (marker for neutrophil granules). These NETs can pass through blood transfusion filters and can be infused into patients, resulting in toxic, prothrombotic and immunomodulatory effects commonly associated with NET functions (Fuchs *et al.*, 2013). Plasma markers of NETs have been found to correlate with diseases involving thrombotic activity. NETs promote thrombosis via the adhesion and aggregation of platelets and erythrocytes, followed by coagulation due to the procoagulant properties of the NETs‘ major constituents (Demers & Wagner, 2014). NET-derived cirDNA enhances the protease activity of coagulation factors and induce thrombin generation in platelet-poor plasma, while histones are cytotoxic to the endothelium and inhibit anticoagulation, resulting in thrombocytopenia (reviewed in (Demers & Wagner, 2014)).

(d) Vital NETosis

Unlike the suicidal form of NETosis discussed above, vital NETosis allows NET release without membrane rupture and loss of conventional living neutrophil functions (Thierry *et al.*, 2016; Yip & Kubes, 2013). Vital NETosis involves the vesicular trafficking of DNA from within the nucleus to the extracellular space. Yip and Kubes (2013) demonstrated that vesicles of DNA budded from the nuclear envelope, passed through to the cytoplasm and fused with the plasma membrane, delivering the NET out of the cell without requiring membrane perforation. Vital NETosis occurs at a rapid rate, compared to suicidal NETosis that requires several hours, and is specifically mediated by Toll-like receptor, TLR4, on platelets that facilitates the activation of neutrophils. The release of mtDNA with or instead of nuclear-derived chromatin can serve as a structural backbone of extracellular traps (ETs) produced by neutrophils and eosinophils, without limiting the lifespan of the cells (Boe *et al.*, 2015). Loss of nuclear content may contribute to cirDNA in circulation and does not result in cell lysis or death, but forms anuclear cytoplasts capable of tracking and engulfing living bacteria (Yip & Kubes, 2013).

(e) Neutrophils and cancer

Large quantities of neutrophils in the plasma of cancer patients have been observed and were found to possess pro- and anti-tumour- and metastasis activities (Granot *et al.*, 2011; Kowanetz *et al.*, 2010; Souto, Vila & Bru, 2011; Zawrotniak & Rapala-Kozik, 2013). Neutrophils have been found to favour cancer cell migration by (1) directly interacting with the cells, (2) promoting tumour growth by secreting matrix metalloproteinases (MMP-9) or (3) tumour angiogenesis and neovascularisation (Acuff *et al.*, 2006; Granot *et al.*, 2011; Huh *et al.*, 2010; Masson *et al.*, 2004; Zawrotniak & Rapala-Kozik, 2013). However, activated neutrophils can also exert cytotoxic effects on tumour cells via ROS or defensin release (Granot *et al.*, 2011; Zawrotniak & Rapala-Kozik, 2013). Mishalian *et al.* (2013) have demonstrated that the phenotype of tumour-associated neutrophils is dependent on tumour stage, as neutrophils were more cytotoxic to tumour cells during the early stages of tumour growth, but became a more supportive phenotype at later stages of tumour growth. In mice at the late stage of cancer, high quantities of plasma DNA and citrullinated histones (important marker for NET formation) were detected (Demers *et al.*, 2012; Zawrotniak & Rapala-Kozik, 2013). DNA-based matrices outside cells assemble factors that cancer cells and cancer-associated thrombosis require for triggering adherence, growth and metastasis (Cools-Lartigue *et al.*, 2013; Demers *et al.*, 2012). Tohme *et al.* (2016), for example, determined that increased NET formation following the surgical stress of hepatectomy for metastatic colorectal cancer can promote the reduction of disease-free survival by approximately fourfold. Similarly, a murine model of surgical stress via liver ischemia reperfusion showed increased NET formation correlating with accelerated metastatic disease development and progression. Studies have shown correlations between high cirDNA levels, reduced extracellular DNase (exDNase) levels and cancer development and progression (refer to Section 4(4)) and that NET destruction via DNase treatments or the inhibition of peptidylarginine deaminase (an enzyme essential for NET formation) can inhibit metastasis (Cools-Lartigue *et al.*, 2013; Tohme *et al.*, 2016).

(f) Other forms of extracellular traps

DNA release has been reported to occur in mast cells, eosinophils, basophils and macrophages, but is less likely to occur, and considerably less frequent, than from neutrophils (reviewed in (Yip & Kubes, 2013)). Eosinophil extracellular traps (EETs) could serve as an example of vital ETosis, as eosinophils remain viable after EET release (Thierry *et al.*, 2016; Yip & Kubes, 2013). EETs contain only mtDNA secreted in a catapult-like manner, compared to nuclear- and mtDNA-containing NETs. The type of cell that forms ETs can, therefore, affect cirDNA contents (nuclear versus mtDNA).

Macrophages have been shown to form ETs with typical ET components, including histone-DNA complexes and antimicrobial peptides cathelicidins and other different components, which suggests that macrophage ETs (METs) may consist of different subtypes and varieties (reviewed in (Boe *et al.*, 2015)), including vital ETosis. MET-like structures with mitochondrial-derived DNA as the primary structural backbone have also been identified. Boe *et al.* (2015) noted, however, that, even when stimulated, *in vitro* MET levels remain relatively small, possibly indicating that METs are regulated to avoid interference with other essential macrophage functions. Macrophages also have a key role in the resolution of inflammation and the early stages of tissue repair by clearing dying cells and debris, including ETs and immobilised microbes therein (reviewed in (Boe *et al.*, 2015)). Macrophages, therefore, play an important role in the

prevention of inflammation, autoimmune responses and irreversible tissue damage induced by excessive or persistent ET formation. The clearance process included nucleic acid degradation with DNAse I and opsonisation of NETs by compliment factor C1q. DNAse I levels, however, appear to be insufficient to degrade NETs completely, implying two possible outcomes (Boe *et al.*, 2015): (1) the involvement of other mechanisms to complete degradation or (2) the incomplete degradation may activate complement and further inhibit serum nuclease activities, indicating the possible release of partially digested DNA fragments of NET-DNA into circulation, which may elicit autoimmune responses and add to NET-mediated tissue damage. NET-uptake by macrophages and subsequent stimulation with lipopolysaccharide results in increased secretion of proinflammatory cytokines IL-1β, IL-6 and TNF-α, possibly indicating the preparation of macrophages for an active infection or injury in the immediate area. Macrophages, therefore, can serve as sources for cirDNA (due to the partial clearance of NETs) and is involved in or can induce further causes of cirDNA release, such as inflammation.

4. Causes of circulating DNA release

(1) Phagocytosis-mediated DNA release from dead or dying cells

In vivo studies have shown that DNA is not detectable in blood samples with apoptotic and/or necrotic cells unless macrophages are present, indicating that the presence of large numbers of dead and dying cells is not sufficient to generate a blood DNA response and that, instead, a blood DNA response requires the interaction of dead and dying cells with phagocytes (Choi *et al.*, 2005; Jiang, Reich & Pisetsky, 2003; Pisetsky, 2012). The phagocytes would then either digest the cells (followed by the discharge of the cleaved low molecular weight DNA fragments) or the increased cirDNA could be due to phagocytes failing to clear the dead and dying cells (causing the phagocytes to die, releasing their DNA and the DNA of the engulfed cells into the bloodstream) (Jiang *et al.*, 2003; Pisetsky, 2012) (Fig. 3). Interestingly, *in vitro* experiments have shown a decrease in cfDNA levels in cultures of apoptotic cells with macrophages and high cfDNA levels in cultures with apoptotic cells alone, while subsequent *in vivo* evaluation of the effects of macrophages showed the opposite (Choi *et al.*, 2005). It is suggested that apoptotic cells interact with proteins (e.g. C1q, a subunit of the C1 enzyme complex that activates the serum complement system, and C-reactive protein, produced by the liver in response to inflammation), which causes the cells to behave or appear necrotic. The clearance of necrotic cells via macrophages result in cirDNA release, so the necrotic behaviour or appearance of these apoptotic cells results in modifications in the interactions of these cells with macrophages followed by increased DNA release (Choi *et al.*, 2005). These phenomena could be responsible for Leon *et al.* (1977)'s observation of low levels of cirDNA in cancer patients treated with radiotherapy. Radiation would cause the necrotic or apoptotic destruction of not only the cancer cells, but also the phagocytes responsible for the clearance of the irradiated cancer cells. The repercussion of the resulting absence of interaction between phagocytes and the irradiated cells will be the low levels of cirDNA despite the high levels of apoptotic or necrotic cells, as observed by the *in vivo* studies of Choi *et al.* (2005). Other reasons for the low levels of circulating apoptotic DNA during radiation treatments are two different forms of cell death, namely mitotic catastrophe (discussed in Section 5(1)) and accelerated senescence (discussed in Section 4(3)).

(2) Autophagy: Clearance of cell degradation

Macroautophagy is the sequestering of portions of the cytoplasm into double-membraned vesicles (autophagosomes) that fuse with lysosomes (autolysosomes) to promote the degradation of the contents (Rello-Varona *et al.*, 2012) (Fig. 3). It is a process mainly reserved for the quality control and turnover of cytosol or cytoplasmic organelles in mammalian cells, sparing the nuclei. Autophagic removal of nuclei could be lethal to cells or result in aneuploidy, though nuclear macroautophagy of multinuclear cells (e.g. hepatocytes) have yet to be identified. Mitochondria, however can be eliminated via autophagy (mitophagy), which could result in the release of mtDNA into the plasma due to the DNA being broken down or escaping the breakdown process (Oka *et al.*, 2012; Rello-Varona *et al.*, 2012).

Autophagy is said to play a tumour suppressive role in the early stages of tumorigenesis (De Bruin & Medema, 2008). In later stages of tumorigenesis, however, autophagy serves as a protector for established tumours and the inhibition thereof will result in cell death via apoptosis and tumour regression (Mathew *et al.*, 2007). Autophagy suppression in healthy tissues, on the other hand, impairs cellular survival in metabolic stress, which promotes increased DNA damage, gene amplification and aneuploidy that may, in turn, promote tumorigenesis (De Bruin & Medema, 2008; Mathew *et al.*, 2007). Autophagy may, therefore, not necessarily be a source of cirDNA through direct nuclei degradation, but can indirectly promote cirDNA release by either killing cells with compromised organelles and proteins, protecting established tumours from stressful conditions or by promoting tumour development and DNA damage when suppressed in healthy cells.

One of the reasons why macroautophagy does not directly affect nuclei is their size. Micronuclei, on the other hand, can be degraded by autophagy as shown by the studies of Rello-Varona *et al.* (2012), indicating that the multinucleated giant cells that forms during mitotic catastrophe can be processed via autophagy and the ability of autophagy to degrade the nuclear envelope and chromatin hints that mitotic catastrophe, through autophagy, may serve as sources for cirDNA due to the micronuclei either being broken down or escaping the breakdown process. Should the micronuclei escape autophagy, mitotic catastrophe would serve as the source for circulating DNA through their micronuclei. However, should the micronuclei be subjected to autophagy, then autophagy will become the source of circulating DNA and no longer be categorised as a cause. If not to participate in the clearance of damaged and degraded cells, autophagy can be used as a source of energy via self-digestion. Malignant cells could activate autophagy as a survival mechanism (Thierry *et al.*, 2016). This idea is corroborated by Van Niekerk *et al.* (2016), showing that the reduction in appetite and subsequent reduction in nutrient intake during infection or sickness results in upregulated autophagy targeting the pathogens. The illness-related appetite loss is likely due to energy produced by autophagy during microbial digestion. Autophagy can, therefore, serve as a source of tumour DNA and exogenous DNA.

(3) Aging and cellular senescence

Total cirDNA, unmethylated cirDNA, RNase P-coding cirDNA and *Arthrobacter luteus* (Alu) repeat (discussed in Section 5(6)(d)) cirDNA levels have been found to differ between nonagenarians and young controls, indicating that cirDNA levels could possibly increase with age concomitantly with increased cellular senescence and cellular death, as

well as decreased clearance and phagocytic capabilities (Jylhävä, 2013; Jylhävä *et al.*, 2011). Wu *et al.* (2002) reported that cirDNA levels were slightly higher in young individuals (<20 years) and in the elderly (>70 years for women and 60-70 years for men), forming a u-shaped distribution of cirDNA levels with increasing age (Jylhävä, 2013; Wu *et al.*, 2002). Although the function and pathological roles of cirDNA are still obscure, elevated cirDNA levels may increase blood viscosity (leading to problems with microcirculation) and become immunogenic (leading to autoimmune reactions), common features in aged individuals (Jylhävä *et al.*, 2011). Interestingly, when Jylhävä (2013) investigated the association between cirDNA contents and measures of functional performance it was determined that total and unmethylated levels of cirDNA reflected the overall frailty, as higher total cirDNA levels and unmethylated cirDNA were found to be associated with lower body strength, cognitive impairment and decreased capabilities in daily functioning and mobility. MtDNA copy number, on the other hand, reflected only the physical aspect of frailty (discussed in Section 5(3)(b)).

CirDNA has been shown to damage healthy cells or can act as a mutagen by integrating into the recipient cell‘s genome (Basak, Nair & Mittra, 2016; Gravina, Sedivy & Vijg, 2016; Mittra *et al.*, 2015). Fragmented DNA and chromatin isolated from blood of cancer patients and healthy subjects were administered to a variety of cells both *in vitro* and *in vivo* and were found to be taken up and localised in cell nuclei within 30 minutes (Mittra *et al.*, 2015). The integration of these DNA samples into host cell chromosomes induced a cellular DNA damage-repair-response, resulting in double-stranded DNA breaks and the activation of apoptotic pathways. The chromatin DNA induced significantly greater effects than the fragmented DNA and both the fragmented and chromatin DNA isolated from cancer patients were more active than that of the healthy subjects, indicating that the physiological effects of cirDNA can be dependent on the DNA origin and characteristics. In the case of aging, the concomitant increase of cirDNA levels with cellular senescence, cellular death and decreased clearance and phagocytic capabilities implies that the majority of the cirDNA originates from dead and dying cells that may, in effect, be able to serve as endogenous DNA damaging agents that can further harm healthy tissues, again leading to increased cirDNA levels.

The link between senescence and aging is becoming more widely accepted as the phenotype becomes better defined. Many of the characteristic features of senescence can be observed in cells exposed to various types of cellular stress, including the loss of telomeric DNA at each cell division which could trigger a sustained DNA damage response (DDR) and permanent cell cycle arrest (replicative senescence) (Chandler & Peters, 2013). Accelerated senescence, induced when cells are exposed to oxidative stress, genotoxic agents or oncogenic mutations, is also expected to cause persistent DDR irrespective of telomere status. Persistent DDR is essential for senescence-associated secretory program generation (where senescent cells secrete a variety of inflammatory cytokines, growth factors, chemokines, proteases and extracellular matrix components), which in the long term might damage tissue function and/or cause acute or chronic inflammation (Adams, 2009; Chandler & Peters, 2013), leading to cell death and resulting in the release of DNA and mtDNA into the circulation. Uncontrolled senescence and/or the accumulated effects of properly controlled senescence can promote the degenerative and cancerous pathologies of aging (Adams, 2009). Senescence is said to limit the proliferation and renewal of adult tissue stem and progenitor cells (noted in neurons, melanocytes and pancreatic islet cells), but appears to increase hematopoietic stem cells with age (another possible main source for cirDNA in plasma and

serum (discussed in Section 5(2)), although these cells are functionally defective (Adams, 2009). The involvement of telomere loss in the induction of endoreplication (discussed in Section 5(6)(b)) may also implicate senescence in the release of nucleic acids into circulation with increasing age.

(4) The action of DNases

DNase may serve as a homeostatic mechanism to regulate cirDNA levels in biological fluids. DNase activity clears the environment from endogenous apoptotic and necrotic DNA and foreign DNA (Gahan, 2012; Velders *et al.*, 2014). Several studies have shown that the common observation of low cirDNA levels in healthy subjects is accompanied with high levels of DNase activity and increased cirDNA levels with decreased DNase activity has been detected in systemic lupus erythematosus patients and several cancers (Cherepanova *et al.*, 2008; Gahan, 2012; Tamkovich *et al.*, 2006; Velders *et al.*, 2014). Significantly increased cirDNA levels in healthy trained subjects are also sufficiently reduced by adaptations of endogenously expressed DNase activity to regain homeostasis (Velders *et al.*, 2014). NET formation, for example, maintains immune homeostasis within healthy organisms during exercise in order to prevent chronic inflammation (Beiter *et al.*, 2015). To ensure this, exercise-triggered NET release is counterbalanced by a concomitant increase in serum DNAse activity.

DNase can, however, also cause the release of cirDNA rather than being a clearance mechanism. NET-resistance in bacteria, fungi and other pathogens has been detected in the form of extracellular DNase (exDNase) release. ExDNase results in the breakdown of the DNA framework of NETs, not only facilitating the release and systemic dispersal of the pathogens (Hawes *et al.*, 2015), but also resulting in the release of the broken down NET-DNA fragments into circulation. NET-resistant pathogens may, therefore, serve as both exogenous sources (from the pathogens themselves (refer to Section 3(1))) and endogenous sources (from the breakdown of NETs and, possibly, other sources of DNA in the surrounding area affected by the released ExDNase) of cirDNA in patients with infections. Buchanan *et al.* (2006) were the first to demonstrate NET resistance in pathogenic bacteria. M1 serotype strains of pathogen Group A *Streptococcus* (GAS), associated with invasive infections such as necrotising fasciitis, expresses Sda1, a potent DNase. Buchanan and colleagues found a correlation between the marked virulence attenuation of GAS *sda1* mutants and the degradation of NETs both *in vitro* and *in vivo*. Several other clinically significant pathogens, including beta-haemolytic streptococci and *Staphylococcus aureus*, have also been found to release DNases (Ferreira *et al.*, 1992; Heins *et al.*, 1967) and it is, therefore, postulated that DNases provide a mechanism for NET resistance.

Several factors regarding DNase‘s cirDNA clearing functions have not yet been determined: (1) whether or not endogenous DNA fragments resulting from DNase activity (e.g. NET-DNA fragments) have any physiological or damaging effects, (2) whether or not DNase release can also degrade cirDNA from endogenous sources other than cellular breakdown mechanisms (apoptosis, necrosis) and ETs, e.g. actively released DNA complexed with lipoproteins (refer to Section 3(5)) or encapsulated in vesicles (refer to Section 3(6)), that may have physiological functions (e.g. intercellular messaging (Gahan & Stroun, 2010)) and (3) whether or not the breakdown of these functional DNA fractions can result in indirect negative effects. Resolving these queries may provide significant insight regarding the biological functions of cirDNA.

(5) Sepsis

Sepsis is caused by the host‘s response to bacteria in the blood. CirDNA levels are increased in sepsis patients and levels above 800 ng/ml have been associated with poor prognosis. Hamaguchi *et al.* (2015) determined that the majority of the cirDNA originates from the host cells and that the amount of bacterial DNA in the blood is negligible, suggesting that the cirDNA of sepsis patients is mainly derived from the host cells.

NET formation has been considered as a source for the increased cirDNA levels (Lögters *et al.*, 2009; Margraf *et al.*, 2008). Some bacteria (e.g. *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*) have the ability to circumvent NET-mediated killing that may result in overwhelming NETosis or a reduced clearance capacity of NETs, both of which are detrimental to sepsis patients and contribute to ongoing inflammation, organ damage and/or exhaustion of the immune system (De Jong *et al.*, 2014; Kaplan & Radic, 2012). In this regard cirDNA is seen as a DAMP, which is released during inflammatory stress and triggers the host immune response (as described in Section 3(a)) (De Jong *et al.*, 2014; Pisetsky, 2012). It has also been proposed that reactive oxygen species (ROS) production can activate a caspase-independent pathway in neutrophils, leading to the release of NETs (Beiter *et al.*, 2011; Breitbach *et al.*, 2012). However, Hamaguchi *et al.* (2015) found that the increase of cirDNA may not be derived from NETs produced by neutrophils, but from other types of host cells. Cecal ligation and puncture operated mice were used and NET formation studied using the citrullination of histone H3 (a characteristic of NET formation). Western blotting and fluorescence-activated cell sorting (FACS) analysis showed that citrullinated histone H3 was barely increased under septic conditions, suggesting a lack of involvement of NETs in cirDNA production under severe *in vivo* bacteremic conditions. It was speculated that necrotic tissue or apoptotis at the infection site or of endothelial cells could serve as potential sources for sepsis cirDNA (refer to Section 3(2) and Section 3(3)).

(6) Oxidative stress

ROS is a product of normal cellular metabolism with a dual role as both a beneficial molecule at low to moderate concentrations (e.g. intercellular communication and defence against infectious agents) and inducing oxidative stress at higher concentrations (e.g. damaging cellular lipids, proteins and DNA). ROS is a byproduct of mitochondrial ATP production that is tightly regulated by antioxidant activity (Galley, 2011). Once the antioxidant defences become overwhelmed, significant damage of lipids, proteins and DNA (both nuclear and mitochondrial) occurs in both the mitochondria and the cells. Oxidative stress has been implicated in multiple pathologies, including cardiovascular disease, cancer, neurological disorders, diabetes, arthritis , aging and sepsis (reviewed in Valko *et al.* (2007) and Galley (2011)) and causes or is involved in NETosis and cell death via apoptosis, serving as a cause for cirDNA release in the form of both normal and oxidised nuclear, mitochondrial and apoptotic DNA fragments in various pathological conditions.

Oxidative stress-mediated mtDNA damage results in a cycle of ROS production and further damage, resulting in eventual cell death termed mitochondrial catastrophe or toxic oxidative stress. The inner mitochondrial membrane becomes permeable, resulting in the activation of the caspase cascade via the release of cytochrome c and apoptosis-

inducing factor that triggers apoptosis. Mitochondrial ROS also drives NETosis both after *in vitro* ribonucleoprotein-containing immune complex stimulation and in the pro-inflammatory low-density granulocyte subset in lupus and chronic granulomatous disease patients *ex vivo* (Lood *et al.*, 2016). Oxidative stress in the mitochondria resulted in the oxidation of both released genomic DNA within the NET-like structures and mtDNA. Oxidised genomic DNA leads to cyclic GMP-AMP synthase (cGAS)-STING-dependent type I IFN and IL-6 induction. The externalisation of oxidised mtDNA, on the other hand, exhibits potent pro-inflammatory and interferogenic properties via TLR9, inflammasome activation (refer to Section 5(3)(a)) and by engaging the cGAS-STING pathway through a B-cell lymphoma (Bcl2) homologous antagonist/killer (Bak)/Bcl2-like protein 4 (Bax)-dependent process. The ROS-dependent extrusion of oxidised mtDNA was confirmed when the inhibition of ROS reduced the relative amount of mtDNA as compared to chromosomal DNA in released NETs. To support the extrusion of mtDNA the levels of both intracellular and NET-derived mtDNA were analysed and it was confirmed that intracellular mtDNA levels decreased concomitantly with increased NET-derived mtDNA levels.

(g) The bystander effect

Ionising radiation results in the development of oxidative stress that induces apoptosis in radiosensitive cells (Ermakov *et al.*, 2009). DNA released from apoptotic cells interacts with DNA-binding receptors of neighbouring cells and results in the activation of lymphocyte signalling pathways associated with ROS and reactive nitrogen species (RNS) synthesis. CirDNA released from apoptotic cells during X-radiation, therefore, serves as a significant stress-signalling factor or DAMP, that induces secondary oxidative stress accompanied by apoptosis in adjacent cells. This study of the effects of information transfer from targeted cells exposed to damaging agents of physical or chemical nature to surrounding, non-irradiated cells is commonly referred to as the bystander effect (Ermakov *et al.*, 2009; Ermakov *et al.*, 2013; Ermakov *et al.*, 2011).

Ermakov *et al.* (2011) determined that low dose irradiation (10 cGy) results in a significant increase in both apoptosis and double- and single-stranded DNA breaks in the DNA released into the extracellular space during the bystander effect. These DNA breaks develop due to both direct damage afflicted via the radiation and the oxidation of DNA bases due to the synthesis of ROS and RNS. It was determined that the concentration of DNA isolated from the growth medium of cells exposed to low dose irradiation were 20 % higher and contained more oxidised bases of guanine (8-oxoG) than that of unirradiated cells. Oxidised DNA fragments are not just limited to the apoptosis induced by irradiation. Ermakov *et al.* (2013) listed a wide range of diseases (including myocardial infarction, rheumatoid arthritis, Leber‘s hereditary optic neuropathy, Parkinson‘s and Alzheimer‘s disease and multiple sclerosis) and cancers (including breast, prostate, lung, epithelial ovarian carcinomas and uterine myoma) that present with increased 8-oxoG levels accompanying cell death and summarised multiple cases of ROS synthesis and increased TLR9 expression induction by oxidised *in vitro* cfDNA exposure in support of the bystander effect principle.

The bystander effect is not only capable of transferring damaging information, as adaptive responses toward low-dose ionising radiation have been reported to occur via similar mechanisms (Ermakov *et al.*, 2013). The development of particular variants of cellular responses (damaging or adaptive) is dependent of the amount of irradiation, the amount of

cells, the origin of the cells and their stage in the cell cycle. Mitchell *et al.* (2004) observed that first treating C3H 10T½ cells with a priming dose of 2 cGy allowed the neighbouring non-irradiated cells to develop an adaptive response against subsequent co-culture with irradiated cells, resulting in improved bystander cell survival. Priming doses (2 cGy) five hours before the administration of a 4 Gy challenge dose does not, however, promote the survival of the cells being irradiated, indicating that an adaptive response during irradiation can protect neighbouring bystander cells from subsequent treatments, but not the targeted cells undergoing irradiation. Unfortunately, Mitchell *et al.* (2004) did not elucidate the causative factor of the adaptive responses within the cultures. However, should DNA released from the cells be involved here, as in the case of the damage-inducing bystander effect, it may be possible that this adaptive response could result from actively released DNA fractions from either surviving irradiated cells or bystander cells in closest proximity to the irradiated cells.

(7) Sterile Inflammation

Other than through microbial infection at trauma sites (discussed in Section 3(1), Section 3(4) and Section 4(5)), inflammation can also occur under sterile conditions through (1) the release of pro-inflammatory mediators in cases where mast cells and nerves are stimulated, (2) the triggering of haemostatic mechanisms when the trauma causes bleeding, or (3) through cell death (a potent stimulator of sterile inflammation) (Rock & Kono, 2008). Sterile inflammation serves a protective role, possibly counteracting pathological processes by removing potentially damaging cell debris and soluble injurious agents by increasing blood flow and fluid leakage to dilute and drain away the agents and recruiting phagocytes. Tissue repair at sites of damage is also catalysed by promoting cell division and the reestablishment of blood supply, processes that are stimulated by the mediators produced by inflammatory cells (Rock & Kono, 2008). NETs also form during sterile inflammation (refer to Section 3(8)). In systemic lupus erythematosus patients, for example, NET formation correlates with increased cirDNA levels and the presence of antibodies against NET-associated proteins (Zawrotniak & Rapala-Kozik, 2013).

(h) Acute versus chronic inflammation

The inflammatory response does not only affect damaged areas and dead or dying cells, normal surrounding tissues can also be killed (Rock & Kono, 2008). Acute inflammatory responses and the involvement of neutrophils are particularly detrimental. Macrophage-rich chronic inflammatory infiltrates are thought to underlie the pathogenesis of many chronic diseases and a number of sterile processes can stimulate these chronic inflammatory responses, including sunburn and cigarette smoke. To date, cirDNA has been identified and quantified in the plasma and/or serum of patients with diseases involving inflammation (Bai *et al.*, 2015; Frank, 2016; Koffler *et al.*, 1973; Leon *et al.*, 1977; Nishimoto *et al.*, 2016; Tan *et al.*, 1966). Acute and chronic inflammation result in the release of nuclear DNA and mtDNA (discussed in Section 5(3)(a)) into circulation via cellular degeneration, turnover and/or NET formation (discussed in Section 3(8)) and the quantification of these DNA levels have long been thought to be useful in either diagnosing or measuring the progression of inflammation-related diseases.

(i) Obesity and chronic inflammation

Obesity has been found to stimulate chronic sterile inflammation, a central pathobiology of obesity and related complications, in adipose tissue that is strongly associated with insulin resistance development (Nishimoto *et al.*, 2016). Enhanced inflammation, along with other obesity-related factors such as oxidative stress, induces adipose tissue cell degeneration and turnover. Nishimoto *et al.* (2016) recently determined that obesity is associated with the release of cirDNA into the plasma of mice and humans. CirDNA released by obesity-related adipocyte degeneration promotes partial macrophage activation through recognition by TLR9 which contributes to the development of adipose tissue inflammation and insulin resistance in both *in vitro* and *in vivo* conditions. Direct correlations between cirDNA levels and fat mass was identified in mice and humans and in differentiated 3T3-L1 adipocytes similar correlations were detected between cirDNA and visceral obesity. However, the cirDNA levels of obese individuals were lower compared to that of other inflammatory diseases, due to the more chronic and low grade levels of inflammation involved in obesity compared to that of other diseases.

(8) Exercise

High-intensive, excessive or exhaustive exercise results in cirDNA accumulation in plasma and the similarities between trauma-, sepsis- and exercise-induced inflammatory responses suggested that strenuous exercise can also increase cirDNA levels (Breitbach *et al.*, 2012). High physical activity can be associated with a leukocyte inflammatory response, mechanical and metabolic muscular damage and DNA damage due to oxidative stress (refer to Section 4(6)), which increases cirDNA levels. However, Breitbach *et al.* (2012) proposed that cirDNA release during exercise may be independent of inflammatory markers like leukocyte oxidative burst, leukocyte or muscle cell apoptosis (Breitbach *et al.*, 2012). These contradictions may indicate that different forms or intensities of exercise trigger different forms of cirDNA release and is better observed when comparing chronic and acute exercise cirDNA characteristics.

Chronic exercise-induced cirDNA release is associated with inflammatory mechanisms due to chronic diseases, indicating that apoptosis and necrosis may be the source for this cirDNA (Breitbach *et al.*, 2012). Chronic resistance exercise, which involves progressively increased training volume, in excess causes a proportional increase in plasma DNA concentrations, indicating that circulating DNA may serve as a marker for monitoring and measuring overtraining in athletes (Fatouros *et al.*, 2006). Acute exercise, on the other hand, results in acute increases and subsequent decreases in cirDNA and seems to be more connected to stress factors (Beiter *et al.*, 2011), cytokines, changes in blood components, increased temperature, energy deficiency or mechanical impairment. During acute exercise, all leukocyte subsets proliferate and release cytokines and antibodies in response to stress factors (Breitbach *et al.*, 2012). The activated lymphocytes and neutrophils infiltrate damaged muscle cells during the time significant cirDNA increases have been determined (within three minutes after 15 minutes of exercise, reaching peak levels after 20 minutes of exercise until 10 minutes post-exercise). After exercise lymphocytes in circulation decrease rapidly below pre-exercise levels (Atamaniuk *et al.*, 2008) with a concomitant decrease of cirDNA levels. After muscle repair and adaptation the immunological reaction is terminated by the downregulation of increased leukocytes via apoptosis (Atamaniuk *et al.*, 2008; Mooren *et al.*, 2002). Other spontaneous cirDNA release mechanisms (e.g. leukocyte oxidative burst, NET

formation (discussed in Section 3(8)) and microvesicle- (discussed in Section 3(6)) or active DNA release (discussed in Section 3(5)) from outer cell membranes) are also speculated to be involved due to: (1) the significantly high levels of cirDNA forming so quickly (within 3 minutes) (Beiter *et al.*, 2011; Breitbach *et al.*, 2012) and (2) the fact that cirDNA release via apoptosis has not yet been shown to occur within minutes (Breitbach *et al.*, 2012). Glycolysis has recently been shown to play a significant role in *in vitro* cfDNA release patterns (Aucamp *et al.*, 2017), which may indicate that high glycolytic activity during exercise can be involved in the rapid release of actively released cirDNA. Breitbach *et al.* (2012), on the other hand, proposed that exercise affects the equilibrium between intracellular DNA, extracellular surface bound DNA and plasma DNA (discussed in Section 3(5)(a)), resulting in elevated plasma cirDNA levels for several hours or days after exercise. Acute long-lasting exercise, e.g. an ultramarathon, will then result in the return of high equilibrium to baseline levels within 24 hours, whereas chronic exercise in the form of periodic repetitive high-intensity exercise will result in chronic or persistent high equilibrium levels.

The significant and rapidly fluctuating cirDNA levels that arise from acute exercise provides important insight regarding the development of sample collection guidelines for cirDNA research. It is apparent that environmental temperatures (e.g. during summer versus winter) during and physical activity or energy levels (e.g. exercising before clinic appointment, walking to clinic and/or climbing stairs and whether the subject has sufficient energy for this physical activity (e.g. by eating prior to the activity)) prior to blood sampling can affect collected cirDNA levels and characteristics, leading to an undesirable background of cirDNA originating from cell damage and/or immunological activity.

Exercise is said to have anti-inflammatory effects via the reduction of visceral fat mass, an increase in the production and release of anti-inflammatory cytokines from contracting skeletal muscles and/or the reduction in the expression of monocyte and macrophage TLRs (Nasi *et al.*, 2016). This correlates with: (1) the abovementioned concept of acute exercise causing temporary bouts of cirDNA release through immunological means (rather than through inflammation and cell degradation); (2) Breitbach *et al.* (2012)'s proposition that cirDNA release during exercise is independent of inflammatory markers; (3) Beiter *et al.* (2011)'s findings that accumulating cirDNA is solely caused by genomic cirDNA and not by mtDNA and, as mtDNA is commonly regarded as a DAMP agent that induces inflammatory reactions (discussed in Section 5(3)(a)) and the lack thereof will pertain to the anti-inflammatory effects of exercise. Strenuous exercise that causes muscle overuse or traumatic injuries has been speculated to release mtDNA into the circulation during apoptosis (induced by stress on the muscles, joints and soft tissues, cellular or tissue damage and exercise-induced oxidative stress). However, Nasi *et al.* (2016) detected decreased-to-normal mtDNA levels in professional volleyball players, who did experience either muscle overuse or traumatic injuries during two volleyball seasons, thereby supporting the theory that regular physical activity can be associated with protective anti-inflammatory effects. No significant correlations were detected between circulating mtDNA and lean mass, body fat or weight, factors that were also speculated to contribute as sources of mtDNA.

(9) Cancer

Earlier methods of cancer cirDNA detection were limited to indirect measuring methods such as radioimmunological (Leon *et al.*, 1977) or actinomycin-D-binding tests (Carpentier *et al.*, 1981; Stroun *et al.*, 1987). However, these tests were likely to not detect DNA in nucleoprotein complexes and could not be used to characterise DNA as the plasma DNA could not be purified. Stroun *et al.* (1987) were the first to extract, purify and characterise DNA from nucleoprotein complexes in the plasma of acute leukaemia, plasmocytoma and lymphoma, various abdominal tumours, metastases from unknown primary carcinoma and lung and breast cancer patients, while no cirDNA could be obtained from plasma samples of healthy subjects. Despite the method not being sensitive enough to detect low plasma DNA levels, the finding of extractable amounts of DNA in samples of cancer patients and the lack thereof in healthy subjects correlated with previous findings of elevated cirDNA levels in malignancies compared to healthy subjects (Carpentier *et al.*, 1981; Shapiro *et al.*, 1983) and, therefore, suggested some correlation between cirDNA and malignancies. The DNA isolated from the patients with malignancies was confirmed to be of human origin, is of low molecular weight, resistant to RNase and pronase, sensitive to DNase I and was composed of sizes ranging from less than 0.5 kb to 21 kb. Whether the DNA originated from activated host lymphocytes or from tumour cells could, however, not yet be determined.

Subsequently, Stroun *et al.* (1989) determined whether the DNA synthesis and strand separation properties of cirDNA from cancer patients shared similarities with that of DNA from malignant cells observed by Beljanski, Bourgarel and Beljanski (1981). Stroun *et al.* (1987)'s method for the extraction of nucleoprotein complex-DNA was used and DNA was extracted from chronic and acute lymphocytic leukaemia, acute myoblastic leukaemia, lung, kidney and prostatic cancers with metastases, metastases from unknown primary tumours, various abdominal tumours, pancreatic cancer and ovarian neoplasia patients. Quantification and characterisation results of the isolated DNA from malignant patients (and the lack thereof in healthy subjects) correlated with that of Stroun *et al.* (1987). The *in vitro* DNA synthesis pattern (induced by carcinogenic drug or chemical exposure) of the cirDNA from seven cases of patients with malignancies was analysed. In five of the cases the tests showed increased *in vitro* DNA synthesis patterns typical of neoplastic DNA, while the remaining 2 cases showed no synthesis changes indicative of the presence of normal DNA in the plasma of these cancer patients. These results were corroborated with hyperchromicity tests, which showed increased UV absorbance at room temperature in reaction with carcinogens in the five cases where increased DNA synthesis was detected and a lack thereof in the two cases where DNA synthesis was unaffected. It was proposed that the normal DNA of the two cases were from nonmalignant host cells from the immune system in response to the malignant cells.

CirDNA levels were also found to differ between patients with metastases and patients with localised tumours (Gahan *et al.*, 2008; Leon *et al.*, 1977). CirDNA in cancer patients could be from two sources, the tumour cells and the surrounding tissue cells (Fleischhacker & Schmidt, 2007). Circulating tumour cells, likely due to cancer metastasis, can also be a source for cirDNA. However, Stroun *et al.* (2001) contradicted the idea that circulating mutated DNA comes from circulating tumour cells due to the scarcity of tumour cells in the Ficoll layer, which is used as a normal control in microsatellite analysis (Anker *et al.*, 1997). The presence of tumour nucleic acids with tumour-associated epigenetic alterations or driver mutations have also been reported in various cancers, e.g. mutations in *KRAS*, *CDKN2A*, *TP53* and

SMAD4 genes (Chen *et al.*, 2016; Fleischhacker & Schmidt, 2007; Garcia-Murillas *et al.*, 2015; García-Olmo *et al.*, 2004; Jahr *et al.*, 2001; Takai *et al.*, 2015; Warton, Mahon & Samimi, 2016).

Cell senescence is considered as a natural defence mechanism against cancer, promoting the immune clearance of oncogenic cells (Chandler & Peters, 2013). *In vitro* studies have shown that genetic alterations of cancer cells (especially alterations in p53 and p16, RAS mutations and retinoblastoma protein tumour suppressors) can allow the cells to escape senescence (Adams, 2009; Dimri, 2005; Roninson, Broude & Chang, 2001). In cells undergoing RAS-induced senescence, knockout of retinoblastoma protein tumour suppressors allowed some of the cells to continue to synthesise DNA (Chandler & Peters, 2013). However, the cells did not expand in number and became polyploid, indicating the presence of other barriers of tumour proliferation. The evasion of senescence and development of polyploid cancer cells during certain genetic alterations could be involved in the increase of cirDNA levels in cancer patients (refer to Section 5(6)(b)). Paradoxically, senescence may promote tumour genesis, possibly by secreting matrix metalloproteases, growth factors and cytokines (Chandler & Peters, 2013; Dimri, 2005).

(j) Contradictions regarding the origin of tumour cirDNA

Research provides various contradictions regarding the origins or causes of elevated cirDNA release levels in cancer patients, especially regarding the involvement of cell death or degradation. The most common contradiction is that studies have implicated cell death as a probable source for plasma tumour DNA (Fournié *et al.*, 1995; García-Olmo *et al.*, 2004; Jahr *et al.*, 2001). However, in the early stages of cancer little cell death appears to occur and cirDNA levels are already higher than normal concentrations (Van der Vaart & Pretorius, 2007). Once increases in cancer cell development leads to an increase in cancer burden and cirDNA release, increased cell death rates, along with cancer cell proliferation, becomes a more likely contribution to cirDNA release. Contradictions like this one is most likely due to: (1) the complexity of the pathophysiology involved in different cancers, (2) researchers trying to generalise cirDNA release mechanisms to cancer in general, rather than treating each cancer type as a different pathological condition (cancers that differ in tissue origin, malignancy and whether or not metastases may be involved, for example, produce different cirDNA levels from either the cancerous cells themselves (Aucamp *et al.*, 2017) or nonmalignant host cells (Heitzer, 2015; Leon *et al.*, 1977; Stroun *et al.*, 1989), so inconsistencies between cancers should not really be that much of a surprise or problem), and/or (3) inconsistencies between research methods due to a lack of established standard operating procedures (choice of sample (e.g. serum vs plasma), sample collection, storage and the extraction, processing and characterisation of cirDNA) (Bronkhorst, Aucamp & Pretorius, 2015; Bronkhorst, Aucamp & Pretorius, 2016a). As mentioned in Section 5(3), laboratory inconsistencies, in particular, can wreak havoc with the successful elucidation of the origins and functional roles of cirDNA, as the chosen method of extraction alone can result in the biased extraction of only certain fractions of cirDNA from samples.

Other particular contradictions include whether certain forms of cell death are specifically involved and whether the presence or absence of clearance mechanisms (phagocytosis) play a role in cirDNA release, the latter of which has already been discussed in Section 3(3)(a) and Section 4(1). Regarding the argument of which cell death mechanism is specifically involved, the disintegration of cells in necrotic parts of actively growing tumours (Bendich *et al.*, 1965) have

been believed to primarily release DNA into the bloodstream. Diehl *et al.* (2005) have proposed that mutant DNA fragments in the circulation are derived from necrotic neoplastic cells engulfed by macrophages. Growing tumours cause problems with vascularisation, resulting in hypoxia in the regions remote from blood vessels (Jahr *et al.*, 2001). Hypoxia plays a large role in tumour growth, invasion and metastasis and cirDNA release is found to be dependent upon hypoxic conditions (Cortese *et al.*, 2014; Thierry *et al.*, 2016). Invasive tumours, therefore, generally have larger necrotic regions than benign tumours. Necrotic cells do not necessarily release DNA into the circulation, but clearance of these cells may be a contributing factor (Diehl *et al.*, 2005). On the other hand, it is believed that hypoxia in tumours do not result in necrosis, but in apoptosis. Hypoxia induced by growing tumours induces p53-dependent or p53-independent apoptosis of tumour cells and of non-tumour cells in the infiltrated tissues (Jahr *et al.*, 2001).

To further complicate the arguments, studies have begun to show that active DNA release (discussed in Section 3(5)) can also be responsible for the presence of cirDNA (Gahan *et al.*, 2008; Stroun *et al.*, 2001). To support this it was also argued that tumours can become resistant to apoptosis via multiple mechanisms, including loss-of-function mutations in tumour suppressor protein p53, loss of functional pro-apoptotic proteins, high expression of anti-apoptotic proteins and modifications in death receptor pathways (De Bruin & Medema, 2008). These cancers continue to proliferate with a concomitant proportional increase in cirDNA (Anker *et al.*, 1999; Anker *et al.*, 1975; Stroun *et al.*, 2001; Van der Vaart & Pretorius, 2008b), indicating that other forms DNA release must be involved.

5. Occurrences that are both a source and cause of circulating DNA release

(10) Mitotic catastrophe

As mentioned in Section 3(3), certain cancers can resist apoptosis and continue to proliferate, which strongly contradicts the general notion of apoptosis as a main source of cirDNA in the plasma samples of cancer patients. However, apoptosis-resistant cancers still undergo treatment-induced cell death. Experiments where apoptotic activity has been inhibited by genetic manipulation (e.g. *BCL2* and multi-drug resistance, *MDR1*, gene expression induction) showed successful inhibition of chemotherapeutic and radiation treatment-induced apoptosis, but increased morphological markers of mitotic catastrophe and accelerated senescence (Roninson *et al.*, 2001). Mitotic catastrophe refers to aberrant mitosis in drug-treated or irradiated cells that leads to the formation of large non-viable cells with several micronuclei, the formation of nuclear envelopes around individual clusters of missegregated chromosomes (Fig. 3). Micronucleated cells that result from mitotic catastrophe differ morphologically from apoptotic cells, forming large cells containing uncondensed chromosomes instead of cells with shrunken cytoplasm and condensed chromatin. A cell cannot undergo mitotic catastrophe without prematurely entering mitosis (Vakifahmetoglu, Olsson & Zhivotovsky, 2008). The abrogation of G1 and/or G2 checkpoints is, therefore, an essential step. Cells that undergo mitotic arrest can either (1) die through apoptosis, (2) escape mitosis and become tetraploid or (3) undergo endocycles and become polyploid, followed by death via apoptosis or necrosis. Cells that escape mitotic arrest also become tetraploid and can either arrest at G1 and die through apoptosis or undergo endoreplication and die through necrosis. Cells that undergo premature mitosis can also die via mitotic catastrophe independent of apoptosis (Roninson *et al.*, 2001).

Mitotic catastrophe has been characterised as the main form of cell death in radiation therapy (Dewey, Ling & Meyn, 1995; Roninson *et al.*, 2001) and is a prominent response to various anticancer drugs. The micronucleated cells do not undergo nuclear fragmentation as seen in apoptosis, but their accumulation of multiple micronuclei subsequently shows features resembling necrotic cell death (Vakifahmetoglu *et al.*, 2008). Mitotic catastrophe, therefore, does not form the characteristic ladder pattern or DNA breaks characteristic of apoptosis (Roninson *et al.*, 2001), but may result in the release of larger DNA fragments into circulation. Additionally, the resulting multinucleated giant cells can be temporarily viable, but eventually die (Vakifahmetoglu *et al.*, 2008). These factors could explain Leon *et al.* (1977)'s observations of low levels of cirDNA in cancer patients treated with radiation therapy. Mitotic catastrophe can also, on the other hand, promote tumour survival as studies have shown that these multinucleated, polyploid giant cells may overcome states of growth arrest and even undergo de-polyploidisation (discussed in (Vakifahmetoglu *et al.*, 2008)). This de-polyploidisation, as mentioned in Section 5(6)(b), may also serve as a source for cirDNA.

(11) Hematopoietic cells

Lui *et al.* (2002) hypothesised that cirDNA may be of hematopoietic origin due to the fact that blood cells are in the closest proximity to plasma. To support this, he used a sex-mismatched bone marrow transplantation model to study the contributions of hematopoietic to non-hematopoietic cells to cirDNA (Lui *et al.*, 2002; Ziegler *et al.*, 2002). Paired buffy coat and plasma samples were collected from 22 sex-mismatched bone marrow transplantation patients and matching serum samples were obtained from seven of these patients. The percentage of Y-chromosome DNA in female patients with bone marrow from male donors and male patients with bone marrow from female donors were determined and it was concluded that cirDNA is predominantly of donor (hematopoietic) origin. A recent study by Tug *et al.* (2015) further implicated hematopoietic cells as an origin for exercise-induced cirDNA, similar to Lui *et al.* (2002)'s findings under normal conditions. Blood plasma samples were taken from sex-mismatched hematopoietic stem cell transplantation and liver transplantation patients after short incremental exercise until volitional exhaustion and the relative contribution of bone marrow- and non-bone marrow-derived cells to the cirDNA pool was determined. It was determined that cirDNA released during short bouts of exercise originates predominantly from cells of hematopoietic lineage.

The higher levels of cirDNA detected in the serum, as opposed to plasma, of *in vitro* studies were determined to be from clotting processes of hematopoietic cells and lysis of white blood cells (Lui *et al.*, 2002; Pinzani *et al.*, 2010). This hematopoietic origin of serum cirDNA was confirmed in later studies by Zheng *et al.* (2012), who performed massively parallel paired-end sequencing of the plasma DNA samples of six sex-mismatched hematopoietic stem cell transplant recipients and one liver transplant recipient and determined that non-hematopoietic cirDNA is shorter than hematopoietic DNA. Quantitative PCR studies of the plasma DNA of 34 non-pregnant and 31 pregnant women by Chan *et al.* (2004) corresponded with studies of Zheng and colleagues as circulating fetal DNA from non-hematopoietic placental cells in maternal plasma was shorter than hematopoietic circulating maternal DNA. The size distributions of both hematopoietically and non-hematopoietically derived DNA demonstrated that both contain a 166 bp peak (possibly corresponding to 146 bp nucleosomal DNA wrapped around a histone core and an unprotected, nuclease-sensitive 20 bp

linker site (Holdenrieder & Stieber, 2009)) and peaks occurring at a 10 bp periodicity at approximately 143 bp and smaller (possibly corresponding to a remnant of a monochromatosome trimmed of its linker segment) (Zheng *et al.*, 2012). This indicates that apoptosis could be involved in the liberation of cirDNA into the plasma (refer to Section 3(3)). However, non-hematopoietically derived DNA showed lower levels of the 166 bp peak and higher levels of the smaller peaks compared to that of hematopoietically derived DNA. Reasons for the differences in size distribution between hematopoietic and non-hematopoietic DNA could include factors affecting intracellular DNA degradation, DNA release, the amount of time that the DNA spends in the circulation and where in the body the circulating DNA is going (Zheng *et al.*, 2012).

Recently Snyder *et al.* (2016) determined that fragmentation patterns of cirDNA can be used to identify where this DNA originated from. It was theorised that the unique epigenetic landscape(s) of the tissue(s) of origin may be detectable in the cirDNA due to the fact that cirDNA fragment patterns are dependent on their association with nucleosomes and that nucleosome positioning varies between cell types. Deep sequencing was used to develop maps of genome-wide *in vivo* nucleosome occupancy and it was determined that the cirDNA nucleosome occupancies correlated with cellular nuclear architecture, gene structure and expression. With this unique method of detecting the origins of cirDNA without the use of genotypic differences between contributing cells or tissues, it was observed that the nucleosome spacing of cirDNA in healthy subjects correlated most strongly with the epigenetic features of lymphoid and myeloid cells, further collaborating Lui *et al.* (2002)'s theory that hematopoietic cell death is predominantly responsible for the presence of cirDNA in healthy individuals and showing that apoptosis may not be the only method by which hematopoietically derived DNA can be released into circulation.

(12) Cell-free circulating mitochondrial DNA

A mammalian cell contains multiple copies of the mitochondrial genome, a double-stranded circle of ~16.5 kb (Chiu *et al.*, 2003; Robin & Wong, 1988; Wallace, 1982; Wallace, 1999). Mitochondrial genome mutations have been associated with aging and multiple diseases, disorders and malignancies, including neuropathy, neuromuscular diseases, neurodegenerative disorders and various tissue cancers (Chiu *et al.*, 2003; Copeland *et al.*, 2002; Wallace, 1999; Wallace, 2005). MtDNA can be released from mitochondria into the cytoplasm and extracellular environment during cellular clearance or repair processes, particularly autophagy (discussed in Section 4(2)), apoptosis (discussed in Section 3(3)) or necrosis (discussed in Section 3(2)) (Zhang *et al.*, 2016). The presence of circulating mtDNA was first reported by Zhong, Holzgreve and Hahn (2000), who identified mtDNA in DNA extracted from 25 healthy subjects and 16 type 2 diabetes mellitus patients' plasma and serum samples. A to G substitution at bp 3243 in the mitochondrial $tRNA^{Leu(UUR)}$ gene, a very common mitochondrial gene mutation, was also identified in the plasma and serum samples of the diabetes patients at higher concentrations than that in blood leukocytes. It was proposed that the amount of mutant mtDNA measured in the serum and plasma may reflect the average degree of heteroplasmy in different types of cells instead of only leukocytes. Since then multiple studies have confirmed the presence of mtDNA in the circulation of cancer patients and implicated circulating mtDNA in disease-, damage- or injury-, exercise- (refer to Section 4(8)), age-related inflammatory responses (refer to Section 4(3)) and neutrophil extracellular traps (NETs) (refer to Section 3(8)).

Centrifugation and filtration studies of the plasma samples of healthy subjects by Chiu *et al.* (2003) have shown that circulating mtDNA is present in plasma samples in both intact cell-free mtDNA and particle-associated forms. Plasma samples prepared by two centrifugation steps were compared with samples processed with additional filtration, to concentrate and measure free-form mtDNA, and ultracentrifugation, to demonstrate that a fraction of the circulating mtDNA was pelletable. Significant reductions in mtDNA concentrations in both filtered and ultracentrifuged samples were detected, supporting the existence of significant fractions of particles containing mtDNA that were pelletable and filterable. Significant amounts of mtDNA were still present in the samples after filtration and ultracentrifugation, suggesting that non-particle-associated mtDNA was also present in plasma samples. The filtration of samples with different pore size filters showed that mtDNA-containing particles existed in different sizes and were possibly of different natures. It was proposed that the particles to which the mtDNA were associated to may be internal and external mitochondrial membrane fragments (Chiu *et al.*, 2003; Thierry *et al.*, 2016).

Circulating mtDNA is not always correlated with circulating nuclear DNA levels in certain pathological conditions, providing potentially unique pathophysiological information distinct from that of circulating nuclear DNA (Zhang *et al.*, 2016). The lack of protection of mtDNA, due to the absence of histones, may imply that circulating mtDNA fragments should be shorter than circulating nuclear DNA. Thierry *et al.* (2016) reported that circulating mtDNA is present in large amounts in the blood. They have also determined that tumour and non-tumour circulating mtDNA concentrations of xenografted mice are elevated and that human tumour circulating mtDNA is more fragmented than mouse non-tumour circulating mtDNA. Testicular germ cell cancer patients presented with higher levels of 79 – 220 bp circulating mtDNA fragments compared to control subjects, with particularly high levels of the 79 bp fragments (Ellinger *et al.*, 2009). Recent research corroborates the very short lengths of circulating mtDNA, peaking at 42 bp (Zhang *et al.*, 2016).

This relates quite interestingly to work on the methodology of cirDNA research. For example, it is possible that the different forms of mtDNA may be due to the different processing methods themselves rather than being of physiological origin. Great care must be taken when considering extraction methods, as studies have shown that simple differences in experimental steps can greatly affect the resulting yield and content of cirDNA. Bronkhorst *et al.* (2015) showed that changes in sample storage and processing can, for example, affect the amount of cell-free DNA extracted from cell culture growth medium. Malentacchi *et al.* (2015) showed similar issues when providing multiple laboratories with plasma samples and the simple instruction of quantifying the plasma cirDNA present. Applied-Biosystems (2015) also revealed that the choice of extraction method can affect the contents of the resulting cirDNA sample, as using the KingFisher systems resulted in the absence of 2 000 bp DNA fragments that are, however, present in DNA extracted with the MagMAX Cell-Free DNA Isolation Kit from Thermo Fisher Scientific and NucleoSpin gel and PCR cleanup kit from Machery Nagel (Bronkhorst *et al.*, 2016b). It is obviously a major issue that different analytical protocols cause differences in results. This is not only important because it might explain away several inconsistencies between studies, but also because it emphasises how important it is to really carefully document exact experimental protocols in this field.

(k) Mitochondrial DNA as DAMPs and NETs

MtDNA has been shown to contain inflammatogenic unmethylated CpG motifs (Bliksøen *et al.*, 2016; Burnham *et al.*, 2016) and to bind to Toll-like receptor TLR9 once released from dead or dying cells, similar to bacterial and viral DNA (Bliksøen *et al.*, 2016; McIlroy *et al.*, 2015; Nasi *et al.*, 2016). The fragmentation profiles of microbial and mtDNA in plasma have been found to be very similar, forming ultrashort cirDNA fragments with lengths shorter than 100 bp (as observed by Zhang *et al.* (2016)), indicating a possible similar degradation process. MtDNA can, in effect, serve as a DAMP that can trigger innate immunity when released during cellular injury through the activation of TLR9, TLR4 and formyl peptide receptor, FPR1, in monocytes or neutrophils and results in a potent inflammatory reaction (Burnham *et al.*, 2016; McIlroy *et al.*, 2015; Nasi *et al.*, 2016; Oka *et al.*, 2012). TLR9, in particular, mediates the interferon regulatory factor pathway and the MyD88-dependent pathway, which culminates in activation of NF-κB and transcription of its downstream genes involved in cell cycle regulation, apoptosis and inflammation (Bliksøen *et al.*, 2016). The inflammatory reactions produce cell damage that releases more mtDNA, resulting in a vicious cycle (Nasi *et al.*, 2016). Platelets have also been found to release intact mitochondria into circulation, as free organelles or in vesicles, that are internalised by leukocytes and processed via bactericidal secreted phospholipase A_2, releasing proinflammatory lipid mediators and mtDNA (Boudreau *et al.*, 2014).

Tissue injury at the time of trauma is an important but non-modifiable factor for post injury inflammation-associated complications. Surgical interventions on trauma patients result in secondary tissue injury that releases DAMPs. McIlroy *et al.* (2015) showed that absolute plasma levels of mtDNA decline in the immediate postoperative period (up to seven hours postoperatively) and increased intraoperative fluid administration was correlated with decreased postoperative levels of mtDNA. MtDNA release was found to remain sustained at elevated levels for at least five days following major orthopaedic interventions on trauma patients. These plasma mtDNA concentrations were independent of tissue necrosis markers, ruling out necrosis as a source of circulating mtDNA. Apoptosis was disregarded as a source of circulating mtDNA, because apoptotic cells are thought to be efficiently and rapidly removed by phagocytosis, preventing the release of DNA. Instead, it was proposed that mitochondrial NETs, the extrusion of intracellular material to the surrounding extracellular environment in order to concentrate antibacterial substances and snare invading microorganisms (Lögters *et al.*, 2009; Mesa & Vasquez, 2013), were responsible for the release of mtDNA into the circulation after major trauma and subsequent surgical interventions (McIlroy *et al.*, 2015; McIlroy *et al.*, 2014). Mitochondrial NETs are said to serve as mechanisms to avoid apoptosis (an example of vital NETosis, refer to Section 3(8)(a)), as there is no implication of nuclear destruction or cell death in the formation of these structures (Yousefi *et al.*, 2008), or used as an amplification device using DNA through TLR9 to produce cellular activation in surrounding cells (Mesa & Vasquez, 2013).

(l) Mitochondrial DNA and aging

MtDNA plasma levels gradually increase after 50 years of age and a role for familiar/genetic background in controlling circulating mtDNA levels has also been proposed, as the mtDNA values of two members of the same sibling relationship older than 90 years were found to be directly correlated (Pinti *et al.*, 2014). MtDNA can modulate the production of pro-

inflammatory cytokines, tumour necrosis factor (TNFα), interleukins (IL6 and IL1a) and regulated on activation, normal T expressed and secreted proteins (RANTES), contributing to the maintenance of low-grade chronic inflammation, a common trait in elderly people. As a result, mtDNA copy number is found to be directly correlated with low-grade chronic inflammation (Pinti *et al.*, 2014) and frailty (Jylhävä, 2013). The correlation between mtDNA copy number and patient frailty was theorised to be due to mtDNA depletion in skeletal muscle or a more generalised catabolism of mitochondria in various tissues. MtDNA abundance declines with age in various tissues, such as the skeletal muscle (Short *et al.*, 2005) and neurons (Blokhin *et al.*, 2008). Jylhävä (2013) determined that mtDNA release into circulation is not due to immunological processes; but instead related to cellular metabolism and maintenance. The decline in cellular mtDNA may, in turn, result in the proportional decrease in plasma mtDNA content and Jylhävä (2013)'s finding of a lack of difference in mtDNA copy number between nonagenarians and young controls may be due to this proportional decrease. Therefore, as tissue mtDNA loss continues with increasing age, the plasma mtDNA levels remain increased until the cellular mtDNA is significantly low or depleted, resulting in a subsequent and gradual decline in plasma mtDNA, thus explaining both Pinti *et al.* (2014)'s observation of increased mtDNA levels after 50 years of age and Jylhävä (2013)'s observation of no difference between mtDNA levels of nonagenarians (over 90 years of age) and younger subjects.

(13) Pregnancy

Intact fetal cells and fetal nucleic acids are said to circulate freely in maternal blood (Bianchi, 2004). It was hypothesised that DNA may be liberated into the maternal blood by the destruction of fetal cells in the maternal circulation due to immune reactions. However, intact fetal cells in maternal blood are rare (with one millilitre of whole maternal blood in normal gestations containing approximately one nucleated fetal cell) and it is unlikely that hematopoietic or other fetal cells can account for the volume and turnover of fetal DNA. There was also no correlation between the amount of fetal nucleated erythrocytes and cell-free fetal DNA (cffDNA) levels (Bianchi, 2004; Zhong *et al.*, 2002). Lo *et al.* (1997), on the other hand, hypothesised that the rapidly growing fetus and placenta possessed tumour-like qualities that provided the possibility for large quantities of fetus DNA to circulate in the maternal plasma and serum similarly to that of tumour DNA in cancer patients (Leon *et al.*, 1977). CffDNA has also been detected in amniotic fluid, maternal urine, maternal cerebrospinal fluid and maternal peritoneal fluid, with the amount of amniotic fluid fetal DNA being two hundred times that of the amount in the maternal plasma (Angert *et al.*, 2004; Bianchi, 2004; Chan *et al.*, 2012). Bianchi (2004) speculated that DNA could, therefore, be transferred directly across the placenta or membranes via a concentration gradient. The presence of male DNA in female brains indicates that cffDNA and cells can also cross the blood brain barrier (BBB) (likely due to changes in BBB permeability during pregnancy), gets distributed to multiple brain regions and potentially persists across the human lifespan, as the oldest female reported with male DNA detected in the brain was 94 years old (Chan *et al.*, 2012).

CffDNA is present in high concentrations from as early as the seventh week of gestation and increases in concentration as the pregnancy progresses, with a remarkable increase during the last eight weeks of pregnancy (Bianchi, 2004; Lo *et al.*, 1998a). Maternal plasma DNA also increases with gestation. Chan *et al.* (2004) determined that the cirDNA of

pregnant women were longer than that of non-pregnant women (possibly due to changes in DNA source, release or degradation due to hormonal or other physiological changes) and that the length of cffDNA (< 313 bp) was shorter than that of the maternal DNA (313 – 798 bp) present in maternal blood. The short lengths of cffDNA hint towards apoptosis as their source rather than necrosis (Gahan, 2013). A study by Lo *et al.* (2010) determined that the most significant difference between fetal and maternal DNA in maternal plasma is the reduction in the 166 bp peak relative to the 143 bp peak, likely due to fetal cirDNA consisting of more molecules in which the ~20 bp exposed and nuclease-sensitive linker fragment has been trimmed from a nucleosome. It was also determined that the entire fetal and maternal genomes are represented in the maternal plasma at a constant relative proportion.

The size and abundant cellular activity of the placenta makes it a logical source of cffDNA and the correlation between fetal DNA and human chorionic gonadotropin has been confirmed, indicating that the trophoblasts of the placenta are a likely source for fetal DNA. Placental-specific mRNA is also readily detectable in maternal plasma (Bianchi, 2004). In normal pregnancies, grams of placental material are shed daily into the maternal bloodstream without eliciting inflammation (Taglauer, Wilkins-Haug & Bianchi, 2014). Pregnancy pathologies or abnormalities, e.g. placenta increta and confined placental mosaicism (where the placenta has a different genotype than the fetus), also lead to an increase in cffDNA. Additionally, it was found that cffDNA is still detectable in maternal blood after therapeutic abortion, where the expulsion of the placenta is incomplete. However, placental volume did not correlate with fetal genetic material levels, indicating that other processes independent of placental size may be involved, including apoptosis (Bischoff, Lewis & Simpson, 2005) or possibly a combined mechanism of apoptosis/necrosis (named aponecrosis) (Taglauer *et al.*, 2014). Correlations between cffDNA levels, gestational age and the apoptosis of placental trophoblast cells and fetal membranes have also been reported (discussed in detail in (Phillippe, 2015)). Cell senescence may also be involved in the release of fetal DNA and maternal DNA into the mother‘s circulation. Phillippe (2015) hypothesised the involvement of the loss of telomere sequences in the release of cffDNA through trophoblast and chorion cell apoptosis and decreases in telomeric DNA in gestational tissues during gestation have been reported (Gielen *et al.*, 2014; Menon *et al.*, 2012; Phillippe, 2015).

Elevated cffDNA levels have been observed in pregnancies complicated by preeclampsia and preterm labour patients (Bianchi, 2004; Wataganara & Bianchi, 2004), likely due to the destruction of fetal cells by the maternal immune system or due to apoptosis of placental and fetal cells (Scharfe-Nugent *et al.*, 2012; Wataganara *et al.*, 2004). These elevated cffDNA levels may serve as DAMPs that trigger an inflammatory reaction that results in spontaneous preterm birth (Scharfe-Nugent *et al.*, 2012). Fetal DNA is hypomethylated and may be detected by TLR9, resulting in NF-κB activation and subsequent inflammatory reactions (as described in Section 5(3)(a)) and the induction of preterm birth (Yuen *et al.*, 2010).

(14) Neuronal functions

Learning, post-trial sleep and circadian oscillations have been found to modulate intense DNA turnover in the adult mammalian brain (reviewed in (Giuditta, Grassi-Zucconi & Sadile, 2016)). The presence of hyperploid neuronal DNA, the more compact structure of neuronal nucleosomes, and the occurrence of unexpected DNA enzymes in brain tissue

has also been highlighted (reviewed in (Giuditta, 1983)). A recent review by Giuditta *et al.* (2016) now implicates metabolic DNA (discussed in Section 3(5)) in brain functions, as DNA fractions characterised by an elevated turnover not involved in cell division or DNA repair has been identified in neuronal cells.

Giuditta *et al.* (2016) provided a comprehensive review of the activity-dependent modulation of brain metabolic DNA reported during: (1) Axonal regeneration: injury to mouse, rat and rabbit hypoglossal nerves, mouse facial nerves and lesions of rat lateral geniculate nucleus, mouse spinal cord and mouse cerebellum resulted in a marked increase in the number of glial nuclei of the hypoglossal nucleus that contained newly synthesised DNA; (2) Inducing changes in neural activity: prolonged saline consumption, prolonged exposure to unavoidable electric foot shocks, raising rats in an enriched sensory environment or intermittent exposure to cold environments causes increased glial DNA synthesis; (3) Learning: passive avoidance task training results in significantly increased and persistent newly synthesised brain DNA levels in mice compared to quiet or shocked controls. Injection of mice and neonatal chicks with 5-I-deoxyuridine and mice with sodium nitrite and hydroxylamine before and/or after certain forms of training (passive avoidance task, imprinting, spatial learning, light cue for food retrieval and/or water maze training) resulted in impaired memory retention; (4) Circadian oscillations of sleep and waking: adults rats exposed to natural light for a week showed peaks in newly synthesised kidney DNA during resting periods, but peaks in newly synthesised cerebral cortex DNA at the beginning of active periods. Four week old rats born and raised under artificial lighting conditions showed brain data with a 12 hour ultradian period rather than a 24 hour period, which serves as part of a normal sleep rhythm at that age. The kidney data, however, maintained their circadian periodicity. Raising rats in a normally lit, enriched environment resulted in sharper circadian oscillation in brain DNA synthesis during active periods, but impoverished conditions result in the loss of circadian rhythm as DNA synthesis remained abnormally high throughout resting periods.

To summarise Giuditta *et al.* (2016)'s conclusions, brain metabolic DNA is significantly modulated by the quality and intensity of neural activities that can be distinguished on the basis of their specificity (e.g. supraoptic nucleus and posterior pituitary stimulated by saline drinking and the neurons and glia involved in axon regeneration) or pervasiveness (circadian oscillations, exposure to enriched or impoverished environments or exposure to a large variety of formal learning protocols).

(15) Genetic processes

Fig. 4 briefly summarises mechanisms of the genetic processes, rereplication, endoreplication, gene amplification and transposons, which may be involved in cirDNA production and/or release.

(m) Rereplication

Rereplication occurs when newly assembled replication forks replicate parts of the genome that have already been replicated, resulting in replication bubbles within replication bubbles (Ullah, Lee & DePamphilis, 2009). This is normally caused by perturbations to the molecular mechanisms that control the "once and only once" replication firing during a normal diploid S phase (Lee, Davidson & Duronio, 2009). In eukaryotes DNA replication initiates at

replication origin sites once recognised by the origin recognition complex (ORC). The ORC binds DNA in late mitosis or early G1 phase synergistically with cell division cycle 6 (Cdc6), followed by the recruitment of the MCM2-7 complex (a replicative DNA helicase) by Cdt1 (replication licensing factor) to the replication origins to form the pre-replicative complex (pre-RC) at the licensed origin (Gómez, 2008; Hook, Lin & Dutta, 2007). Typically, replication initiation occurs once and only once per cell cycle (Gómez, 2008; Hook *et al.*, 2007; Truong & Wu, 2011; Ullah *et al.*, 2009). Cells inactivate pre-RC formation once the S phase is initiated to prevent relicensing and re-initiation, preventing rereplication within the same cell cycle. After mitosis, the pre-RC is de-repressed for the origins to be licensed again for the next cycle. Rereplication can, however, be triggered when previously replicated sites are re-initiated when induced by the overexpression of licensing factors (e.g. Cdt1 and Cdc6) or by depletion of licensing inhibitors (e.g. geminin or Emi1) (Gómez, 2008; Hook *et al.*, 2007; Ullah *et al.*, 2009), e.g. in cancers (Klotz-Noack *et al.*, 2012; Tatsumi *et al.*, 2006; Vaziri *et al.*, 2003).

Consequences of rereplication include the accumulation of single-strand DNA, activation of DNA damage checkpoints, head-to-tail fork collision and regeneration of small double-stranded fragments of rereplicated DNA, which could lead to mitotic catastrophe (refer to Section 5(1)) (Hook *et al.*, 2007; Truong & Wu, 2011) and genomic instability that can contribute to cancer (Lee *et al.*, 2009) (Fig. 4). Because rereplication is not a normal process of mammalian growth and development, apoptosis of the affected cells is triggered, possibly releasing the rereplicated DNA fragments into circulation (Ullah *et al.*, 2009). Cdt1 overexpression alone can cause rereplication, which is augmented by Cdc6 overexpression. Upregulation of these two proteins has been demonstrated in tumorigenesis models. Cdt1 is also suggested to induce genomic instability and aneuploidy, leading to the formation of tumours (Hook *et al.*, 2007).

On the other hand, it has been speculated that rereplicated DNA can contribute to the regulation of DNA replication and/or transcription (Gómez & Antequera, 2008) (Fig. 4). The activation of DNA replication origins during the S phase in normally proliferating cells generates short DNA fragments that undergo several rounds of replication in a single cycle, producing double-stranded 100 – 200 bp DNA molecules with 5‘ attached RNA primers (Gómez, 2008; Gómez & Antequera, 2008). These rereplicated fragments derive from the nucleosome free-region that surrounds the transcription start sites of active promoters associated with the DNA replication origins (Gómez, 2008). Nucleosomes are suggested to mark the boundaries of the over-replicated regions. The overproduction of these short DNA fragments is strictly linked with the time of firing of specific DNA replication origins during the S phase, which suggests that this occurrence is a constituent step during the activation of properly licensed DNA replication origins. This form of controlled rereplication of short genomic fragments falls below the level of detection of DNA damage checkpoints. What is yet to be determined is whether the overproduction of short DNA molecules plays a role in the activation of DNA replication origins or whether it is a byproduct of replication initiation events. The physical overlapping between the overreplicated regions and transcription initiation sites indicates a possible mechanistic link exists between these processes (Gómez & Antequera, 2008). It is also speculated that these short DNA molecules that do not elongate into mature transcripts or replicons might serve as dynamic signalling mechanisms to highlight and perhaps contribute to maintaining the essential regulatory regions in the genome.

Gómez and Antequera (2008) also determined that rereplication of short DNA fragments are not due to DNA repair, are independent of bulk DNA and do not adopt the conformation of extrachromosomal circular DNA described for several genomic regions in mammalian cells under conditions of stress and genomic instability. It was also determined that this phenomenon is not limited to cell cultures, it can also occur in organisms. These characteristics are strikingly similar to that of Pelc's metabolic DNA and virtosomal DNA (discussed in Section 3(5)), which also form independently from DNA repair, are different from and independent of bulk DNA and are complexed with RNA polymerase (in the case of virtosomal DNA). There is, however, one critical difference between these rereplicated short DNA fragments, metabolic DNA and virtosomal DNA. Metabolic and virtosomal DNA are independent of active cell proliferation and can, therefore, be found in both dividing and non-dividing cells. For the rereplication of short DNA fragments, on the other hand, active cell proliferation is a requirement (Gómez & Antequera, 2008). It does not, however, imply that rereplicated DNA fragments cannot contribute to metabolic and virtosomal DNA fractions in dividing cells. Whether rereplicated DNA fragments are actively released by cells has not been determined, but they are likely to be released during cell damage and degradation. Their dependence on cell proliferation and short fragment sizes can also implicate rereplication as a source of the fractions of small cirDNA fragments observed in the circulation of cancer patients (discussed in Section 4(9)) and the shorter forms of cffDNA of developing fetuses (discussed in Section 5(4)).

(n) Endoreplication

Endopolyploidy occurs due to variations of the canonical G1-S-G2-M cell cycle that repeatedly replicates the genome without cell division (Fox & Duronio, 2013; Hieter & Griffiths, 1999; Lee *et al.*, 2009). One form of endoreplication is the developmentally controlled endocycle or reduplication (Ullah *et al.*, 2009), which consists of discrete periods of the S phase and G phase resulting in cells with a single polyploid nucleus (Fig. 4). A key feature of the endocycle is that DNA content increases by clearly delineated genome doublings, an important distinction from rereplication where uncontrolled, continuous re-initiation of DNA synthesis within a given S phase occurs, resulting in increases in DNA content without clearly recognisable genome doublings (Lee *et al.*, 2009). Another form of endoreplication occurs through endomitosis, where cells enter but do not complete mitosis (Fox & Duronio, 2013; Lee *et al.*, 2009) (Fig. 4). The same regulatory proteins that drive typical cell division cycles are used to drive endoreplication (Fox & Duronio, 2013). Endoreplication is essential for normal organismal development, where it is used as part of terminal differentiation to provide nutrients and proteins needed to support the developing egg or embryo. Increasing DNA content by endoreplication sustains the mass production of proteins and high metabolic activity necessary for embryogenesis (Lee *et al.*, 2009). Cells also use endoreplication as part of terminal differentiation to support specialised functions, facilitate growth (by increasing cell size) and to provide key functions to the adult organism. In cases where energy sources are limited or rapid growth is required, increasing cell volume without division is more advantageous (Hieter & Griffiths, 1999; Lee *et al.*, 2009). Endoreplication can also be used for growth and tissue regeneration during conditions that would otherwise prevent proliferation by bypassing the controls that maintain genomic stability through diploidy, thereby promoting homeostasis despite dire conditions (Lee *et al.*, 2009). When genome damage is detected in developing or regenerating tissues, endoreplication is utilised to increase tissue mass without proliferating the cells in order to prevent the segregation of damaged chromosomes (Fox & Duronio, 2013).

Telomere shortening or dysfunction is a potent endoreplication-promoting mechanism that induces senescence or apoptosis in many cells (Fox & Duronio, 2013; Gorla, Malhi & Gupta, 2001; Lee *et al.*, 2009), except in hepatocytes (which do not undergo apoptosis during telomere dysfunction). Senescent hepatocytes can, however, still regenerate when damaged via endoreplication (Gorla *et al.*, 2001; Lee *et al.*, 2009). Retinoblastoma proteins (RB) and p53 serve as tumour suppressors and transcription repressors during senescence (which prevents damaged and potentially oncogenic cell proliferation) (Chandler & Peters, 2013). Regulation of RB could provide a mechanistic link to continued endoreplication despite senescence and/or DNA damage (Fox & Duronio, 2013). Inhibition or loss of RB contributes to polyploidisation in hepatocytes and following telomere crisis in human fibroblasts and mammary epithelial cells. Not all cells develop this endoreplication phenotype during RB loss, indicating that tissues exhibit differing capacities for endoreplication (Fox & Duronio, 2013). Endoreplication of senescent cells instead of proliferation during RB loss could imply that there is a second barrier involved in the regulation of cell cycle machinery, namely the p53-dependent upregulation of mitotic gene repressors (Adams, 2009; Chandler & Peters, 2013). CirDNA could form due to telomere-induced senescence and/or apoptosis. Due to the connection between telomere shortening and aging, one can assume that endoreplication may be a favoured mode of tissue repair in aged individuals (Fox & Duronio, 2013), which could explain why increased age is accompanied with increased levels of cirDNA.

Polyploidy is also ubiquitous in tissues and organs after various types of hypertrophic stimulation, including myocardial cells (during hypertension), mammary glands (during lactation), endometrial cells (during pregnancy), lymphocytes (during infections) and multiple tumours and cell lines (during oncogenesis) (Gorla *et al.*, 2001). Tumour tissues and non-cancerous cells have similar switches to endoreplication to avoid apoptosis when responding to genotoxic insults or mitotic catastrophe (discussed in Section 5(1)) (Lee *et al.*, 2009). Polyploidisation could serve as a precursor to aneuploidy that may contribute to oncogenesis (Fox & Duronio, 2013; Lee *et al.*, 2009).

Programmed endoreplication in normal polyploid tissues is usually permanent and the cells or tissues are unable to revert back to mitotic cell cycles (Fox & Duronio, 2013). Normal endoreplication is therefore associated with senescence and differentiation, whereas polyploidy promotes mitotic progression in many cancer cells. Unscheduled endoreplication promotes tumour production due to the occurrence of chromosome instabilities (caused by the missegregation of chromosomes during mitosis) (Fox & Duronio, 2013). At low frequency some of the polyploid cancer cells can revert back to mitotic cell cycles (Lee *et al.*, 2009). Normal polyploid cells can also resume mitotic cycles, but exhibit chromosome instabilities similar to that of cancerous polyploid cells (Fox & Duronio, 2013), e.g. polyploid liver cells can revert to mitotic cycles that become error prone, producing aneuploid cells (cells with an imbalance in chromosome content) (Duncan *et al.*, 2010; Fox & Duronio, 2013). Studies suggest that the transition of liver cells from endoreplication to polyploidy to aneuploidy represents a mechanism for the generation of a genetically diverse pool of cells, which might be amplified under stressful conditions (Duncan *et al.*, 2010; Fox & Duronio, 2013). In a mouse liver disease model of tyrosinemia-induced injury, for example, cells with a specific aneuploidy containing an extra disease-resistance gene copy can be amplified to suppress liver injury (Duncan *et al.*, 2012; Fox & Duronio, 2013). It is possible that the reverse of polyploid cells back to mitotic cell cycles and the induction of mitotic catastrophe of cells that develop error prone mitotic cycles (discussed in Section 5(1)) can also form cirDNA.

(o) Gene amplification

DNA amplification results in an increase in copy number of a discrete chromosomal DNA region (Mukherjee & Storici, 2012) (Fig. 4). Amplified DNA regions can consist of genes whose proteins are essential for cells and other closely positioned DNA sequences associated with other functionally important genes (Mandrioli *et al.*, 2010). DNA amplification can also form double minutes, circular extrachromosomal elements that replicate autonomously and lack centromeres and telomeres (Mukherjee & Storici, 2012). Double minutes segregate randomly during mitosis and are very unstable, except when they provide selective advantage to cells by serving as carriers for extra copies of oncogenes or drug resistance genes. CirDNA could be amplified gene fragments transported between cells to amplify the production of certain proteins to enhance certain functions of an organ. CirDNA could also be amplified gene fragments released by cells after they were used to amplify a specific function in the cell.

The replication of specific gene sequences instead of the whole genome represents an evolutionary advantage, as the amplification of unnecessary DNA sequences are avoided (Mandrioli *et al.*, 2010). However, DNA amplification is a major source of genetic variation that is not necessarily pathogenic, but could lead to polymorphisms between individual genomes in organisms (Mondello, Smirnova & Giulotto, 2010; Mukherjee & Storici, 2012). DNA copy number increase is also a major molecular mechanism that drives oncogenesis in many variations of cancer and affects tumour progression and clinical outcome (Mondello *et al.*, 2010). Cancer cells could use gene amplification to produce high levels of oncogenes in order to promote metastasis or high levels of drug resistance genes, which would result in the overproduction of certain proteins in response to chemotherapy (Mondello *et al.*, 2010; Mukherjee & Storici, 2012). DNA amplification is also associated with several neuropathies and can affect susceptibility to certain diseases, including systemic lupus erythematosus (Mukherjee & Storici, 2012).

Mukherjee and Storici (2012) demonstrated that DNA amplification can be driven by small DNA fragments. The homology tracts shared between the small DNA fragments and the target chromosomal DNA serves as the boundaries of the amplicon region. DNA fragments are generated by DNA metabolic processes (DNA replication, repair and recombination), can form following reverse transcription of cellular RNAs into cDNAs, can originate from the uptake of chromosomal degradation products from cell death (necrosis or apoptosis) or lysis or can be immunostimulatory GC-rich DNA, dsRNA, antisense oligonucleotides or exogenous DNA taken up by cells (Mukherjee & Storici, 2012). The small DNA fragments produced during the activation of DNA replication origins observed by Gómez (Gómez, 2008; Gómez & Antequera, 2008) (discussed in Section 5(6)(a)) can also be a possible source for DNA amplification, due to the fragments‘ production and accumulation during the cell cycle in the nucleus. Cells therefore have multiple sources of short DNA fragments that could serve as mediators of gene amplification, including cirDNA.

(p) Transposons

Of the human genome, 44 to 45 % consists of over four million annotated transposon copies belonging to at least 848 families and subfamilies of elements, making transposons major components of human genes and chromosomes (Batzer & Deininger, 2002; Mills *et al.*, 2007). However, a more recent study determined that as much as 66 to 69 % of the

human genome could be repetitive or repeat-derived (De Koning *et al.*, 2011). Most of these transposons are inactive, with only 35 to 40 subfamilies of the retrotransposons Alu, long interspersed element (LINE-1), SINE-VNTR-Alus (Short interspersed nuclear element – variable number of tandem repeats – Alu, or SVA) and elements of the human endogenous endovirus K family (HERV-K) being active in recent human history (Mills *et al.*, 2007). Alu, LINE-1 and SVA elements and their subfamilies are considered the most active transposons and have been shown to cause diseases by integrating into human genes. HERV-K has yet to be documented to cause human diseases, indicating that these insertions are either rare or non-existent. New insertions occur at a rate of one insertion per 10 to 100 live births, indicating that humans can have up to 60 to 600 million private transposon insertions, which is equivalent to one insertion per 5 to 50 bp of the human genome (Iskow *et al.*, 2010; Mills *et al.*, 2007).

Gahan and Stroun (2010) mentioned transposons and retrotransposons as possible sources for cirNAs. Transposons are discrete pieces of DNA with the ability to change their positions in the genome by transposition, where these pieces are either copied (retrotransposons that produce intermediary RNA copies of themselves) or removed (DNA transposons) and then inserted at a new position in the genome (Bull & Adamatzky, 2013) (Fig. 4). New positions can be targeted very specifically or more or less arbitrarily (Bull & Adamatzky, 2013). CirNAs may contain transposon elements or even serve as transposons and/or retrotransposons, facilitating the transfer or copying of DNA fragments from one cell to another. DNA transposons are not active in the human genome (Belancio, Roy-Engel & Deininger, 2010), but may be horizontally transferred from other organisms to the human genome.

Recent sequencing of *in vitro* osteosarcoma cfDNA samples has revealed that DNA actively released by cultured osteosarcoma cells consists primarily of satellite DNA (satDNA) and transposable elements (TE) (unpublished results). In particular, it has been determined that ERV (K) class II, MaLR, TcMar-Tigger, ALUs, L1 and satellites were overrepresented, while LTR elements and DNA elements were underrepresented in the cfDNA compared to the human genome. The presence of satDNA and TEs in the cfDNA (1) may serve a specific function and is, therefore, deliberately released by cells, (2) may be byproducts of cellular processes, such as DNA elimination, and are incidentally biologically active, or (3) may be biologically inert byproducts. However, a strong correlation between the level of TE occurrence in the actively released DNA and TE activity and/or reactivation capacity (processes, such as DNA demethylation, can reactivate retrotransposons (Bourc'his & Bestor, 2004)) has been observed during the research, implying that these molecules are functional.

Retrotransposition could be a possible source for circulating RNA. Cells that repress apoptosis or have defects in certain DNA repair pathways (common features of cancerous cells) show higher levels of retrotransposition (Belancio *et al.*, 2010), but these RNAs remain within the nucleus (Skipper *et al.*, 2013) and the cells themselves remain intact, indicating that the cells could not release these RNAs unless there is an active release mechanism situated in the cell that could release the retrotransposon RNAs from the nucleus and the cell into the circulation.

6. Most abundant versus most functional circulating DNA fractions: Where the focus of circulating DNA research should lie

Many studies have focused on the identification of the main origin of cirDNA and two specific contenders for main origins are cellular breakdown mechanisms (particularly apoptosis and necrosis) and active DNA release mechanisms (Bronkhorst *et al.*, 2016b; Dakubo, 2016; Delgado *et al.*, 2013; Gahan, 2006; Hendy *et al.*, 2016; Holdenrieder *et al.*, 2005; Lichtenstein *et al.*, 2001; Morozkin *et al.*, 2011; Stroun *et al.*, 2001; Ulivi & Silvestrini, 2013; Van der Vaart & Pretorius, 2007; Van der Vaart & Pretorius, 2008a). At first it appeared that this continuous controversial argument is due to their generalised involvement in the release of DNA into circulation from the majority of biological features (as seen in Table 1 and Fig. 2). However, by categorising the list of biological features into putative sources of cirDNA and causes of cirDNA release it appeared that a seemingly unintended lack of consensus regarding the very definition of the term "main origin" developed once active DNA release was introduced. Since then opinions regarding the main origin of cirDNA split into two main groups: (1) those in favour of cellular breakdown, who argue that DNA debris from cell damage and/or death is the most abundant fraction of the cirDNA and, therefore, must be the main origin of cirDNA; (2) those in favour of active DNA release, who argue that the newly synthesised and purposefully released DNA fraction of cirDNA is likely the most functional fraction, making active release the main origin of cirDNA. Abundance versus function is, therefore, where the core of the "controversy" lies. By simply removing the concept of finding a "main origin" and rather classifying the two fractions separately as most abundant and most functional fractions of cirDNA, discerning which of the two should be regarded as the more important fraction becomes easier and more target or goal oriented.

The confusion that the most abundant and most functional concepts caused when searching for a main origin is completely understandable and became even more confusing once it was discovered that the most abundant cellular breakdown DNA fraction can induce damaging effects, making them both abundant and functional. As mentioned in Section 4(3), Mittra *et al.* (2015) and Basak *et al.* (2016) showed that fragmented DNA and chromatin isolated from the blood of cancer patients and healthy volunteers induce apoptosis *in vitro* and *in vivo*. Studies that focus on the bystander effect during X-radiation have shown similar damaging consequences when DNA released from irradiated cells are given to non-irradiated recipient cells (discussed in Section 4(6)(a)). Exposing untreated HUVECs for three hours to the cfDNA isolated from the growth medium of irradiated cells resulted in a similar induction of apoptotic activity and double stranded DNA breaks than that of HUVECs irradiated for three hours (Ermakov *et al.*, 2011). This apparent functionality (which one would only expect from the functional, actively released DNA fraction) and the fact that actively released DNA is indeed at significantly lower concentrations compared to that of the cellular breakdown DNA fraction, implies that the overabundance of cellular breakdown DNA fraction should make the actively released DNA fraction negligible (Holdenrieder *et al.*, 2005; Jahr *et al.*, 2001; Lichtenstein *et al.*, 2001). In effect, the common argument of apoptosis and other cell degradation forms being the main origins of circulating DNA is easily corroborated.

However, this says nothing about the biological purpose of circulating DNA, as DNA release via cellular breakdown is either (1) merely a byproduct of waste management, (2) imperfect waste management (as damaged cells, apoptotic

bodies and micronuclei, for example, can escape phagocytosis) or (3) the result of an overwhelmed waste management system (as seen in Fig. 3). There is, therefore, no intended physiological purpose for this majority fraction of cirDNA, regardless of its damage-inducing properties. Moreover, Mittra *et al.* (2015)'s treatment of recipients with the DNA of healthy volunteers induced significantly less damage than that of the cancer patients. As seen during the review (Table 1 and Fig. 2) and in our cell culture research (Aucamp *et al.*, 2017; Bronkhorst *et al.*, 2016b; Bronkhorst *et al.*, 2016c), cells have the potential to release both actively released DNA and DNA from cellular breakdown processes and cellular breakdown levels are increased in cancer patients (discussed in Section 4(9)). It is, therefore, possible that the cirDNA of the healthy volunteers contained higher levels of actively released DNA compared to DNA of cellular breakdown processes compared to the DNA from cancer patients, resulting in the lower levels of cellular damage. However, their study focused solely on the damaging effects of the administered DNA and other possible effects by actively released DNA fractions were not considered. Garcia-Olmo *et al.* (2015) contributed here as their results have shown that newly synthesised and/or spontaneously released virtosomes from non-dividing cells reduced tumour growth and metastasis, but had little effect on normal dividing fibroblasts. This not only shows that actively released DNA induced biological effects other than the damaging effects seen by Mittra and colleagues and during the bystander effect studies, but that these effects may also be cell- or tissue-specific and, perhaps, beneficial.

Four other very important factors discussed in Section 3(5) that corroborate the concept that there is a purpose for actively released DNA compared to DNA from cellular breakdown mechanisms, are that actively released DNA is (1) newly synthesised (2) only from living cells, (3) complexed to proteins or lipoproteins, encapsuled into vesicles and/or adhered to cellular membranes in order to protect it from nucleases after synthesis and (4) their levels are controlled or regulated, reaching maximum levels that cannot be exceeded (refer to Section 3(5)(a) and Section 3(5)(b)) as seen with hormones in the body, but not with the DNA of cellular breakdown mechanisms. Therefore, although actively released DNA makes up a relatively small fraction of the total circulating DNA, it appears that those small levels are purposefully managed and can elicit biological effects seemingly just as effectively as the damage induced by the majority fraction of DNA released by cellular breakdown mechanisms. With this in mind it is possible that the overabundance of DNA released by cellular breakdown mechanisms overwhelms recipient cells with DNA debris (in the form of DNA-chromatin complexes and/or nucleosomes) with intrinsic pro-inflammatory (Rock & Kono, 2008) and or DAMP properties, thereby causing the damaging effects as an inflammatory or immunological side effect and not under instruction.

The decision on which fraction and aspect of cirDNA to scrutinise for research will depend on the objectives. For example, research regarding prognostics or treatment efficiency (e.g. traumatic injuries or tissue damage) may benefit from analysing only the fluctuation of the cirDNA fraction derived from cellular destruction processes. Conversely, oncologists may learn more about the diagnostics and cancer status of an individual by analysing both the characteristics and fluctuation of DNA derived from both dead and living cells. Several *in vitro* experiments indicate that analysis of the DNA that is actively released by cancer cells could be used as multi-purpose biomarkers, providing information on the origin of the cancer, the dynamics of the tumour, its malignancy status, as well as the potential of developing metastases. In addition, by focusing on the functional fraction of cirDNA, keeping in mind its capacity to act as an

intercellular messenger, could provide deeper insight into role of cirDNA in the pathogenesis of cancer, and other diseases. However, as the cirDNA research field is dominated by the clinical perspective, which focuses solely on the apoptotic / necrotic fraction of cirDNA, the idea of characterising actively released DNA has been tarnished. As a result, the utility of basic and *in vitro* research has not been questioned seriously. Therefore, we would like to suggest that researchers / clinicians will benefit greatly by adopting a more holistic (or systems) view of cirDNA research, taking into consideration all of the biological pathways in which cirDNA is involved and also considering the utility of integrating *in vitro* and *in vivo* research.

7. Utilisation of *in vitro* models to solve *in vivo* problems

To study the minority actively released fraction of cirDNA within a living, functioning organism is extremely difficult, as there are many biological systems, factors and mechanisms involved at any given point in time (Fig. 2). Recent research has, however, shown potential in narrowing down more precise origins of cirDNA. Snyder *et al.* (2016) (discussed in Section 5(2)), Jiang and Lo (2016) and Ulz *et al.* (2016) showed that whole genome sequencing and cirDNA fragmentation patterns and sizes can be used to identify where the DNA originated from. However, in living organisms there are not only vast amounts of putative sources capable of releasing DNA into biofluids, but also vast amounts of complex systems of interconnected cellular responses that are capable of influencing or resulting in cirDNA release. In effect, focusing on fragment patterns and sizes can elucidate the origin of cirDNA only to a certain extent, but may become less effective in more serious or complicated diseases. To truly find the exact tissue origin and purpose of cirDNA or track down a tissue-, cancer- or disease- specific biological marker, one will most likely require some form of ~~"c~~losed-circuit" model of the physiological, biological or pathological area in question to remove the ~~"b~~ackground noise" produced by the cirDNA from the rest of the organism. The utilisation of *in vitro* cell culture models have been used for many years for this purpose with great success and we wish to encourage the use thereof to serve as the ~~"c~~losed-circuit" models required in cirDNA research.

The use of cell cultures in conjunction with biofluid samples can promote the *in vivo* translation of *in vitro* results. Our cell culture studies, for example, have shown strong correlations between the fragment sizes and patterns of DNA extracted from the growth medium of several cell lines and that of human plasma samples (Applied-Biosystems, 2015; Aucamp *et al.*, 2017; Bronkhorst *et al.*, 2016b). Many researchers still remain sceptic regarding the ability of *in vitro* models to represent *in vivo* systems, but as technology progresses and evolves to aid and simplify research of cirDNA in biofluid samples (Jiang & Lo, 2016; Lowes *et al.*, 2016), so is technology for *in vitro* cell culture systems evolving to improve their reflection of *in vivo* conditions. The excuse of not using *in vitro* models because they are not properly reflective of *in vivo* conditions are, therefore, becoming increasingly irrelevant.

Fibercell systems, for example, provide the opportunity to produce small-scale three-dimensional cell cultures and co-cultures for large scale cell product (e.g. protein) harvesting, with hollow fibres in bioreactors acting as artificial capillaries to simulate tissue circulation. These systems can be used for protein and exosome production (Whitford, Ludlow & Cadwell, 2015), *in vivo* drug analysis, cell secretome analysis (Chang *et al.*, 2009) and infection models (Cadwell, 2015). For example, this technology has been used to develop a tuberculosis model, approved by the

European Medicines Agency, for the development of drugs and the selection of treatment combinations and doses before they are tested in clinical trials (Gumbo *et al.*, 2015; Hughes, 2015). This model has a forecasting efficiency within 94 % of the clinical values observed in subsequent clinical trials, making this *in vitro* model as efficient as a clinical trial.

Three-dimensional cell culture techniques that produce spheroids can also present with the characteristics of tissue biopsy samples (Antoni *et al.*, 2015; Pampaloni, Reynaud & Stelzer, 2007; Wrzesinski & Fey, 2013) and can simulate organ-like characteristics, functions and responses to therapy better than primary cell lines (Ramaiahgari *et al.*, 2014). Rather than growing in monolayers, spheroids form from cell aggregates via various scaffolding/matrix or scaffold-free methods, allowing cells to re-establish communication networks between one another and the extracellular matrix that are necessary for maintaining tissue specificity and homeostasis (Pampaloni *et al.*, 2007). A culture method by Wrzesinski and Fey (2013) can be a significant benefit as this specific method (1) utilises rotating bioreactors to develop spheroids without the use of any synthetic scaffolds or matrices and (2) requires development for 21 days in rotating bioreactors, allowing the cells to re-establish physiological conditions that become suppressed by trypsinisation. Furthermore, most of the spheroids' physiological and metabolic functions have been shown to remain stable for a further 21 days. Wrzesinski and Fey (2013)'s method for 3D spheroid development, therefore, efficiently overcomes the physiological restrictions of *in vitro* cell culture methods and can be cultured for several weeks.

8. Conclusions

(1) It is evident that there are various different potential sources of cirDNA and causes of cirDNA release in the mammalian body:
   i. Healthy cells
   ii. Normal physiological occurrences (pregnancy, senescence, aging, endoreplication, gene amplification, the presence of transposons and retrotransposons in the genome, NETosis and other immunological processes)
   iii. Diseases and disorders (viral, fungal, and bacterial infections, parasitic infestation, cancer, disorders that involve or cause tissue breakdown)
   iv. Environmental and/or lifestyle factors (e.g. sources of nutrition, water, inhalation of pollen),
   v. Strenuous or excessive exercise
   vi. Abnormal genetic occurrences (e.g. rereplication that can cause genetic instability and subsequent cell death, releasing the genetic content into the circulation)

(2) The main characteristic that these sources and causes of cirDNA have in common is the release of cirDNA via cellular breakdown processes, such as apoptosis and necrosis, and/or active release mechanisms.

(3) These various sources and causes also have the ability to interact or affect one another, producing a cascade of different effects that can release DNA into circulation.

(4) It is clear that the physiological environment, function and life cycles of healthy cells and the pathophysiology of the relevant disease or disorder strongly affect the release of DNA into circulation.

(5) It is also unlikely that cirDNA will come from a single source, but from combinations of various sources and causes depending on an organism's health, age, activities and environment.

(6) By replacing the term "main origins of cirDNA" with that of most abundant and most functional fractions one can provide a clearer picture regarding where future cirDNA research should lie.

(7) The majority of the DNA fraction from cellular breakdown mechanisms reveals processes such as apoptosis and necrosis to be the most abundant fraction of circulating DNA in general. This fraction is by no means useless or "junk" resulting from cellular death as this fraction can induce damaging effects. However, this DNA fraction is mainly an after effect from other processes as it is primarily not purposefully released into circulation.

(8) It is the minority fraction of actively released cirDNA purposefully synthesised, protected from nucleases, actively released into circulation and their concentrations regulated as to not exceed certain levels, that serves as the most functional fraction of cirDNA.

(9) It is the actively released DNA fraction that will provide:

   (i) Insight regarding the true purpose of cirDNA in living organisms.

   (ii) The unique key biomarkers that researchers hope to find for the non-invasive diagnosis and prognosis of many diseases and disorders.

   (iii) Other unique methods of utilisation such as using cirDNA to identify predispositions to diseases, diagnose early stages of cancers, detect early epigenetic changes due to lifestyles and using the concept or mechanism of cirDNA for therapeutic purposes such as immunotherapy or gene therapy.

(10) The use of "closed-circuit" *in vitro* models are strongly encouraged in conjunction with *in vivo* confirmation in order to effectively isolate tissue, target or physiological system-specific actively released DNA.

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10. Conflict of interest

The authors report no declarations of interest.

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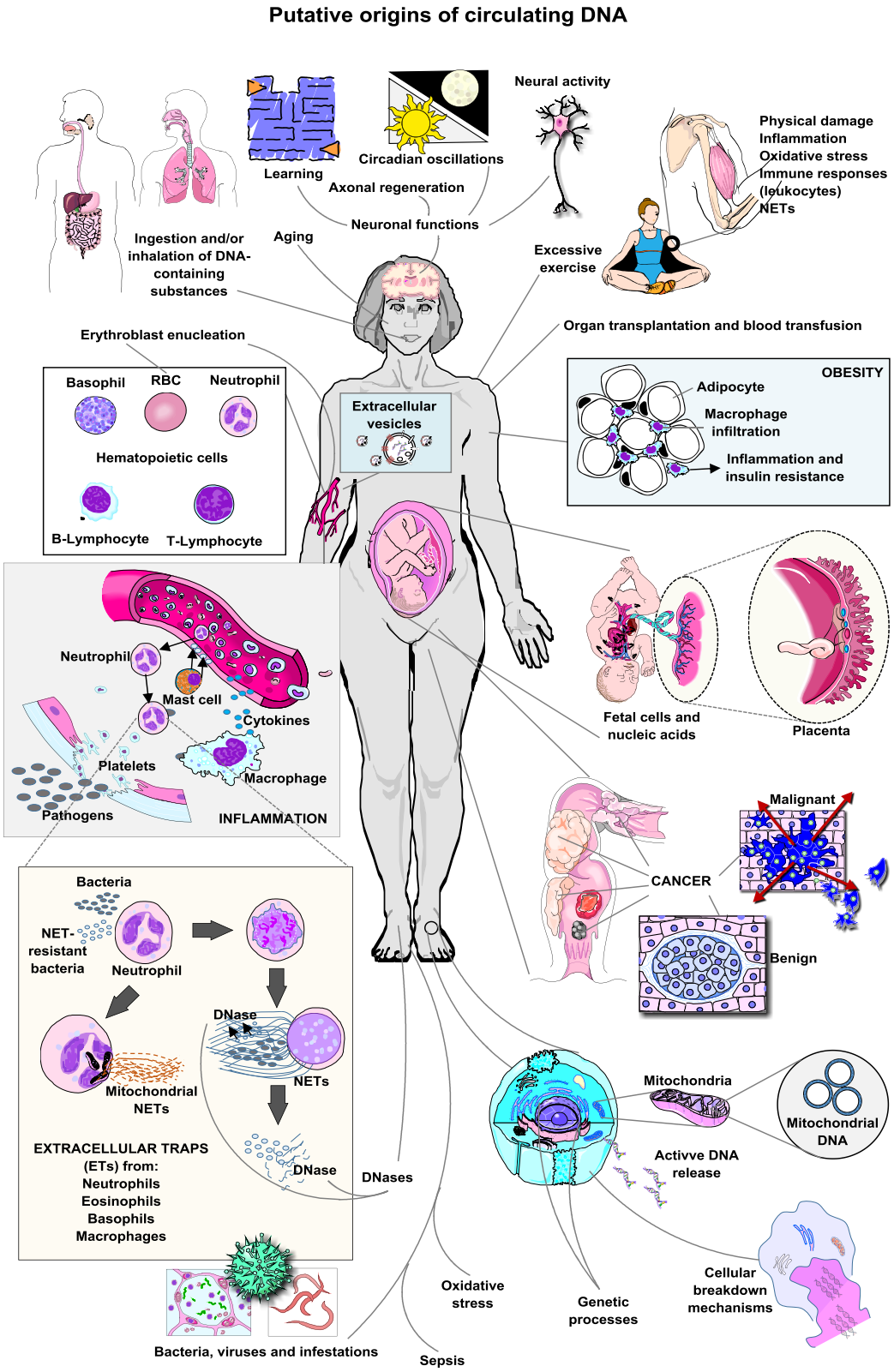

Fig. 1 The putative origins of circulating DNA

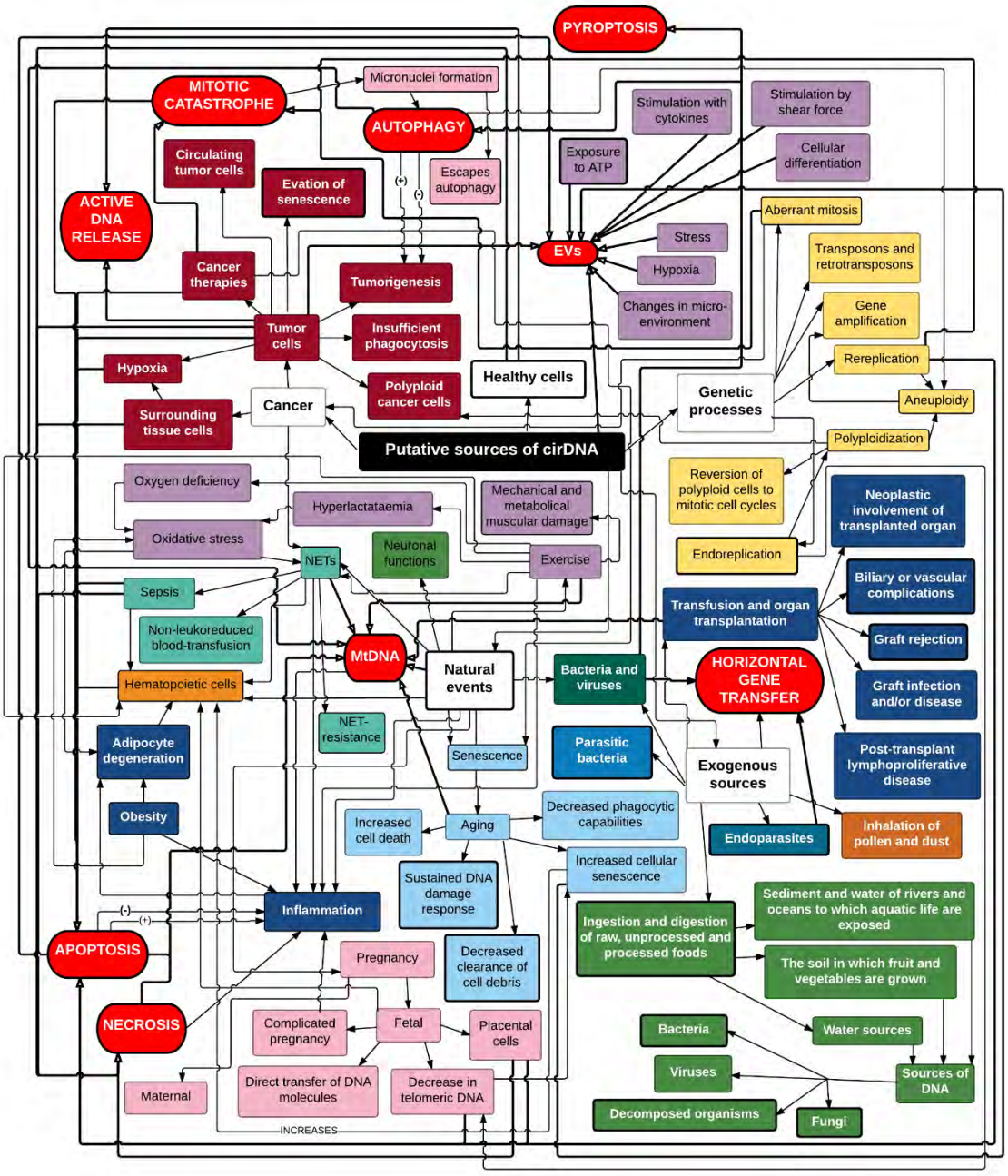

Fig. 2 The complex interactions between biological features that can further contribute to circulating DNA release and complicate both the discovery of novel biological markers and the elucidation of circulating DNA origins and biological functions.

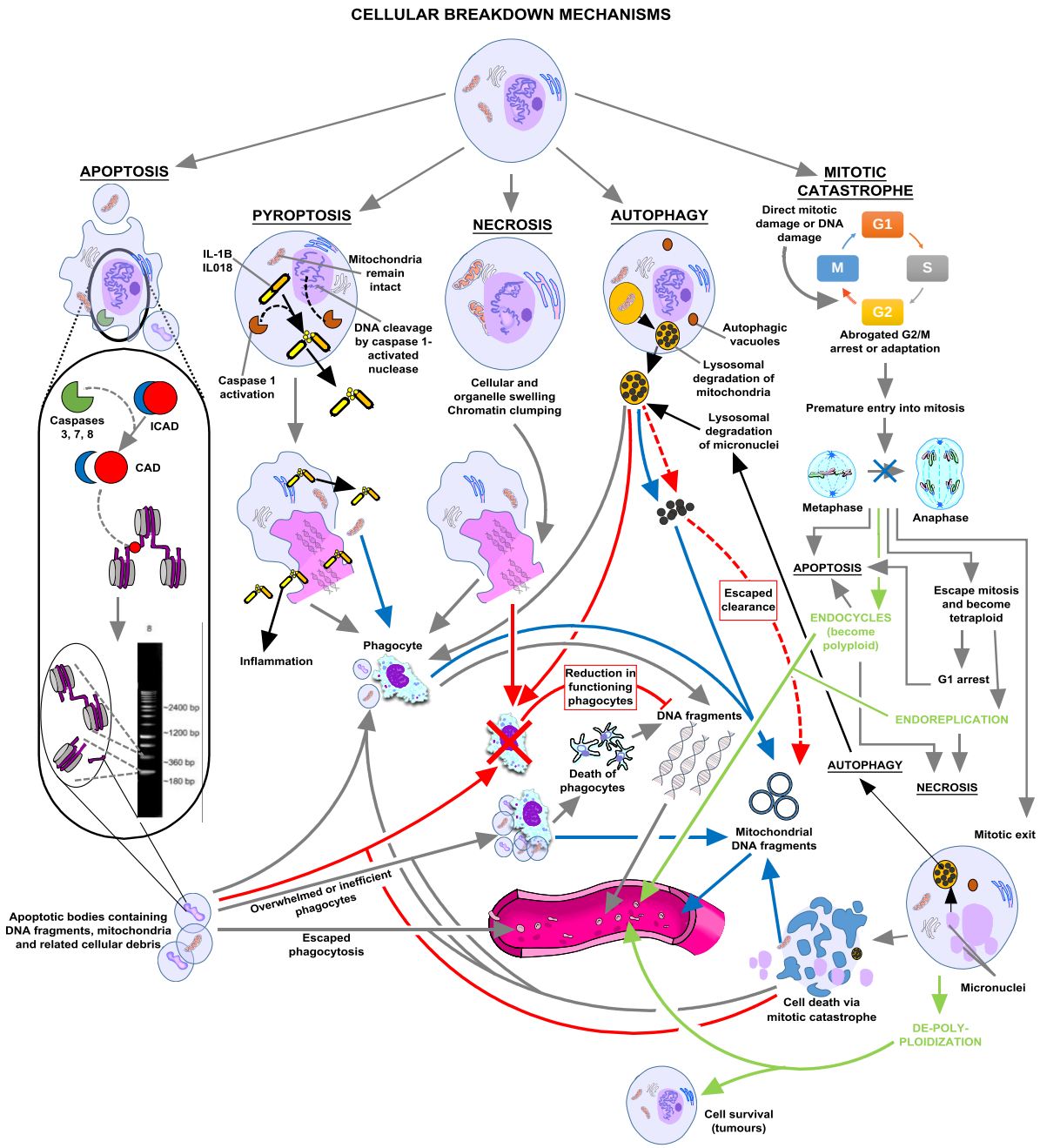

Fig. 3 Circulating DNA release by cellular breakdown mechanisms.

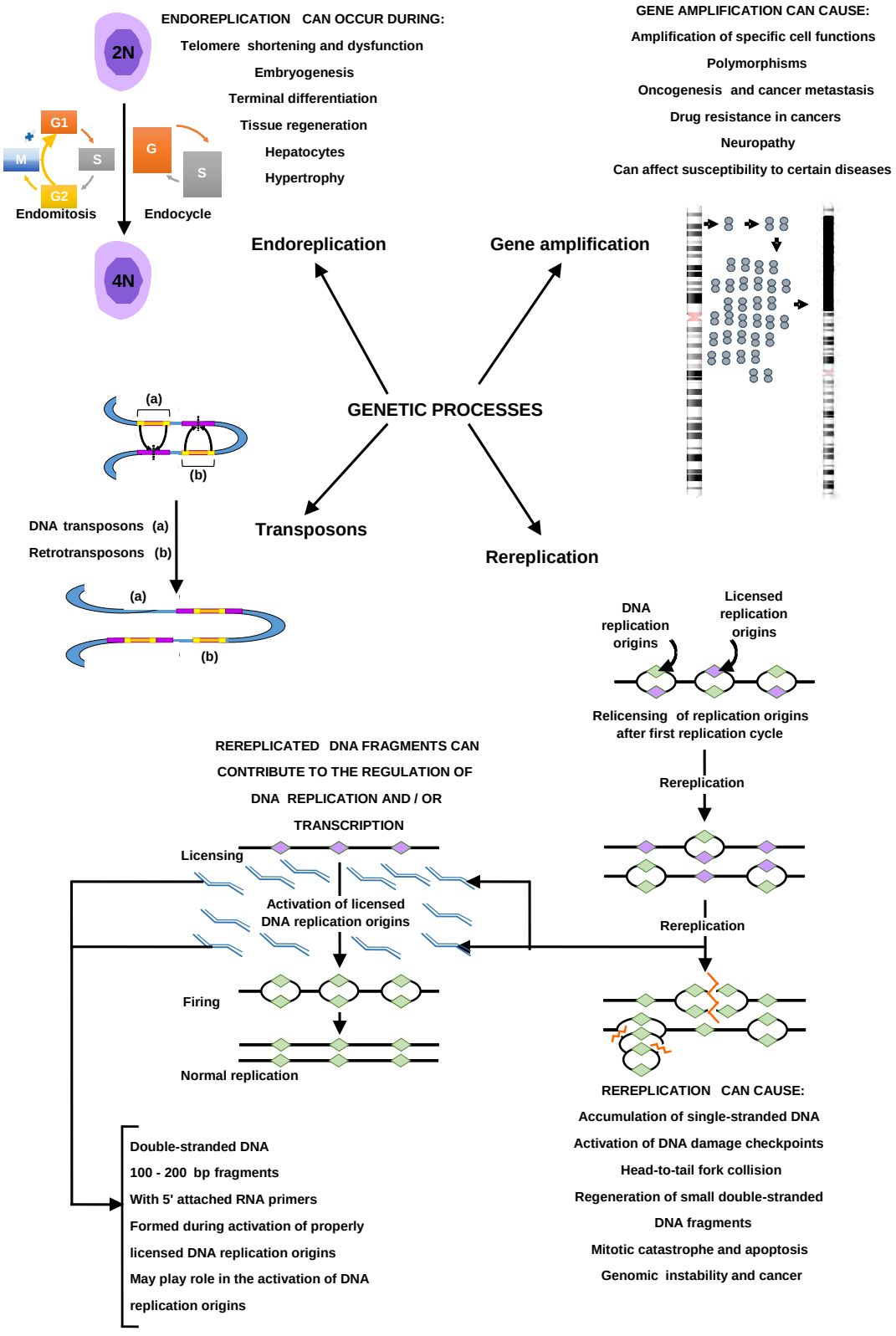

Fig. 4 Summary of genetic processes that can contribute to circulating DNA production and/or release.

Table 1 Categorisation of a comprehensive summary of putative biological features and their circulating DNA release mechanisms as sources of circulating DNA or causes of circulating DNA release from living or dead cells

Biological feature	Mechanisms involved in the release of cirDNA	Live cells	Dead cells	Source	Cause
NECROSIS	Clearance of necrotic cells via macrophages		x	x	
APOPTOSIS	When apoptotic cells escape phagocytosis		x	x	
	When apoptotic cells undergo phagocytosis		x		x
	Apoptotic bodies		x	x	
MITOTIC CATASTROPHE	Cell death via apoptosis		x		x
	Tetraploid cells (if cells escaped apoptosis)		x	x	
	Cell death via necrosis		x		x
	Cell death independent of apoptosis and necrosis		x	x	
	Micronuclei (if micronuclei escaped autophagy)		x	x	
	Cell/tumor survival (see cancer)				
	De-polyploidization	x			x
AUTOPHAGY	Autophagic clearance of micronuclei from mitotic catastrophe		x	x	
	Autophagic clearance of mitochondria		x		x
	Resulting cell death		x		x
	Pro-tumor activity				
	Tumor protection	x			x
	Promotion of tumor development in healthy cells	x			x
	Promotion of DNA damage in healthy cells	x	x		x
	Self-digestion as survival mechanism	x	x	x	
	Digestion of pathogens during infections		x	x	
PYROPTOSIS	Results in degradation of nuclear DNA		x	x	
	Mitochondria remains intact – Autophagic clearance		x		x
	Inflammation-induced pyroptosis		x	x	
	Organ failure		x	x	
ACTIVE DNA RELEASE	(virtosomes, metabolic DNA, DNA-protein complexes)				
	From endogenous cells	x		x	
	From exogenous cells or organisms	x		x	
VESICLES	(e.g. exosomes, microvesicles and excluding apoptotic bodies)	x		x	
ERYTHROBLAST ENUCLEATION	Apoptosis	x		x	
	Autophagic clearance of:				
	Pyrenocytes	x		x	
	Erythroblast mitochondria	x		x	
	Phagocytic clearance of pyrenocyte vesicles by macrophages	x			x
	Escaping phagocytic clearance		x	x	
EXOGENOUS SOURCES	Infections (bacteria, viruses)	x	x	x	
	Infestations (parasites)	x	x	x	
	Food and water	x	x	x	
	Inhalation and absorption	x	x	x	
	Transplantations and transfusions				

	Successful	x		x	
	Complications or rejection		x		x
	Autophagic digestion of pathogens during infection		x		x
	Infection-induced pyroptosis		x		x
HEMATOPOIETIC CELLS	Cell death or damage		x		x
	Healthy functioning cells	x		x	
	Pregnancy	x	x	x	x
	Exercise	x	x	x	x
MITOCHONDRIA	Cell death/injury resulting in mitochondria destruction/release				
	Mitochondria and/or their DNA escape autophagy		x	x	
	Mitochondria and/or their DNA undergo autophagy		x		x
	Cell death induced by mitochondrial DNA		x		x
	Inflammation		x	x	
	Platelets	x		x	
	Inflammation caused by mitochondrial DNA		x		x
	Aging		x	x	x
	Exercise		x	x	x
	Cancer		x	x	x
	NET formation	x	x	x	
AGING	Cell senescence and death		x		x
	Inflammation		x		x
	Mitochondria				
	> 50 years inflammation causes and is induced by mtDNA		x		x
	~ 90 years mitochondria depletion becomes evident		x		x
	Decreased clearance mechanisms, including phagocytosis		x		x
	Defective hematopoietic stem cell formation occurs	x	x		x
	Telomere loss resulting in endoreplication	x			x
NETOSIS	Specific hematopoietic cells (eosino-, baso- and neutrophils, macrophages)	x	x	x	
	Blood transfusions	x	x	x	
	Pro-tumor effects		x	x	x
	Anti-tumor effects		x	x	x
	Mitochondrial DNA				
	Vital NETosis	x		x	
	Eosinophils	x		x	
DNASES	Pathogen NET resistance				
	Endogenous DNA (from NETs and nearby DNA sources)		x		x
	Exogenous DNA (from pathogens)	x	x		x
SEPSIS	Exogenous DNA (from infection – shown to be unlikely)	x	x		x
	NET formation and NETosis	x	x		x
	Necrosis		x		x
	Apoptosis		x		x
	Pyroptosis		x		x
OXIDATIVE STRESS	Involved in various diseases, including:				
	Sepsis		x		x
	Cancer		x		x

	Aging		x		x
	NET formation and NETosis		x		x
	Release of both normal and oxidized nuclear and mitochondrial DNA via:				
	Necrosis		x		x
	Apoptosis		x		x
	NETosis	x	x		x
	In mitochondria oxidative stress induces:				
	Caspase-cascade leading to apoptosis		x		x
	NET formation and NETosis	x			x
	Inflammation		x		x
BYSTANDER EFFECT	Oxidative stress		x		x
	Apoptosis		x		x
	Adaptive response	x			x
INFLAMMATION	Necrosis-induced inflammatory responses		x		x
	Apoptosis-induced inflammatory responses		x		x
	NET formation and NETosis	x	x		x
	Adipose degeneration – low grade chronic inflammation		x		x
	Cancer and inflammatory diseases (phagocytosis processes become overwhelmed)				
	Apoptosis		x		x
	Necrosis		x		x
	Pyroptosis		x		x
	Pyronecrosis		x		x
	Necroptosis		x		x
	Release of pro-inflammatory substances (which includes nucleosomes and DNA-chromatin complexes)		x		x
PREGNANCY	Active DNA release from both mother and fetus	x			x
	From fetus				
	Hematopoietic cells	x	x	x	
	Rereplication producing short DNA fragments	x	x		x
	Placental trophoblasts				
	Active transfer of DNA across placenta/membranes	x		x	
	Apoptosis		x		x
	Inflammation (preeclampsia, preterm labor)		x		x
EXERCISE	Acute exercise without over-exertion				
	Immune reaction (NET formation and NETosis)	x	x		x
	Active DNA release mechanisms (e.g. vesicles)	x			x
	Chronic strenuous or damaging exercise				
	Apoptosis		x		x
	Necrosis		x		x
	Active DNA release mechanisms	x			x
CANCER	From tumor cells				
	Cell death due to increased tumor burden		x		x
	Active DNA release	x			x
	NETs to promote or prevent tumor development	x	x		x

	Rereplication producing short DNA fragments	x	x		x
	From cells surrounding the tumor				
	Cell death due to increased tumor burden		x		x
	NETs in response to tumor development	x	x		x
	Inflammation		x		x
	Therapy-induced cell death (e.g. apoptosis, pyroptosis)		x		x
	Senescence				
	Polyploidy formation when escaping senescence	x			x
	Tumor genesis promotion via senescence	x			x
	Mitochondria				
	Cell death		x		x
	NET formation and NETosis	x			x
	Inflammation		x		x
DNA REREPLICATION	Induces genomic instability resulting in tumorigenesis (see cancer)		x		x
	Induces apoptosis		x		x
	Formation of short DNA fragments during DNA replication or transcription	x	x	x	
DNA ENDOREPLICATION	Polyploidization				
	To evade apoptosis and mitotic catastrophe	x		x	
	Tumorigenesis due to chromosome instability	x	x		x
	De-polyploidization	x		x	
	Error prone mitotic cycles resulting in mitotic catastrophe		x		x
	Telomere shortening-induced endoreplication				
	Apoptosis		x		x
	Senescence	x	x		x
GENE AMPLIFICATION	Active release of amplified gene fragments	x		x	
	Release of amplified gene fragments through cell death/damage		x		x
	Double minutes	x		x	x
	Tumorigenesis	x	x		x
	Increases disease susceptibility	x	x		
TRANSPOSONS	Endogenous retrotransposons	x		x	
	Exogenous DNA transposons	x	x	x	

Chapter 3: Method optimization and standardization

3.1 Selection of appropriate cell lines for cfDNA analysis

In this study, a human bone cancer (osteosarcoma) cell line (143B), obtained from the American Type Culture Collection (ATCC® CRL-8303™), was used as the primary cell culture model for the development of a standard operating procedure and characterization of cfDNA. The selection of this specific cell line was based on its malignancy, stability and historical success in cell culture work performed both at our laboratory, as well as those of other research groups. For follow-up investigations (Articles **IV** and **VI**), several other cell lines were selected based on differences in tissue of origin, growth rate and cancer status, and is summarized in the table below:

Table 1: Cell lines used in this study

Cell line	Abbreviation	Code	Cancer status	Notes
Rhabdomyosarcoma	RD	(ATCC® CCL-136™)	Malignant	Large muscle tumor cells
Fibroblast	FIBRO	N/A	Non-malignant	Skin cells
Melanoma	A375	(ATCC® CRL-1619™)	Malignant	Skin cancer cells
Keratinocytes	HaCat	(AddexBio)	Non-malignant	Immortalized human skin cells
Cervical adenocarcinoma	HeLa	(ATCC® CCL-2™)	Malignant	HPV-induced cancer cervix epithelial cancer cells
Human embryonic kidney	HEK-293	(ATCC® CRL-1573™)	Non-malignant	Adenovirus-transformed cells
Hepatocellular carcinoma	HepG2	(ATCC® HB-8065™)	Malignant	Cancerous liver epithelial cells

3.2 Development of a robust preanalytical workflow

The main purpose of this study was to investigate the biological properties of the cfDNA present in the growth medium of cultured cells. To do this, it was necessary to assess its fluctuation, in conjunction with structure, over different time periods (Article **V**). Subsequently, comparisons needed to be made between different cell lines (Article **VI**). The final objective was to sequence this DNA, which required relatively high concentrations (Article **VII**). Furthermore, it is a long term goal to further develop these protocols while expanding the scope of these studies.

However, since the use of *in vitro* cell-culture models for the study of cfDNA is largely unchartered territory, there was not enough literature from which an appropriate methodology could be confidently adapted. Furthermore, many pilot experiments that were performed in the early stages of this study identified some challenges concerning *in vitro* cfDNA analysis that needed to be overcome. One of the first stumbling blocks was the relatively low levels of cfDNA in the growth medium of cultured cells compared to that of human blood. It was necessary to detect small fluctuations in these concentrations. Therefore, sufficient amounts of cfDNA needed to be isolated from a relatively large volume of growth medium. A related issue of concern was that cfDNA levels in culture medium seemed to be sensitive to some experimental or methodological changes, and external perturbations. Examples include: (i) due to different structures of cfDNA, the application of different centrifugal forces during sample processing can fractionate cfDNA to some degree causing either sample gain or loss; (ii) since the plastic of different tube types have different adsorption interactions with DNA (binding to tube walls) the inconsistent use of tubes throughout experiments can potentially influence downstream analyses; (iii) fluctuations in composition or temperature of growth medium, phosphate buffered saline or trypsin also seemed to have some effects.

Therefore, the general success of this study was highly dependent upon the development of a robust preanalytical process that is effective, precise, and repeatable. As such, it required careful consideration of numerous variables that may cause confusion. Early in the enquiry it was recognized that there are many points of contact between the methods and materials used in translational cfDNA studies and those that were intended to be used for *in vitro* cfDNA analysis in this study. However, as mentioned in Chapter 2, there is an alarming lack of analytical consensus among different groups doing applied cfDNA research. Since it was an important aim of this study to demonstrate that *in vitro* cfDNA research can be used in conjunction with bio-fluid based analyses, the development of a standard preanalytical workflow for *in vitro* cfDNA analyses was done in parallel with evaluating the implications thereof for *in vivo* studies. These results are reported in Article **III**. The hope for combining these two different lines of thought into one coherent stream, is that it could contribute information that will help with the optimization, standardization and consolidation of the preanalytical techniques used in both *in vitro* and *in vivo* studies, and also demonstrate that *in vitro* studies can facilitate *in vivo* studies. A separate investigation was done to establish the most suitable housekeeping gene to use for cfDNA quantification (Article **IV**). Although β-Globin was identified as the second most stable of the eight genes tested, it was used for quantification instead of ACTB, because β-Globin is used the most widely for cfDNA quantification. Furthermore, in cases where it was not necessary to use PCR, cfDNA was quantified with the Qubit Fluorometer.

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According to the studies reported in Articles **III** and **IV**, along with insight from pilot studies, a standard operating procedure was established and maintained throughout the study, and is given in the Methods and Materials sections of Articles **V**, **VI**, and **VII**.

Article III

Cell-free DNA: Preanalytical variables

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Invited critical review

Cell-free DNA: Preanalytical variables

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ABSTRACT

Since the discovery of cell-free DNA (cfDNA) in human blood, most studies have focused on diagnostic and prognostic uses of these markers for solid tumors. Except for some prenatal tests and BEAMing, cfDNA analysis has not yet been translated to clinical practice and routine application appears distant. This can be attributed to overlapping factors: (i) a lack of knowledge regarding the origin and function of cfDNA, (ii) insufficient molecular characterization, and (iii) the absence of an analytical consensus. In this review, we address the latter determinant and focus specifically on quantitative analysis of cfDNA. While the literature reports limited value for a single quantitative assessment, cfDNA kinetic assessment will be an essential component to qualitative characterization. In order to establish quantitative analysis for accurate kinetic assessments, process optimization and standardization are crucial. This report elucidates the most confounding variables of each preanalytic step that must be considered for optimal analysis.

Contents

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1. Introduction

1.1. Background

The first half of the 20th century marked a new point of departure in the explanation of numerous biological phenomena, especially heredity and disease. Three discoveries stand out as prominent milestones. First, in 1928 Fred Griffith demonstrated the transmission of a pathogenic property from heat-killed bacteria to a living non-pathogenic strain by interchanging culture medium [1]. Second, in 1944 Avery and co-workers showed that such an acquisition of pathogenicity can be ascribed to the transmission of genetic material [2]. These observations not only enabled others to infer the role of DNA in heredity, but also provided the first evidence for the occurrence of DNA beyond the confinement of cells. Last, in 1948 Mandel and Metais discovered the presence of cell-free DNA (cfDNA) in the plasma of healthy and diseased humans [3]. However, little attention was drawn to this pivotal discovery, probably because it preceded the structural clarification of DNA.

Two decades later, Tan et al. observed increased cfDNA in the blood of patients with systemic lupus erythematosus vs healthy subjects [4]. In 1973, Koffler et al. made the same observation, but also showed that patients with other afflictions such as leukemia, rheumatoid arthritis and malignant tumors also present with increased levels of cfDNA [5]. At this point, with the relative non-invasiveness of blood sampling in mind, many began to wonder whether quantitative analysis of cfDNA may have diagnostic value. This question was answered partially in 1977, when Leon et al. showed that the amount of cfDNA in the blood of patients with different types of cancer is notably higher than in healthy subjects. They also noticed that this phenomenon is more pronounced in patients suffering from metastasis. Moreover, after these patients received radiation therapy most showed a significant reduction in cfDNA levels, which generally correlated with improvement in clinical condition [6]. These results were the first that suggested the potential of cfDNA to serve as a marker for the detection and therapy monitoring of disease. Since these analyses could potentially replace more expensive and invasive screening methods, cfDNA research started to attract the attention of many biologists, especially oncologists.

1.2. Translation of cfDNA analysis to clinical practice

Except for a few groups that have found no correlation between cfDNA levels and clinicopathological data [7,8], the observations made by Leon et al. have been extensively corroborated [9–12]. However, despite showing great promise as potential biomarkers, quantitative analyses of cfDNA have not yet been translated to clinical practice and routine application seems distant. This struggle can be attributed to three overlapping factors, and can be summarized as follows: (i) a lack of knowledge regarding the origin and function of cfDNA, (ii) insufficient molecular characterization, and (iii) the absence of an analytical consensus. The purpose of this work was to develop a perspective on the necessity of an analytical consensus, and to gather information and to produce data that will be useful considerations for the optimization of cfDNA measurements and development of a standard operating procedure.

In the case where data from individual studies are compared, the concentration of cfDNA in the blood of cancer patients is generally higher than in normal subjects [13–16]. Indeed, a comprehensive review by Fleischacker et al. summarizing more than 50 assessments by different research groups indicates that this holds true in most cases [17]. However, when comparing results from a collection of studies it is clear that there is a major confliction of data. CfDNA in the plasma of cancer patients ranges between just a few ng/mL to several thousand ng/mL, which overlaps with the concentration range of healthy humans [17–21]. To emphasize these inconsistencies, van der Vaart and Pretorius generated a graphical representation of the different yields of cfDNA reported in the literature. This way the cfDNA concentrations of individuals in over a hundred groups, categorized as healthy, cancer afflicted, and unhealthy individuals that do not have cancer, could be compared. After careful inspection it is apparent that there is a significant overlap of cfDNA concentrations between the different groups, making it nearly impossible to establish a normal reference range for any type of cancer, or to establish a cut-off value for diagnosis [22].

The reason for this overlap can be ascribed to two factors. Firstly, it has been demonstrated that cfDNA levels are not constant, but fluctuate within healthy and diseased individuals when measured over both long and short periods [23,24]. This could be attributed to a web of cellular responses to various environmental cues and stressors (such as the circadian rhythm, heavy smoking, non-malignant diseases, exercise, heart dysfunction and medicinal status, for instance). Since all cells release cfDNA, the total amount of cfDNA in the blood of cancer patients is thus not only representative of tumor derived DNA, but also of DNA released by "healthy" cells under different conditions. Additionally, cfDNA released by malignant cells is not necessarily just aberrant. This could cause false positive/negative results [22]. However, a further consideration of this matter falls outside the scope of the task. Secondly, there is a total lack of an analytical consensus and no standard operating procedure in place. The disparities between research groups mainly arise in the selection of DNA isolation and quantification methods. It has been demonstrated that the amount of cfDNA extracted by different methods may vary as much as 50% [25]. However, quantitative variation of cfDNA is not only caused by using different methods for the same type of measurement, but is also observed when the same extraction methods are utilized for duplicate samples by different groups [26].

Recent work by Malentacchi et al. exemplifies this state of affairs. They reported the findings obtained by the SPIDIA-DNAplas project, one of the subprojects of the EU SPIDIA (Standardization and improvement of generic pre-analytical tools and procedures for in vitro diagnostics.) The former is based on the implementation of an external quality assessment scheme for the evaluation of the influence of the pre-analytical phase on cfDNA measurements. This included questionnaire responses of 56 participating laboratories from 21 European countries on current laboratory policies regarding blood collection tube type, sample size, time between venipuncture and processing, time between processing and DNA isolation, storage temperature of plasma, and the DNA isolation and quantification methods. This revealed a remarkable difference in approach between the different laboratories [27].

Furthermore, Malentacchi et al. prepared plasma samples which were then sent to each of these laboratories for cfDNA extraction, after which it was sent back for quantification and integrity evaluation. It was found that the cfDNA concentrations ranged between 2.87 and 224.02 pg/μL (with a median of 23.15 pg/μL and an interquartile range of 22.27). Moreover, only 12.5% of the laboratories obtained non-fragmented cfDNA [27].

1.3. Is the use of cfDNA as a biomarker feasible?

It is clear that a single quantitative assessment is of limited value, and it's unlikely that it could be used as a stand-alone biomarker for disease. However, since the association between elevated levels of cfDNA and cancer became known, many observations have been made indicating that cfDNA also possess unique qualitative characteristics that can be exploited for the same purpose. Firstly, tumor-associated molecular characteristics, such as tumor suppressor genes, oncogenes, microsatellite alterations, methylation changes, copy number aberrations, single nucleotide mutations and chromosomal rearrangements have been detected in the cfDNA of cancer patients [22,28]. Unfortunately, none of these properties have thus far been correlated with a specific disease. Secondly, it has been demonstrated that the relative size distribution of cfDNA correlates with clinical condition, i.e., whether one is afflicted with cancer or not. It was also shown that the different size groups exhibit unique molecular characteristics [28]. However, similar to quantitative analyses, fragmentation values of cancer patients overlap with

values of control subjects. Although Jiang et al. are in the process of reconciling the discrepancies between studies, it is as yet a partial victory. Lastly, a clear line of causality between the transfection-like uptake of tumor-derived cfDNA by normal cells and the development of metastasis has been drawn. This was demonstrated to occur in vitro [29] and in vivo [30]. If further corroborated, this would be a remarkable new paradigm in how cancer progression occurs.

These studies have set the stage for others to investigate the relevance of cfDNA analysis in the diagnosis and prognosis of diseases and conditions other than cancer. For instance, elevated levels of cfDNA have been associated with a myriad of other diseases/conditions such as diabetes [31], myocardial infarction, stroke, organ transplant rejection [32], inflammation [33], sepsis [34,35], septic shock [36], aging [37], cell death in elderly patients [38], fatigue [39,40], and traumatic injuries [41]. With regard to strenuous exercise and traumatic injuries, cfDNA levels correlated with recovery, which suggests a new method to quantify cellular damage, or in the case of traumatic injury serve as a predictive marker of fatal outcome. Furthermore, high concentrations of fetal DNA has been observed in the blood of pregnant woman that present symptoms of placenta accreta [42], gravidarum [43], and pre-eclampsia [44]. In addition, Lo et al. showed that the entire fetal genome is represented by maternal plasma-derived cfDNA, providing a novel way for non-invasive determination of the mutational status of a fetus [45]. Considering the matters discussed above, it seems likely that quantitative analysis of cfDNA will be very useful when assessed in conjunction with rigorous qualitative characterization. Indeed, a thorough analysis of the kinetics of cfDNA concentration will certainly make an analysis more clinically relevant.

To confidently use quantitative analysis for these purposes, the reliability and universal equivalence of procedures is imperative. Although many have conceded that this is very important, no adequate attempt has yet been made to reconcile the discrepancies between the various methods used. The lack of head-to-head comparative studies between techniques has undoubtedly led to the development of in-house procedures that are prejudicial to the smooth translation of cfDNA analysis to clinical practice [46]. For this reason, the purpose of this work was to scrutinize the literature in order to identify possible variables that may affect quantitative measurements of cfDNA at each preanalytical step (summarized in Fig. 1.), and then to determine experimentally whether slight modifications of the former correspond to changes in cfDNA concentration. At our laboratory we are developing a eukaryotic cell culture model to study possible factors that may hinder the translation of cfDNA analyses to clinical practice. This is achieved by evaluating the characteristics and composition of cfDNA released by cells into the culture medium. Out of necessity, the isolation and quantification of cfDNA had to be optimized and standardized. After doing so, results became much less confusing and experiments became repeatable. Although we use growth medium as a source of cfDNA instead of blood, there are many points of contact between the methods used. The results obtained will thus be relevant to both in vitro and in vivo studies.

2. Preanalytic factors that affect cfDNA measurements: literature survey

2.1. The choice of matrix: plasma or serum

Publications in various clinical fields have demonstrated that cfDNA is remarkably more abundant in serum samples than in plasma samples [47]. When these differences in yield were further evaluated, it became apparent that not only are higher levels of DNA consistently detected in serum, but that serum shows notably more variation between patients [22]. The elevated levels of DNA may be due to genomic DNA contamination. It has been reported that clotting in the collection tube results in the lysis of leukocytes and hematopoietic cells [22,48–50], and that the fragment length of DNA in serum is significantly greater than those found in plasma [51]. Considering that plasma samples show less variability and are less likely to suffer from cellular contamination, it is safe to recommend its use as a source of cfDNA for all analyses, especially for mutation analysis given that the lower levels of background wild-type DNA would appease the detection of mutated DNA. It may also be of particular interests that the vesicles with which cfDNA is associated (virtosomes, HDL, argonaute 2, exosomes) are capable of binding to fibrin and fibrinogen [52]. The removal of this fraction from blood to obtain serum could thus result in the loss of crucially informative cfDNA. Furthermore, platelets have been shown to be the main producer of microparticles that play a key role in thrombin generation. Defibrination of plasma would thus result in the loss of platelet-derived microparticles, and consequently the loss of more cfDNA [53]. Although it has been established that plasma is more suited for cfDNA analysis, and Thierry et al. demonstrated that plasma is undoubtedly a worthier source of tumor-derived DNA, studies regarding the clinical significance of cfDNA are in many cases still based on serum samples [54]. It is thus necessary to stress once again that, keeping in mind these discrepancies between plasma and serum, it is advisable to rather use plasma and not to compare results between these two sources.

2.2. Sample processing

An absolute prerequisite for quantitative and qualitative analyses of cfDNA is uncontaminated starting material. In other words material free from cells that may release cellular DNA at any preanalytical step. Van Wijk et al. [55] and Poon et al. [56] have, however, found plasma to be contaminated with fetal cells. The artificial increase of cfDNA during blood processing was then pointed out by Chiu et al., when they showed that total DNA concentration was influenced greatly by the blood processing protocol followed. They went on and demonstrated that microcentrifugation or filtration is required prior to centrifugation in order to rid the plasma of all cellular DNA [57]. Herrera et al. then observed a dramatic difference in yield between banked (819 μg/L) and fresh (13 μg/L) samples from esophageal cancer patients. This difference was ascribed to the fact that banked samples were subject to only one centrifugation step at 1600 × g for 5 min, while the fresh samples were centrifuged twice for 10 min at 1600 × g [58]. Contrarily, Lui et al. observed no difference in DNA yield between samples that were centrifuged once or twice. When further subjecting samples to multiple centrifugation steps, still no change in DNA yield was observed [50].

2.3. The effect of anticoagulants

EDTA, heparin and citrate are the most commonly used anticoagulants. Provided that blood is processed before 6 h after collection, none of these anticoagulants affect the amount of cfDNA isolated. When, however, processing is delayed for longer than 24 h, EDTA surmounts heparin and citrate [59]. Although it has been previously suggested that heparin could inhibit PCR [60], and its use was contraindicated by Messaoudi et al., Lam et al. observed no effect on RT-PCR.

2.4. Storage conditions

It is known that between venipuncture and blood processing, cfDNA concentrations increase over time. The point at which time delay causes an abrupt spike in concentration is still wrapped up in contention. Some authors have observed this increase to ensue completely within 2 h [61], while others have reported 24 h [48]. Results by Messaoudi et al. indicate that DNA concentration remains stable up until 4 h, but increases dramatically after 6 h of delay [21]. In contrast, Jung et al. observed no increase in concentration after 8 h of storage [62]. In addition, others have reported that delaying processing for 6 h did not have a significant effect on concentration [49,50]. The results of all experiments were not influenced by storage temperature. Although this is also the case with plasma, time of storage before DNA extraction has no effect. For instance, storage at − 80 °C for two weeks or at − 20 °C for four weeks and

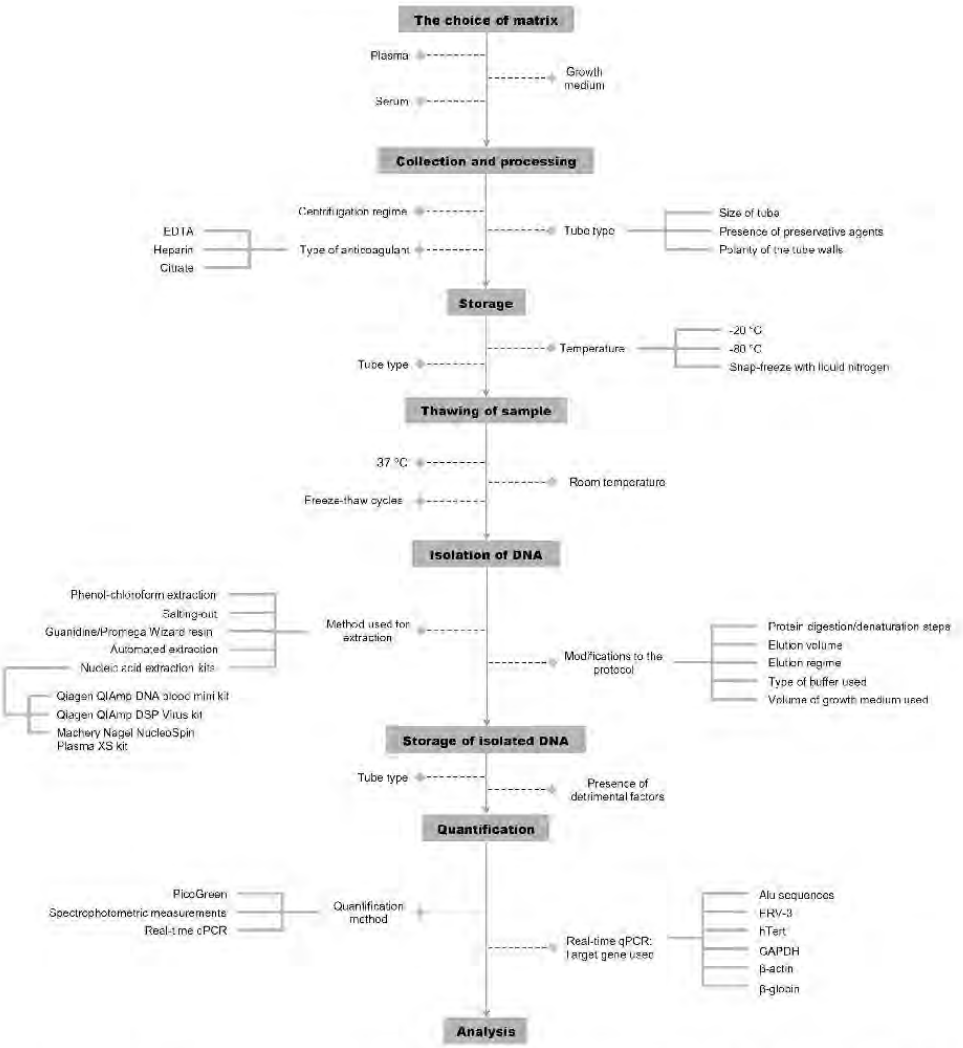

Fig. 1. Preanalytical factors that affect quantitative measurements of cfDNA. These variables are discussed in detail in Section 2. The most confounding variables were then selected and scrutinized further experimentally. These results are summarized in Figs. 2–5.

3 freeze-thaw cycles had no significant effect on DNA concentration. In the case of the latter, however, DNA was observed to be more fragmented. This is not true for isolated cfDNA stored in elution buffer [49].

2.5. Type of DNA isolation method

Due to the wide range of different cfDNA extraction methods that are currently available, selection of the most efficient and cost effective is a difficult and arduous task. In the case of low DNA concentrations, it is further exaggerated. The extraction methods that are most frequently used by researchers include the phenol chloroform method, salting-out, Guanidine/Promega Wizard resin, automated extraction with the Kingfisher (ThermoLifeSciences) and MagNA Pure (Roche) robotic systems, and the QIAamp DNA Blood Mini Kit from Qiagen. The latter method has been reported to recover 82–92% of cfDNA from serum [63], with a relative standard deviation of 14.37% [64]. However, when Legler et al. compared nine extraction kits they found the QIAamp DSP Virus Kit to deliver the highest yield, although results obtained by the QIAamp DNA blood mini kit were comparable. The lesser amount of cfDNA obtained by the QIAamp DNA blood mini kit was ascribed to the fact that it was designed for the extraction of large fragments of genomic DNA [65]. This was also reported by other independent groups [66]. It is noteworthy that Clausen and coworkers also found the QIAamp DSP Virus Kit to transcend the QIAamp DNA blood mini kit in terms of yield and also proposed that an extraction system tailored for viral DNA may favor small fragments of DNA, which should be essential for cfDNA extraction [67]. Furthermore, it has been demonstrated that the NucleoSpin Plasma XS Kit from Macherey-Nagel is superior to the QIAamp DNA blood mini kit in terms of yield, purity and efficiency

of small DNA fragment retrieval. Moreover, the kit was said to have fewer steps with shorter durations [68]. This finding is supported by a study done by Fleischhacker et al., in which they have compared 3 spin column extraction kits, namely the QIAamp DNA Blood Midi Kit from Qiagen, the NucleoSpin Kit from Macherey-Nagel and the MagNA Pure isolation system from Roche Diagnostics. The automated roach system produced 2–3 times more DNA than the MN columns and 5–10 times more than the Qiagen columns [26]. It stands out that the two most preferable methods are the QIAamp DSP Virus Kit and NucleoSpin Plasma XS Kit from Macherey-Nagel.

2.6. Modifications to the DNA isolation protocol

Xue et al. stated that protein digestion/denaturation is one of the most important steps during the isolation of cfDNA. Using serum samples, they compared the denaturing capabilities of the protease included in the QIAamp Blood Kit with proteinase K (400 μg/mL). They found incubation for 1 h with proteinase K, as opposed to no incubation, to greatly increase the recovery of DNA, and also beats the protease, from Qiagen, with regard to yield. Moreover, they showed that longer incubation time (2 h) and higher incubation temperature (50 °C) showed no further effects on DNA yield. Furthermore, Xue et al. examined the effects of using different volumes of elution buffer on the yield of cfDNA. As the elution volume was incrementally increased, so did the yield of DNA. At a certain point (300 μL), however, a plateau is reached. When they used distilled water instead of AE elution buffer, or pre-warmed the elution buffer at 37 °C or 50 °C, no significant difference in yield was observed [61].

2.7. Types of tubes used

Swinkels et al. found a correlation between the size of collection tubes and thus the amount of blood collected and the degree of cellular DNA contamination in plasma. Regardless of storage temperature, cellular DNA could still be removed prior to DNA isolation by centrifugation at 16,000 × g for 5 min [69]. The influence of the different kinds of collection tubes has been noted. Gautschi et al. reported that the use of either Vacutainer or S-Monovette tubes to isolate cfDNA from plasma or serum did not affect yield [12]. The same was observed when EDTA and cell-free DNA™ blood collection tubes were compared for samples in which blood was processed promptly after venipuncture [70]. When, however, a preservative agent is added to inhibit blood cells from releasing genomic DNA during longer periods of storage, the cell-free DNA™ blood collection tubes perform better [71]. It has been reported that part of a sample can be lost due to adsorption on tube walls [72]. DNA in the double helix form is very hydrophilic. Polypropylene, the main constituent of the most generally used plastic microtubes, however, is a hydrophobic polymer and can bind DNA [73]. The interaction of DNA with polypropylene and its subsequent conformational changes, complete denaturation, and strand separation has been demonstrated [74,75]. Moreover, this interaction was shown to be more pronounced with short fragments of DNA [76]. Other tubes, such as polyallomer tubes have been shown to be more suitable for the storage of isolated DNA [73]. These tubes, however, are five times more expensive than polypropylene tubes which may turn out to be immoderate considering the large amount of tubes that are typically used.

In light of this, several other less-expensive tubes have been tested, only to reveal that alternative tube types, such as polyethylene, results in even more loss of sample due to adsorption. As adsorption to the walls was shown to be more pronounced at higher ionic strengths, it is advisable to maintain relatively low ionic strength as well [72]. To avoid binding of DNA to tube walls as a confounding variable in experiments, pollyallomer tubes can be used, or one type of tube should be used for all extractions.

2.8. The presence of detrimental factors in isolated DNA samples

Anchordoquy examined the detrimental effects of metal contaminants in DNA samples on the integrity of the DNA. They observed that during freezing, trace amounts of metal ions such as cupric and ferrous iron destabilized lipid/DNA complexes, causing damage to DNA. An attempt was made to preserve DNA integrity by means of adding DTPA and BPS, which are chelating agents. ROS formation was successfully reduced, leading to the inhibition of iron-induced damage [77]. It was also found that the same form of DNA protection is exerted by spermine. It, however, is only sufficient at low concentrations of metals. At higher concentrations spermine actually induced DNA damage. The damaging effects of spermine decreased as the ionic concentration of the medium increased, and also by the addition of metal-chelating agents [78].

2.9. Quantification of cfDNA

Malentacchi et al.'s SPIDIA-DNAplas project revealed that laboratories utilize different methods for the quantification of cfDNA, including fluorescent (PicoGreen) and spectrophotometric measurements and qPCR. Spectrophotometric measurement was identified as the most commonly used among the participating laboratories [27]. Sample concentrations can be measured directly, producing an A260/A280 ratio which provides DNA purity estimates. However, there are many factors that can potentially interfere with measurements, including PCR primers, dNTPs, RNA and aromatic organic compounds, e.g. phenol [79]. In other words, the type of sample and the extraction method may influence cfDNA concentrations when using spectrophotometric quantification. Similar to SYBR-Green, PicoGreen is a fluorochrome that selectively binds double stranded DNA, resulting in an increase in fluorescence. Very low fluorescence of the unbound PicoGreen allows for minimal background and it is less sensitive, but still susceptible, to interference from PCR primers, proteins, etc. [79]. However, fluorescent analysis of DNA concentrations cannot distinguish between different DNA sources. The presence of bacterial, fungal or viral infections may, for example, influence DNA concentrations when measured via PicoGreen. This means that PicoGreen will also be insensitive to the presence of apoptosis or any other form of damage or genomic DNA contamination of samples, making qPCR the method of choice when quantifying specific DNA. It is therefore critical that the clinical context of the sample and purpose of the analysis of the DNA concentration must be considered before deciding on using PicoGreen. The use of PicoGreen does, however, correlate well with qPCR in routine clinical chemistry use and is more cost effective and less time consuming [80].

Quantifying gene expression levels in cfDNA samples, particularly housekeeping genes, through qPCR to measure the total amount of cfDNA are becoming more common in cfDNA research. Different target genes have been evaluated with qPCR, such as Alu sequences, ERV-3, human telomerase reverse transcriptase (hTert), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and β-globin. Of these, β-globin is the most popular [22]. Fleischacker et al. demonstrated that the yield of cfDNA is doubled when β-globin, as opposed to both ERV and GAPDH, is used for quantification [26]. However, studies have shown that housekeeping gene expression can change in cell lines when treated with mood stabilizers [81]. It is, therefore, possible that other substances or stressors may induce similar changes. Differences in expression have also been identified between different organs and tissues, as well as interindividual differences [82]. These and other variations that are not caused by the disease or disorder in question may be contributing to the varying cfDNA levels detected by various studies for a certain disease or disorder. Care must, therefore, be taken when choosing a gene to target for cfDNA quantification. Screening samples of healthy (control) target tissues or patients for stably expressed genes, keeping possible interindividual expression

differences in mind and obtaining a detailed clinical history of patient medication use, diet etc. should be considered when using qPCR as a cfDNA quantification method.

3. Preanalytic factors that affect cfDNA measurements: experimental investigation

3.1. Methods and materials

3.1.1. Cell culturing

To evaluate the effect of methodological variables on quantitative measurements of cfDNA, culture medium of the human bone cancer (osteosarcoma) cell line 143B (ATCC® CRL-8303™) was used as a source of cfDNA. Given that DNA levels in growth medium fluctuate much like cfDNA in the blood of humans, we could use it as a model to evaluate the effect of different variables on both high and low concentrations of cfDNA. Cells were cultured in T75 flasks in Dulbecco's Modified Eagle's medium (DMEM) (HyClone; #SH30243.01) supplemented with 10% fetal bovine serum (Biochrom; #S0615) and 1% penicillin/streptomycin (Lonza; #DE17-602E) at 37 °C in humidified air and 5% CO_2. After the cells have reached the necessary confluency, the culture medium was removed, processed and stored at −80 °C in 15 mL tubes.

3.1.2. Extraction of cfDNA

CfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the instructions described by the PCR clean-up user manual. Briefly, samples were removed from the −80 °C freezer and thawed at 37 °C and then vortexed and centrifuged briefly. For each biological replicate, cfDNA was extracted in triplicate. For every sample, 600 μL of growth medium was mixed with 1200 μL of binding buffer. Samples were then vortexed, the entire volume of growth media was added to the spin column in small regiments, and centrifuged at 11,000 × g for 1 min at room temperature. The columns were then washed twice, followed by the elution of cfDNA into 20 μL of elution buffer.

3.1.3. Quantification of cfDNA

PCR amplification of cfDNA was measured using a real-time quantitative assay for the β-globin gene. All assays were performed on a Rotor-Gene Q detection system (Qiagen) using a 72 well ring-setup. The reaction mixture consisted of 2 μL DNA and 23 μL master mix, which was composed of 8.1 μL H2O, 12.5 μL TaqMan Universal MasterMix (Life technologies; #1,502,032), 0.4 μL of 10 μM dual fluorescent probe 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3′, and 1 μL of 10 μM forward and reverse primers, respectively. The primers used were: F1, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, and R1, 5′-CCT TGA TAC CAA CCT GCC CAG-3′. These probe and primers were synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific). PCR conditions were set to: 95 °C for 10 min, followed by 45 cycles of 15 s denaturation at 95 °C, 1 min annealing at 60 °C, followed by 30 s extension at 72 °C. Sequence data of β-globin is attainable from GenBank (accession number: U01317). The absolute concentration of the target gene was calculated by using a standard curve. In this study, a standard curve was generated using five-fold serial dilutions of genomic DNA

Table 1
Modifications to standard procedure.

Modification to standard procedure	Description
Modifications to sample handling	
Centrifugation regime	Growth medium was centrifuged for 10 min at different forces (1000, 5000, 10,000 and 20,000 × g). Other samples were subject to two rounds of centrifugation, first at 1000 × g and then transferred to new tubes before the next centrifugation at 5000, 10,000 and 20,000 × g, respectively. After centrifugation all samples were transferred to new tubes.
Growth medium storage temperature	After centrifugation, growth medium was transferred to fresh tubes and stored until cfDNA was extracted. Three storage schemes were tested: −20 °C, −80 °C, and snap-freezing in liquid nitrogen followed by storage at −80 °C.
Growth medium thawing temperature	Prior to cfDNA extraction, the growth medium is thawed. Two approaches were tested: thawing of growth medium at room temperature, and at 37 °C in a temperature controlled water bath for 5 min.
Growth medium storage tube type	After collection and processing, growth medium was stored in three different tubes: 15 mL nuclease free tubes (Ambion), regular 1.5 mL tubes (Eppendorf), and DNA LoBind tubes (Eppendorf).
Modifications to cfDNA extraction protocol	
Treatment with denaturing agents	Prior to cfDNA extraction, growth medium was treated with SDS (0.05%), proteinase K (1.5 mg/mL), and a combination of the two for 30 min at 50 °C, respectively. In the cases where SDS was used, buffer NTB was used instead of buffer NTI. As the kit makes no suggestions regarding the use of proteinase K, buffer NTI was used in this case.
Effect of combining snap freezing with proteinase K	Four different scenarios were compared: (1) cfDNA was extracted from growth medium directly after collection, (2) growth medium was treated with proteinase K immediately after collection, followed by cfDNA extraction, (3) growth medium was snap frozen before cfDNA extraction, and (4) growth medium was snap frozen and then thawed and treated with proteinase K prior to extraction.
Binding buffer type	After thawing, growth medium is mixed with binding buffer before it is added to the spin column. Here, we compared two binding buffers, NTI and NTB. In the case of buffer NTB, the ratio of sample to buffer is 1:5. In the case of extractions where buffer NTI is used, the sample to buffer ratio is only 1:2.
Elution volume	CfDNA was extracted and eluted into 20 μL, 40 μL, 60 μL, and 100 μL of elution buffer, respectively.
Elution regime	CfDNA was extracted and eluted into 20 μL of elution buffer and repeated twice more to have a final volume of 60 μL. This was followed by the elution of DNA into 30 μL elution buffer and repeated once more to achieve a final volume of 60 μL. The former was compared to DNA eluted into 60 μL of elution buffer once.
Elution tube type	To examine the loss of eluted DNA due to adsorption to tube walls, regular 1.5 mL tubes (Eppendorf) were compared with 1.5 mL DNA LoBind tubes (Eppendorf)
Comparing different protocols	
Non-optimized	Media was collected and centrifuged at 1000 × g and transferred to fresh 1.5 mL Eppendorf DNA LoBind tubes and stored at −20 °C. Before extraction, the medium was thawed at room temperature, and no denaturing agent was added thereafter. CfDNA was then extracted and eluted into 20 μL of elution buffer in one step. Samples were stored in 1.5 mL DNA LoBind tubes (Eppendorf).
Optimized	Media was collected and centrifuged at 10 000 × g and transferred to fresh 15 mL tubes (Ambion). The media was then snap-frozen in liquid nitrogen and stored at −80 °C. The samples were then thawed at 37 °C, and incubated with proteinase K (1.5 mg/mL) for 30 min at 37 °C. CfDNA was extracted and eluted into 60 μL of elution buffer in three steps (3 × 20 μL) into regular 1.5 mL tubes (Eppendorf).
QIAmp DSP virus kit	CfDNA was extracted according to the instructions provided by the manufacturer.
Increasing the yield of cfDNA	
Effect of media evaporation	For each replicate, 6 mL of growth medium was aliquot into 2 mL tubes and evaporated in a SpeedVac to a total volume of 2.5 mL.

(50,000, 5000, 500, 50 and 5 pg/μL). Each biological replicate was quantified in duplicate, and triplicates of the standard curve were included in each run (only assays with R^2 values >0.99 for the standard curve were used).

4. Results and discussion

Using the literature survey as a guideline, several changes to the basic protocol were investigated. The details of these modifications are described in Table 1. Results are summarized in Figs 2, 3, 4 and 5.

The data of all graphs are presented as supplementary material in [86].

5. Summary and conclusion

Conventional methods used in the diagnosis and monitoring of cancer, prenatal complications and various other diseases are sensitive, but are not sufficient to overcome inherent limitations, such as the need for large samples of testing material. These procedures are extremely invasive and potentially hazardous. Ideally, diagnostic specimens should be easily accessible. Although the usefulness of blood and urine has been corroborated, molecular diagnosis of certain diseases require biopsies gained by invasive methods. Furthermore, blood derived cells lack specificity and has not proven to be useful for routine use in disease management of patients with solid tumors [83]. Bearing in mind the non-invasiveness of venipuncture, cfDNA analyses could potentially eliminate tissue biopsies, CT scans, chorionic villus sampling and amniocentesis. It is thus no surprise that cfDNA has been the recipient of profuse experimental attention in the past two decades [83]. In this time, quantitative and many qualitative characteristics of cfDNA have been revealed that advocate its potential as a diagnostic, prognostic and theranostic marker for a myriad of diseases.

Unfortunately, the useful application of cfDNA analysis is as yet a partial victory. Barring some prenatal diagnostic tests [84] and BEAMing, a technique used to detect mutations in various genes of cancer patients [85], cfDNA analysis has not yet been translated to clinical

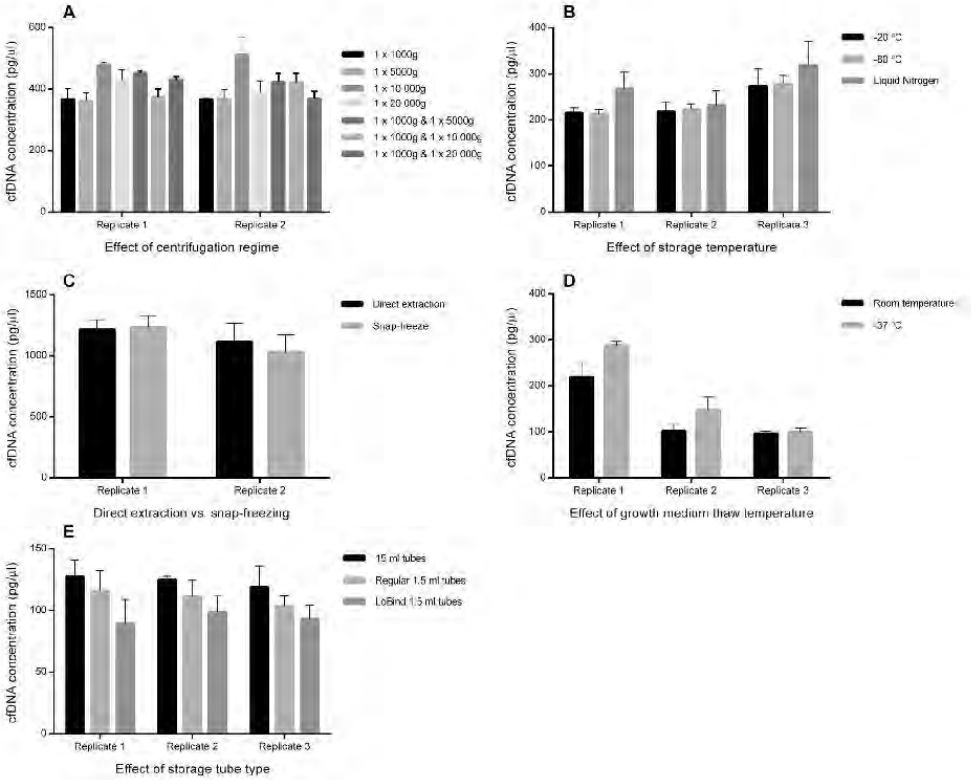

Fig. 2. The effect of sample handling on quantitative measurements of cfDNA. **A**. The effect of varying centrifugal forces and repetition. Samples were centrifuged for 10 min at the different forces. Samples that were subjected to two rounds of centrifugation were first centrifuged at 1000 × g, and then transferred to fresh tubes before the next centrifugation. In both experiments (which were done two months apart), one centrifugation at 10,000 × g delivered the highest yield. **B**. Changes in cfDNA yield during storage. After the medium was centrifuged, it was transferred to fresh tubes and stored until cfDNA was extracted. In all three experiments, storage at −20 °C and −80 °C delivered comparable results, while snap-freezing the samples in liquid nitrogen before storage at −80 °C resulted in an increase of cfDNA yield. It could be argued that this extra step dissociates the DNA from the protein complexes with which it is associated. However, in **C** (results taken from Fig. 3B.) we see that the amount of cfDNA extracted after snap-freezing correlates with the amount of cfDNA that is extracted from medium directly after collection. It can thus be argued that in the case of storage at −20 °C and −80 °C a fraction of cfDNA is lost due to fragmentation. **D**. The effect of medium thawing temperature on the amount of cfDNA extracted. In all experiments, the cfDNA yield was higher when the medium was thawed at 37 °C as opposed to room temperature. Except for the last experiment, the difference was rather significant. This is convenient, given that thawing at 37 °C takes only about 3 min, whereas thawing at room temperature takes at least 10 times longer. **E**. The loss of cfDNA due to tube type. Storage of medium in three different tubes resulted in three different amounts of cfDNA extracted. Storage in nuclease-free 15 mL tubes resulted in the least amount of DNA loss, followed by regular 1.5 mL Eppendorf tubes, with DNA LoBind Eppendorf tubes resulting in the most loss of cfDNA. In the case of the 15 mL tubes, it may be argued that less cfDNA is lost due to a smaller tube area-to-sample ratio than the 1.5 mL tubes. The reason for the most cfDNA being lost in LoBind tubes is unclear.

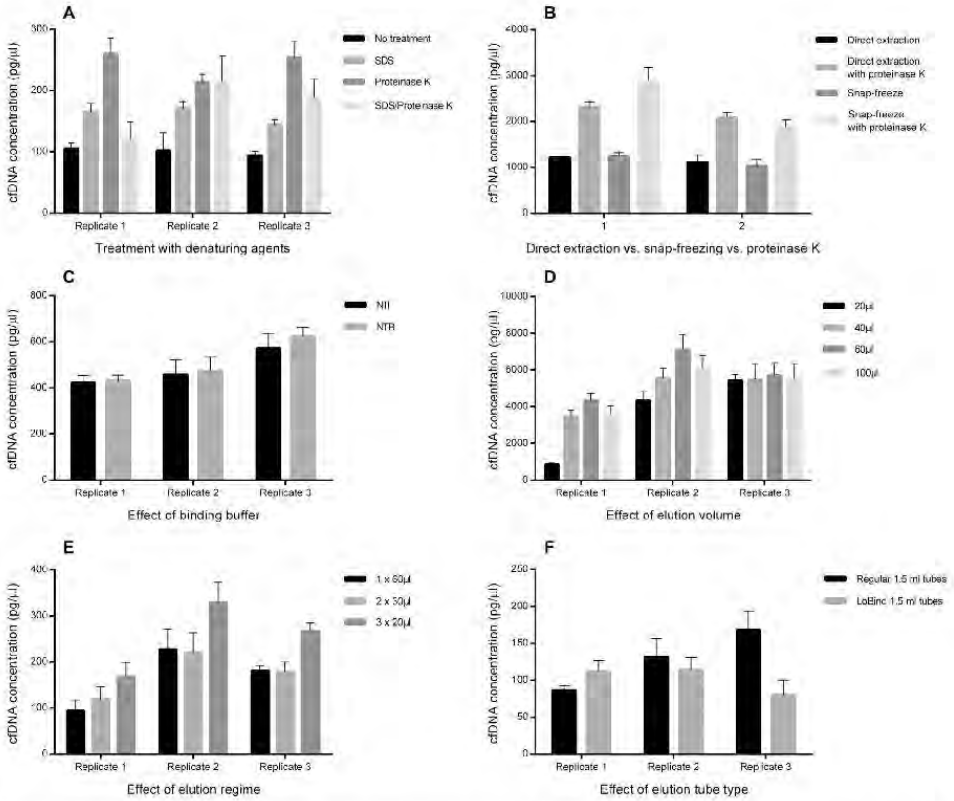

Fig. 3. The effect of modifications to the extraction protocol on quantitative measurements of cfDNA. **A**. The effect of the addition of denaturing agents. The use of SDS (0.05%), proteinase K (1.5 mg/mL), and a combination of the two were evaluated. In the cases where SDS was used, buffer NTB was used instead of buffer NTI. As the kit makes no suggestions regarding the use of proteinase K, buffer NTI was used in this case. The use of SDS increased the yield of cfDNA by approximately 50%, and proteinase K increased the yield by more than 100%, whereas a combination of the two delivered confusing results. **B**. A comparison of cfDNA yield between direct extraction and snap-freezing, with and without the use of proteinase K. Since the medium of all the samples in the previous experiment was snap-frozen, this experiment was done to determine whether the high yields of cfDNA are wholly or only partly due to the addition of proteinase K, and not partly due to snap-freezing. It is clear that, when the averages are calculated, snap-freezing does not increase the amount of cfDNA extracted and that the high yields can be ascribed solely to the addition of proteinase K. **C**. The effect of binding buffer type. In the case of buffer NTB, the ratio of sample to buffer is 1:5. In the case of extractions where buffer NTI is used, the sample to buffer ratio is only 1:2. Out of curiosity, we wanted to compare these two buffers in the absence of SDS. The use of buffer NTB resulted in only a very slightly higher yield of cfDNA than that obtained by the use of buffer NTI. As the use of NTB is more arduous, this convention is not advised. **D**. The effect of elution volume. CfDNA was extracted and eluted into 20 µL, 40 µL, 60 µL, and 100 µL of elution buffer, respectively. In all experiments, DNA yield increased as elution volume increased up to 60 µL where it reached its peak, and declined rather dramatically after that. **E**. The effect of elution regime. CfDNA was extracted and eluted into 20 µL of elution buffer, and repeated twice more to have a final volume of 60 µL. This was followed by the elution of DNA into 30 µl of elution buffer, and repeated once more to achieve a final volume of 60 µL. The former was compared to DNA eluted into 60 µl of elution buffer at once. In each experiment, the yield of DNA was significantly higher when eluted in three steps of 20 µL at a time. **F**. The effect of tube type on the loss of extracted cfDNA. To examine the loss of eluted DNA due to adsorption to tube walls, we compared regular 1.5 mL Eppendorf tubes with 1.5 mL Eppendorf DNA LoBind tubes. In two of the experiments the concentration of cfDNA stored in the LoBind tubes was much lower than that of DNA stored in standard tubes. These results agree with the results obtained in Fig. 2**E**.

practice, and routine application seems distant. This struggle stems from three factors that seem to overlap: (i) a lack of knowledge regarding the origin and biological function of cfDNA, (ii) insufficient characterization of its molecular characteristics, and (iii) the absence of an analytical consensus. Although both of the former points are crucial to the elucidation of the phenomenon of cfDNA, the objective of this work was to address and draw attention to the lack of an analytical consensus, especially as regards quantitative analyses of cfDNA. After careful inspection of the literature it became apparent that it is unlikely that quantitative analyses of cfDNA could be used as a stand-alone biomarker. It is clear, however, that a thorough quantitative assessment of the kinetics of cfDNA would be a strong auxiliary component to qualitative characterization. In fact, we maintain the position that it should make qualitative analyses more clinically relevant. In order to confidently use cfDNA analysis for this purpose, reproducibility and reliability are imperative. This requires the optimization, standardization and equivalence of procedures. Although many have conceded that this is lacking, unusually few publications have addressed it.

By means of a literature study we have identified several methodological variables that affect quantitative measurements of cfDNA, and perhaps some that have not yet been considered (or at least not yet considered as being worthy of attention). Evaluation of these variables using an in vitro model revealed surprising results. Almost every variable tested had a rather dramatic impact on the amount of cfDNA isolated. Indeed, some changes resulted in a significant increase of cfDNA yield. A combination of certain changes should appease cfDNA extraction which may appease gene expression profiling and sequencing which, by the standards of a cell culture model, requires a rather large

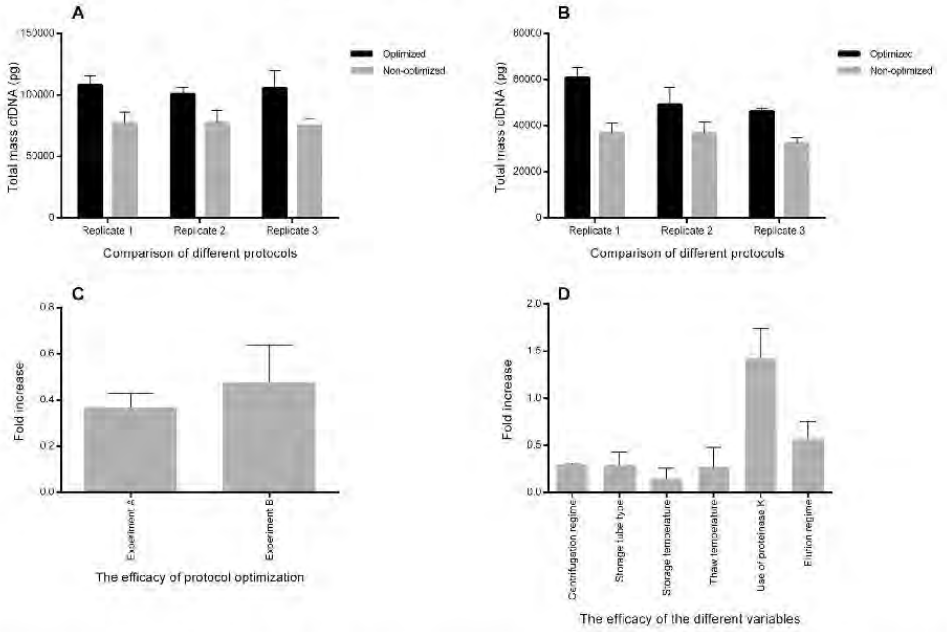

Fig. 4. Comparing different extraction protocols. **A**. The variables that produced the highest increase in cfDNA yield were incorporated into the extraction protocol and compared to a protocol consisting of its less effective counterparts. In the optimized protocol, media was collected and centrifuged at 10 000 x g and transferred to fresh 15 mL tubes. The media was then snap-frozen in liquid nitrogen and stored at −80 °C. The samples were then thawed at 37 °C, and incubated with proteinase K (1.5 mg/mL) for 30 min at 37 °C. CfDNA was extracted and eluted into 60 μL of elution buffer in three steps (3 × 20 μL) into regular 1.5 mL Eppendorf tubes. In the non-optimized protocol, media was collected and centrifuged at 1000 x g and transferred to fresh 1.5 mL Eppendorf DNA LoBind tubes and stored at −20 °C. Before extraction, the medium was thawed at room temperature, and no denaturing agent was added thereafter. CfDNA was then extracted and eluted into 20 μL of elution buffer in one step. Samples were stored in 1.5 mL Eppendorf DNA LoBind tubes. In **B** the former experiment was repeated with three new replicates. In **C**, the average efficacy of the optimized protocols is shown and compared to **D**, which shows the average efficacy of all variables as determined in the separate experiments. Presumably, the optimized protocol should deliver a value close to this sum total (2.99), but that is not the case. Clearly, some of these changes are working against each other.

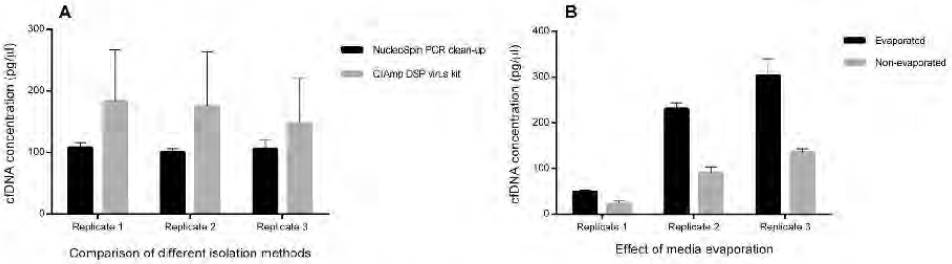

Fig. 5. Increasing the yield of cfDNA. **A**. Comparing the yield of cfDNA delivered by the NucleoSpin PCR clean-up kit and the QIAmp DSP virus kit. The DSP virus kit clearly recovers more cfDNA fragments than the NucleoSpin kit. There was, however, great variation in the yield of cfDNA between the samples in each experiment. This could possibly be due to the non-equal distribution of pressure by the vacuum pump, or some other factor. On the other hand, the NucleoSpin kit was much more consistent. **B**. The effect of media evaporation. For each replicate, 6 mL of growth medium was aliquot into 2 mL tubes and evaporated in a SpeedVac to a total volume of 2.5 mL. The cfDNA in these samples were thus expected to be increased 2.4 fold. The average increases of the replicates were then calculated as 2.33 with a standard deviation of 0.2. This is a loss of approximately 3%, which is rather minor.

amount of cfDNA. However, it is evident that too many changes work against each other. Establishing the optimal combination of changes requires further experimentation. We hope that our findings will be useful considerations when optimizing protocols and setting up a standard operating procedure.

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Article IV

Reference gene selection for in vitro cell-free DNA analysis and gene expression profiling

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Short Communication

Reference gene selection for *in vitro* cell-free DNA analysis and gene expression profiling

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ABSTRACT

Objectives: (i) To optimize cell-free DNA (cfDNA) and mRNA quantification using eight housekeeping genes (HKGs), (ii) to determine if there is a difference in the occurrence of HKGs in the cfDNA and mRNA of normal cells and cancer cells, and (iii) to investigate whether there is some selectivity involved in the release of cfDNA.

Design and methods: cfDNA was isolated directly from the growth medium of 3 cultured cancer cell lines and one non-malignant, primary cell line. At the same time interval, mRNA was isolated from these cells and cDNA was synthesized. CfDNA and cDNA were then amplified with real-time PCR utilizing eight different HKGs.

Results: For all cell lines tested, Beta-actin (ACTB) is the most appropriate HKG to use as a control for cfDNA and mRNA quantification. There was no clear difference in the occurrence of HKGs between cancer cells and healthy cells. Lastly, there is a consistent and distinct difference between the mRNA expression and cfDNA of all cell lines.

Conclusions: This study reveals a new candidate HKG for a robust control in cfDNA analysis and gene expression profiling, and should be considered for optimal analysis. Furthermore, results indicate that cfDNA is selectively released from cells into culture medium.

1. Introduction

It is becoming increasingly clear that diseased individuals present with elevated levels of plasma-derived cell-free DNA (cfDNA) and that these levels correlate with disease progression, treatment, and recovery. This indicates great potential for quantitative analysis of cfDNA as a non-invasive screening tool for the detection and therapy monitoring of several diseases, especially cancer [1,2]. In addition, many *in vitro* studies suggest that the lateral transfer of aberrant cfDNA may be a causative agent in oncogenesis [3] and metastasis [4], while other researchers have shown that transfection with cfDNA derived from normal cells can halt tumor growth [5]. Clearly, studying the kinetics of cfDNA *in vitro* and *in vivo* will have a positive impact on clinical diagnostics, therapy development, and our understanding of pathology. There is currently, however, one substantial drawback. Although elevated levels of cfDNA are generally observed in individual cases, there is a major overlap of values when a collection of studies are compared [1].

This makes it very challenging to confidently conduct and compare different *in vitro* cfDNA studies. Furthermore, with regard to *in vivo* investigations, this makes it virtually impossible to establish a normal reference range for any disease, or to establish a cut-off value for diagnosis. Except for some biological factors (such as the effects of the circadian rhythm, medicinal status, and other external stimuli, for example), this overlap can be ascribed mainly to the absence of a standard operating procedure. In a previous publication, we have addressed this issue thoroughly [1], and there are two variables that distinctly affect quantitative measurements of cfDNA. Firstly, the amount of cfDNA isolated by different methods may vary by as much as 100% [6]. Since other researchers have given this matter a sufficient amount of attention, it need not be discussed any further here. Secondly, when different target genes are used for quantification the amount of cfDNA measured may vary by as much as 50% (this value may be even more) [6]. This is a serious problem but has been badly neglected.

Today, real-time polymerase chain reaction (RT-PCR) is the favored technique for the fast and accurate quantification of cfDNA and examination of mRNA expression. The targeting of housekeeping genes (HKGs) as biomarkers, especially β-globin, in cfDNA quantification has become quite common. In RT-PCR, HKGs are primarily used as internal controls, and a suitable HKG is expected to be expressed in all cells, regardless of tissue type, developmental stage, cell cycle phase or external stimulus. However, this is not always the case as studies have shown notable expression variation between healthy and diseased tissues [7], treated and untreated cell lines [8], as well as inter-individual expression [9]. The prerequisites for internal control HKGs are (i) adequate expression in the target tissues and (ii) minimal expression variability between both the samples and the experimental conditions used [10]. This promotes the normalization of sample differences generated

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during sample preparation. Quite the opposite is required in terms of biomarker detection, as the goal is to search for variations in gene expression to identify pathologies. However, the internal control prerequisites can be used to optimize cfDNA quantification and to study gene expression changes after administering external signals. This study focuses on the utilization of HKG expression analysis in order to identify suitable HKGs for specific cultured cells in order to optimize cfDNA quantification.

2. Materials and methods

In this study, eight different HKGs (see Table 1 for summary) were used to optimize cfDNA quantification and evaluate mRNA expression in 4 cultured cell lines. This included 143B (osteosarcoma), A375 (melanoma), and RD (rhabdomyosarcoma) cells obtained by the American Type Culture Collection (ATCC®), and also a primary skin fibroblast (ZANLP) cell line, which served as a non-malignant control. Cells were cultured and maintained as prescribed by the ATCC. All cells were cultured to 80% confluency in 175 cm^2 flasks (Nunc™), after which the media was renewed and incubated for a further 24 h. After incubation, the media was collected, centrifuged at 10 000 × *g* for 10 min, and stored at − 80 °C. The cells were trypsinated, 5×10^6 cells collected, and pelleted at 300 × *g* for 5 min.

The total RNA was extracted immediately using the NucleoSpin RNA II extraction kit (Machery Nagel) according to the instructions provided by the user manual for the purification of RNA from cultured cells and tissue. The concentration of the RNA was determined and the purity confirmed with the Nanodrop ID-1000 spectrophotometer (Thermo Scientific). The RNA samples were stored at −80 °C. cDNA was synthesized from the extracted RNA samples with the High Capacity RNA-to-cDNA kit (Applied Biosystems) as prescribed by the manufacturer.

For cfDNA extraction, the culture medium was thawed at 37 °C and concentrated using a Savant ISS110 SpeedVac Concentrator (Thermo Electron Corporation) to a third of the original volume. The cfDNA was extracted with the NucleoSpin Gel and PCR Cleanup kit (Machery Nagel) according to the instructions provided by the PCR clean-up user manual, except binding buffer, NTB was used instead of NTI. To evaluate mRNA expression and to assess the occurrence of HKGs in cfDNA, RT-PCR was performed on the Rotor-Gene Q (Qiagen) detection system according to the instructions of the GeNorm kit manual. Except for β-globin (IDT, Whitehead Scientific), the primers and probes were selected from the GeNorm Reference Gene Selection Kit (Primerdesign). Fifteen cfDNA replicates and eight cDNA replicates were prepared. For each cell line, all cDNA samples were included in one run, and cfDNA samples were split into two runs. The thresholds chosen to produce Ct values from the resulting raw fluorescence data was 0.028 for the cfDNA samples (as used in previous quantitative PCR studies) and 0.04 for the cDNA samples.

3. Results and discussion

Regarding cfDNA quantification, Beta-actin (ACTB) was identified as the most appropriate HKG to use as a control for RT-PCR, with β-globin coming in second (Fig. 1 A–D). All other HKGs exhibited considerable variation (large standard deviations), and evaluating their use as controls beforehand is strongly recommended. For mRNA expression profiling, ACTB again appears to be the most appropriate target gene. In this case, β-globin performed poorly. Furthermore, there was little variation in the occurrence of HKGs between the cfDNA of cancer cell lines and that of healthy fibroblasts, showing that studying the occurrence of HKGs may not be the most prudent method to monitor induced changes in cfDNA content under various experimental conditions. Both the cfDNA of the cancer cell lines (with the exception of A375) and healthy fibroblasts did not release HKGs related to cellular energy metabolism (SDHA, ATP5B, and CYC1). However, mRNA amplification showed that they were indeed expressed in the cells, suggesting that there is some selectivity involved in the release of cfDNA. In 2009, Puszyk et al. showed that there is a similar unequal representation of cfDNA sequences in the blood of humans [17].

4. Conclusion

This study reveals that Beta-actin (ACTB) is a strong new candidate reference gene that can be used as a control in both cfDNA analysis and mRNA expression profiling and should be considered for optimal analysis. In addition, results suggest that there is selectivity involved in the release of cfDNA from cultured cancer cells. This may give some insight on the possible origin of cfDNA, knowledge that is currently lacking. We suggest that a similar study should be conducted for *in vivo* experiments, since this will expedite the translation of quantitative cfDNA analyses to clinical practice.

Author contributions

AJB, JA, and JFW contributed equally to the drafting of the manuscript.

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Table 1
Housekeeping genes utilized for *in vitro* cell-free DNA analysis and gene expression profiling.

Symbol	Housekeeping gene	Function	Amplicon size (bp)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Glycolytic enzyme with diverse non-glycolytic functions, including the binding of DNA and RNA, regulation of transcription and facilitating vesicular transport [11].	110 (Intron spanning)
ACTB	Beta-actin	Cell motility, structure and integrity and serves as a major constituent in the contractile apparatus [12].	92 (Single exon)
EIF4A2	Eukaryotic translation initiation factor 4 A2	Involved in the binding of messenger RNA to the ribosome. Plays a vital role in oncogenesis [13].	113 (Single exon)
SDHA	Succinate dehydrogenase	Part of the Kreb's cycle and respiratory chain (complex II) [14].	120 (Intron spanning)
CYC1	Cytochrome C-1	Mobile electron carrier between complex III and complex IV of respiratory chain. Also involved in apoptosis.	145 (Intron spanning)
ATP5B	ATP synthase	Complex V of the respiratory chain which produces ATP using the energy provided by the proton electrochemical gradient [15].	119 (Intron spanning)
TOP1	Topoisomerase 1	Controls the topological states of DNA during transcription.	141 (Intron spanning)
β-GLOBIN	Beta globin	Forms part of the heteromeric hemoglobin protein-complex involved in protein transport [16].	102 (Single exon)

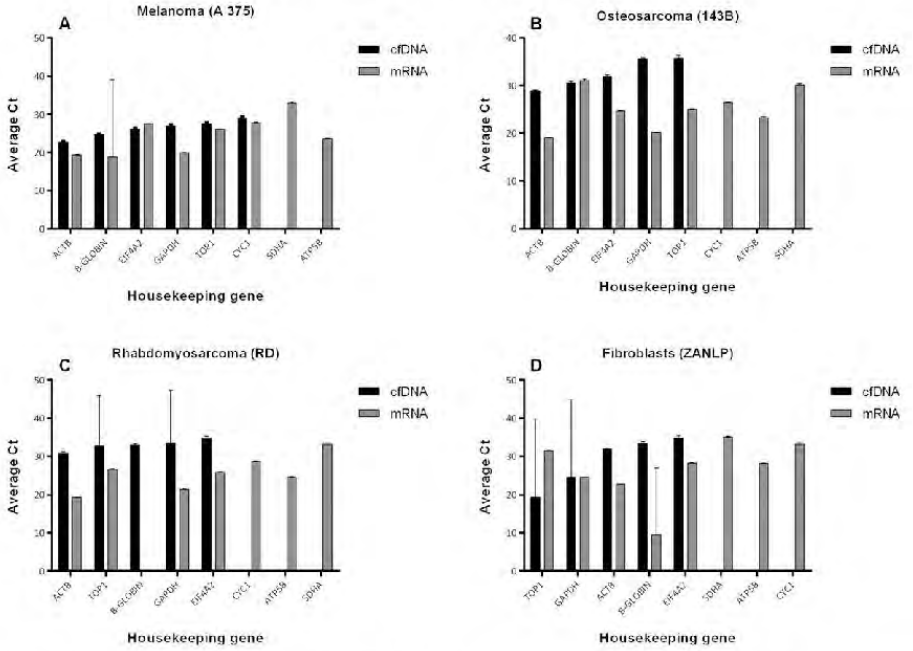

Fig. 1. Average Ct values of HKGs that occur in the cfDNA and mRNA of (*A*) A375, (*B*) 143B, (*C*) RD, and (*D*) ZANLP fibroblast cells. The concentration of cDNA used was kept constant for all four cell lines. The amount of cfDNA used represents the total yield of cfDNA for each cell line. Low Ct values indicate higher levels of initial PCR template and earlier cycle amplification. Error bars indicate standard deviation. Small standard deviations indicate stable expression, while large deviations indicate unstable expression. n = 15 for cfDNA replicates and n = 8 for cDNA replicates.

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Chapter 4: Molecular and biological characterization of cfDNA

Article V

Characterization of the cell-free DNA released by cultured cancer cells

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Characterization of the cell-free DNA released by cultured cancer cells

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ABSTRACT

The most prominent factor that delays the translation of cell-free DNA (cfDNA) analyses to clinical practice is the lack of knowledge regarding its origin and composition. The elucidation of the former is complicated by the seemingly random fluctuation of quantitative and qualitative characteristics of cfDNA in the blood of healthy and diseased individuals. Besides methodological discrepancies, this could be ascribed to a web of cellular responses to various environmental cues and stressors. Since all cells release cfDNA, it follows that the cfDNA in the blood of cancer patients is not only representative of tumor derived DNA, but also of DNA released by healthy cells under different conditions. Additionally, cfDNA released by malignant cells is not necessarily just aberrant, but likely includes non-mutated chromosomal DNA fragments. This may cause false positive/negative results. Although many have acknowledged that this is a major problem, few have addressed it. We propose that many of the current stumbling blocks encountered in *in vivo* cfDNA studies can be partially circumvented by *in vitro* models. Accordingly, the purpose of this work was to evaluate the release of cfDNA from cultured cells and to gauge its potential use for elucidating the nature of cfDNA. Results suggest that the occurrence of cfDNA is not a consequence of apoptosis or necrosis, but primarily a result of actively secreted DNA, perhaps in association with a protein complex. This study demonstrates the potential of *in vitro* cell culture models to obtain useful information about the phenomenon of cfDNA.

1. Introduction

Understanding the processes involved in the generation of cell-free DNA (cfDNA) is critical for inferring its role in biology and pathology, and expediting the translation of analyses to clinical practice. However, despite the apparent ubiquity of cfDNA in biofluids, the elucidation of its origin has only been a partial victory. Excluding exogenous DNA (bacterial, viral and parasitic, for instance), several possible sources and cognate mechanisms have been advocated. Firstly, it was presumed that cfDNA enters the blood following the lysis of cells on the interface between a tumor and circulation. Since it was shown that the concentration of cfDNA in the blood of cancer patients is greater than could be accounted for by the mass of cells present [1], this notion has been abandoned. Secondly, it was proposed that cfDNA may originate from the destruction of tumor micrometastases and circulating cancer cells. However, as regards the mutated cfDNA in the plasma of colorectal cancer patients, no mutated counterpart has been found in the cells of the Ficoll layer where micrometastatic cells should be present [2]. In addition, the fact that this layer is used as a normal control in microsatellite analysis indicates the lack of cancer cells in this material. On the basis of this information, the hypothesis was rendered erroneous [3]. There remain three more possible sources that may account for the occurrence of cfDNA, namely: apoptosis, necrosis, and active cellular secretion [4].

In most cases where plasma or serum DNA is subject to electrophoresis, a ladder pattern reminiscent to that obtained by apoptotic cells is seen [5]. Typically, these fragment sizes relate to multiples of nucleosomal DNA stretches, ranging from 150 to 1000 bp. Jiang et al. have reported a distinct size of 166 bp for the cfDNA of hepatocellular carcinoma (HCC) patients. This observation is consistent with their previous analyses on maternal blood [6], and similar results have been obtained by other independent groups [7–9]. Suzuki and colleagues characterized the cfDNA of healthy humans and demonstrated a size distribution between 61 and 567 bp, with a prominent peak between 161 and 170 bp. Since DNA fragments derived from necrotic cells are normally larger than 10,000 bp [10], these findings suggest an apoptotic origin for the bulk of cfDNA in diseased as well as healthy individuals [11–13]. In contrast, others have observed larger fragments of cfDNA in the blood of cancer patients, which suggests an origin from necrosis [14–18]. An argument against necrosis as the primary pathway for cfDNA release comes from observations that cfDNA levels decrease by approximately 90% following radiation therapy. If necrosis ensued, one would not expect a decline but a surge in cfDNA concentration [19,20]. However, it can also be argued that the reduction of cfDNA

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levels may be due to the inhibition of the cfDNA release pathways of healthy cells by the radiation [18].

On the other hand, many studies indicate that a significant fraction of cfDNA is derived from active cellular secretions, in which newly synthesized DNA in association with a lipid-protein complex is released in a homeostatic manner [21–25]. In 2010, Gahan termed this protein complex "the virtosome" [26]. Since then, other mobile protein complexes such as Argonaute2 [27] and high density lipoprotein [28] have been shown to be associated with nucleic acids and occur in the extracellular environment. Furthermore, cells have been shown to release extracellular vesicles, such as exosomes and prostasomes, which contain highly specific nucleic acids that often relate to the cell from which it originated [29–31].

It is evident that the origin of cfDNA is still elusive. Although most evidence suggests that the release of cfDNA is mainly a consequence of apoptosis, it is becoming increasingly clear that cfDNA may be released by more than one mechanism. It may also be of particular interest that Jiang and co-workers have detected a small amount of mitochondrial DNA and not only demonstrated that the fragments are shorter than that of nuclear DNA but also more abundant in HCC patients than in healthy subjects [32]. Since many of the confounding factors that affect the release of cfDNA *in vivo* are absent in cell cultures, we propose that many of the stumbling blocks encountered in *in vivo* cfDNA studies can be partially circumvented by *in vitro* models. Unfortunately, *in vitro* analyses of cfDNA are currently lacking. For this reason, the aim of this work was to assess the release and composition of cfDNA from cultured human osteosarcoma cells (143B) and to gauge its potential use for elucidating the nature of cfDNA. The release pattern of cfDNA was characterized over time, and the sizes of the cfDNA fragments at each of these intervals were evaluated. Apoptosis, necrosis, and the cell cycle profiles were also investigated at different time intervals using flow cytometry.

2. Materials and methods

2.1. Cell culturing and growth medium processing

The human bone cancer (osteosarcoma) cell line 143B was obtained from the American Type Culture Collection (ATCC® CRL-8303™). Cells were grown in Dulbecco's modified Eagle's medium (Hyclone DMEM/high glucose) (Thermo Scientific; #SH30243.01) containing 4 mM L-glutamine, 4500 mg/L glucose, and sodium pyruvate. It was further fortified with 10% fetal bovine serum (FBS) (Biochrom; #S0615) and 1% penicillin/streptomycin (Lonza; #DE17-602E). Cells were incubated in humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded and cultured in bulk in 175 cm^2 cell culture flasks (Corning; #431080) (36 mL growth medium) and grown to confluency. To detach the cells from the flask surface, they were washed with phosphate-buffered saline (PBS) (Sigma; #SLBJ5110V) and then incubated for 5–10 min at 37 °C with 0.25% trypsin (Lonza; #2 MB258). After trypsinization, the cells were redistributed equally into twelve 75 cm^2 flasks, each containing 12 mL growth medium. The cells were then grown for 12 h, after which the growth medium was renewed. After this time, pairs of flasks were incubated for 4, 8, 12, 16, 20 and 24 h, respectively. At the end of incubation, the growth medium was collected in 15 mL nuclease-free tubes (Ambion; #3108090) and then centrifuged at 10,000 ×*g* for 10 min and transferred to fresh 15 mL tubes. The samples were then stored at − 80 °C until extraction. In concurrence with growth medium collection, the cells were collected by trypsinization, which was then used for extraction and determination of total cellular protein content. This experiment was then also repeated for 28, 32, 36 and 40 h, respectively.

2.2. Extraction and quantification of cellular protein

Total cellular protein was liberated by sonication with the Bioruptor UCD-200 (Diagenode). Before usage, the Bioruptor was cooled to 4 °C using distilled water and ice. Samples were then vortexed and loaded into the Bioruptor wells. Sonication settings were: power, H-position (high); sonication cycle, 30 s on/30 s off; total sonication time, 5–10 cycles. Halfway through sonication, samples were removed and vortexed.

Proteins were quantified with the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies). This method relies on the design of a standard curve with proteins included in the Qubit® Protein Assay kit (0, 200 and 400 ng/μL). Briefly, working solution was prepared by adding a volume of 1 μL of Qubit™ reagent to 199 μL of Qubit™ buffer. For the standards, 10 μL was mixed with 190 μL of working solution in 0.5 mL Qubit® assay tubes. For the cellular protein samples, 2 μL was added to 198 μL of working solution. The tubes were vortexed for 3 s and then incubated for 15 min at room temperature. After incubation, the tubes were inserted into the Qubit® 2.0 Fluorometer and the stock concentration readings were noted. First, to evaluate the precision of the Qubit® 2.0 Fluorometer a stock solution of bovine serum albumin (BSA) (2 mg/mL) was diluted to 0.15, 0.20, 0.25, and 0.30 mg/mL and then quantified. It was then calculated that the Qubit is 93% accurate, which was sufficient for the purposes of subsequent experiments. This way, more tedious analyses like the BCA assay could be circumvented.

2.3. Extraction of cell-free DNA

CfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the manufacturer's PCR clean-up instructions. Briefly, samples were removed from the − 80 °C freezer and thawed at 37 °C in a temperature controlled water bath. After incubation, the samples were vortexed and centrifuged briefly. For each biological replicate, cfDNA was extracted in triplicate. For every sample, 600 μL of growth medium was mixed with 1200 μL of binding buffer. Samples were then vortexed, the entire volume of growth media was added to the spin column in small regiments, and centrifuged at 11,000 ×*g* for 1 min at room temperature. The columns were then washed twice, followed by the elution of cfDNA into 20 μL of elution buffer.

2.4. Quantification of cell-free DNA

PCR amplification of cfDNA was measured using a real-time quantitative assay for the β-globin gene. All assays were performed on a Rotor-Gene Q detection system (Qiagen) using a 72 well ring-setup. The reaction mixture consisted of 2 μL DNA and 23 μL master mix, which was composed of 8.1 μL H_2O, 12.5 μL TaqMan Universal MasterMix (Life Technologies; #1502032), 0.4 μL of 10 μM dual fluorescent probe 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3′, and 1 μL of 10 μM forward and reverse primers, respectively. The primers used were: F1, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, and R1, 5′-CCT TGA TAC CAA CCT GCC CAG-3′. These probe and primers were synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific). PCR conditions were set to: 95 °C for 10 min, followed by 45 cycles of 15 s denaturation at 95 °C, 1 min annealing at 60 °C, followed by 30 s extension at 72 °C. Sequence data of β-globin is attainable from GenBank (accession number: U01317). The absolute concentration of the target gene was calculated by using a standard curve. In this study, a standard curve was generated using five-fold serial dilutions of genomic DNA (50,000, 5000, 500, 50 and 5 pg/μL). Each biological replicate was quantified in duplicate, and triplicates of the standard curve were included in each run (only assays with R^2 values >0.99 for the standard curve were used).

2.5. Fragment size evaluation of cell-free DNA

The size of cfDNA extracted at the different time intervals were analyzed by capillary electrophoresis (CE). This was done on a microchip using the High Sensitivity DNA kit and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. The assay was performed according to the instructions

provided by the manufacturer. After the nucleic acids are separated analogously to CE, they are normalized to a ladder and two DNA markers, and are represented as a virtual band. The software then automatically calculates the size of each band, and is represented as an electropherogram.

2.6. Flow cytometric detection of apoptosis and necrosis using the FITC annexin V assay

The BD Annexin V FITC assay (BD Biosciences) was utilized to determine whether the apoptotic profile of 143B cells differ at specific time intervals. 143B cells were washed twice with PBS and resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/mL at different time intervals (4, 12, 24 and 40 h). 100 μL of this solution was then transferred to 12 × 75 mm round bottom (conical) tubes (BD Biosciences) and mixed with 5 μL of FITC Annexin V and 5 μL of propidium iodide (PI). Samples were then vortexed and incubated in the dark at room temperature for 20 min. After incubation, 100 μL of 1X binding buffer was added to each tube and then analyzed using the BD FACSVerse System and BD FACSuite Software. For a positive apoptosis control, 143B cells were treated with 650 μM hydrogen peroxide for 4 h. Unstained cells were included for settings optimisation of the flow cytometer.

2.7. Flow cytometric detection of apoptosis with the TUNEL assay

An APO-BrdU™ TUNEL assay kit (Molecular Probes, Invitrogen) was used for the detection of DNA fragments as recommended by the manufacturer, with slight modifications. Briefly, an amount of 2×10^6 cells were suspended in 0.5 mL PBS. Cells were counted using the scepter (Merck, Millipore) and fixed by adding the cell suspension to 5 mL of 1% (*w*/*v*) paraformaldehyde (Fluka) on ice for 15 min. After fixation, the cells were centrifuged for 5 min at 300 ×g, and the supernatant was discarded. The cells were washed twice with 5 mL of PBS, and the pellet was resuspended in 0.5 mL of PBS followed by 5 mL of ice-cold 70% (*v*/*v*) ethanol. The cell suspensions were stored in a −20 °C freezer until analyzed for fragmented DNA. On the day of analysis, the samples were resuspended in PBS and 1 mL aliquots (approximately 1×10^6 cells) were removed and placed in 12 × 75 mm flow cytometry tubes (BD Biosciences). The cell suspensions were then centrifuged for 5 min followed by the removal of the remaining ethanol by aspiration. The cell suspensions were washed twice with 1 mL of wash buffer (Apo- BrdU™ TUNEL assay kit), and the supernatants were removed by aspiration. Then, the pellet was resuspended in 50 μL of freshly prepared DNA-labeling solution (Apo-BrdU kit) containing 10 μL reaction buffer, 0.75 μL of TdT enzyme, 8.0 μL of BrdUTP and 31.25 μL dH_2O. The cells were incubated in the DNA-labeling solution for 60 min at 37 °C. The cells were gently shaken every 15 min to keep it in suspension. At the end of the incubation time, the cells were washed twice in 1 mL of rinse buffer (Apo-BrdU kit) by centrifugation at 300 ×g for 5 min at room temperature.

The supernatants were removed by aspiration, and the pellet was resuspended in 100 μL of freshly prepared antibody staining solution containing 5 μL Alexa Fluor® 488 dye-labeled anti-BrdU antibody and 95 μL rinse buffer. Cells were incubated in this solution for 30 min at room temperature in a darkened room. After incubation, 0.5 mL of propidium iodide/RNase A staining buffer (Apo- BrdU™ TUNEL assay kit) was added. Cells were analyzed for DNA fragmentation using the BD FACSVerse System and BD FACSuite Software.

2.8. Flow cytometric cell cycle analysis

Cells were cultured as described in section 2.1. After 12 h of incubation, the point where medium was renewed, 5-ethynyl-2′-deoxyuridine (EdU) was added to a final concentration of 10 μM to each flask and

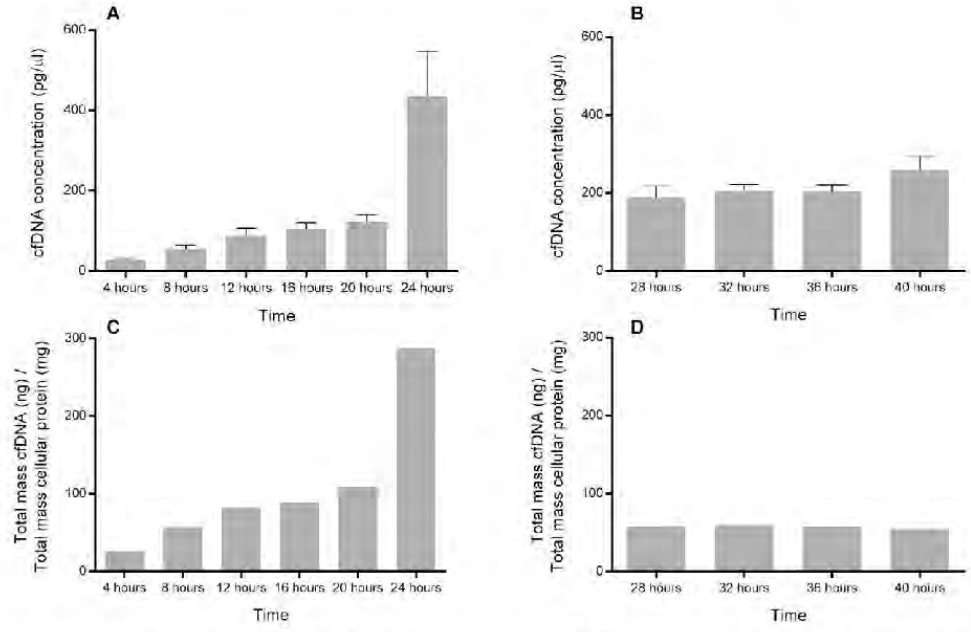

Fig. 1. Time-course characteristics of cfDNA released from 143 B cells. A. Bar graph showing the amount of cfDNA released by 143 B cells after 4–24 h of incubation following medium renewal. B. Bar graph showing the amount of cfDNA released by 143B cells after 28–40 h of incubation following medium renewal. The value of each bar represents the amount of cfDNA in 1 μL of a total of 20 μL of elution buffer obtained from 600 μL of growth medium. C & D Represents the amount of cfDNA released at each time-point normalized in terms of total cellular protein.

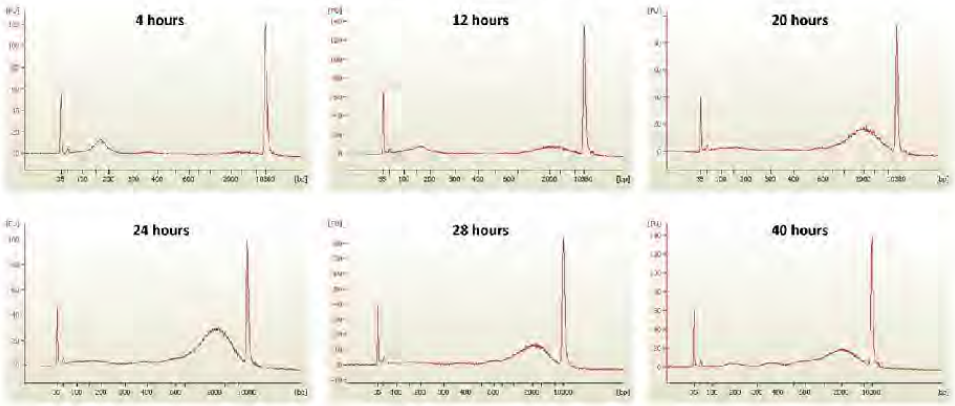

Fig. 2. Capillary electropherograms showing the size of cfDNA isolated after incubation at various times following medium renewal. In each electropherogram two major peaks can be seen, one at 35 bp and one at approximately 10,000 bp. These peaks correspond to the two size markers. The relative fluorescence of these markers is then used to calculate the size of the unknown samples. Thus, any deviation from the baseline, excluding the markers, indicates the size of cfDNA.

mixed well. Detection of EdU incorporation into DNA was performed with the Click-iT® Edu Alexa Fluor® 488 Cell Proliferation Kit (Molecular Probes, Invitrogen) according to the instructions of the manufacturer. Briefly, harvested cells were washed once with 1% BSA in PBS and pelleted by centrifugation at 500 $\times g$ for 5 min at room temperature. The supernatant was removed and the pellet resuspended at a density of 1×10^7 cells/mL in PBS. To fixate cells, 100 μL of the cell suspension was mixed with 100 μL of Click-iT® fixative agent in a 12 × 75 mm flow tube and incubated in a dark room for 15 min. After incubation, cells were washed with 3 mL of 1% BSA in PBS, pelleted, and the supernatant was discarded. The pellet was then dislodged, resuspended rigorously, and mixed with 100 μL Triton® X-100-based permeabilization reagent and then incubated for 30 min at room temperature in a dark room. After incubation, cells were washed with 3 mL of 1% BSA in PBS, pelleted, and the supernatant was discarded. The Click-iT® EdU reaction cocktail was prepared as recommended by the manufacturer and 5 mL was added to each cell pellet. Samples were incubated for 30 min at room temperature in a dark room and then washed with 3 mL of 1% BSA in PBS and pelleted. After the supernatant was discarded 0.5 mL of 1% BSA in PBS was added to the pellet. For staining of cellular DNA, 5 μL of Ribonuclease (RNAse A) was first added to each sample and mixed, after which 2 μL of the Click-iT® EdU CellCycle 488-red (7-AAD) and 2 μL of Propidium iodide was added to each sample and mixed again and then incubated at room temperature for 30 min. After incubation, cells were transferred to ice until analysis with a BD FACSVerse System and BD FACSuite Software.

2.9. Flow cytometry instrumentation and data analysis

Fluorescence of single cells was measured by a FACSVerse™ bench top flow cytometer equipped with blue (488 nm) and red (640 nm) lasers. Events were acquired on BD FACSuiteTM software, version 1 (Becton & Dickson, Mountain View, CA, USA). Amplification of signals were carried out at logarithmic scale and measurement of events plotted on forward light scatter (FSC), side light scatter (SSC), green fluorescent (FL1) and red fluorescent (FL2). A gating strategy was used to distinguish the fluorescently labeled cell population from unstained populations. A total of 10 000 events as defined by gates, were counted. Positive as well as stained and unstained negative controls were included in each analysis. The FACSVerse were calibrated using FACSuite™ CS&T research beads. Data was processed with FCS Express V4 software (De Novo Software, CA, USA). Gates were set on the dot plot FSC and SSC during analysis. The geometric means of fluorescence for all the parameters were calculated from the respective histograms or two parameter fluorescence dotplots.

Fluorescence results were expressed in arbitrary units as mean fluorescence intensity (MFI) and cell counts were expressed as percentage. Flow cytometry samples were analyzed blind, and the researcher responsible for analyzing flow cytometry data was blinded to the objectives and outcome of the experiments.

2.10. Treating growth medium with denaturing agents

cfDNA was isolated as described in section 2.3, except prior to extraction the growth medium was incubated with SDS (0.05%), proteinase K (1.5 mg/mL), and a combination of the two for 30 min, respectively. In the cases where SDS was used, buffer NTB was used instead of buffer NTI. As the kit makes no suggestions regarding the use of proteinase K, buffer NTI was used in this case. Since the medium of all the samples in the previous experiment was snap-frozen, an experiment was done to determine whether the high yields of cfDNA are wholly or only partly due to the addition of proteinase K, and not partly due to snap-freezing. To do this, four different scenarios were compared: (1) cfDNA was extracted from growth medium directly after collection, (2) growth medium was treated with proteinase K immediately after collection, followed by cfDNA extraction, (3) growth medium was snap frozen

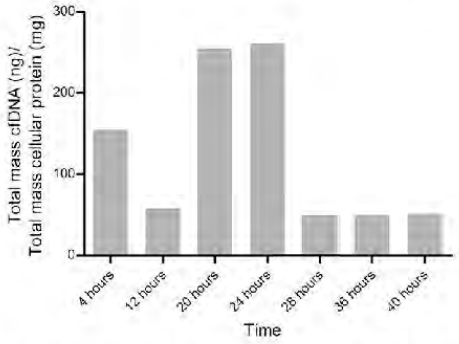

Fig. 3. Bar graph showing the amount of cfDNA released by 143B cells after 4–40 h of incubation following medium renewal. cfDNA was quantified by capillary electrophoresis using an Agilent 2100 Bioanalyzer.

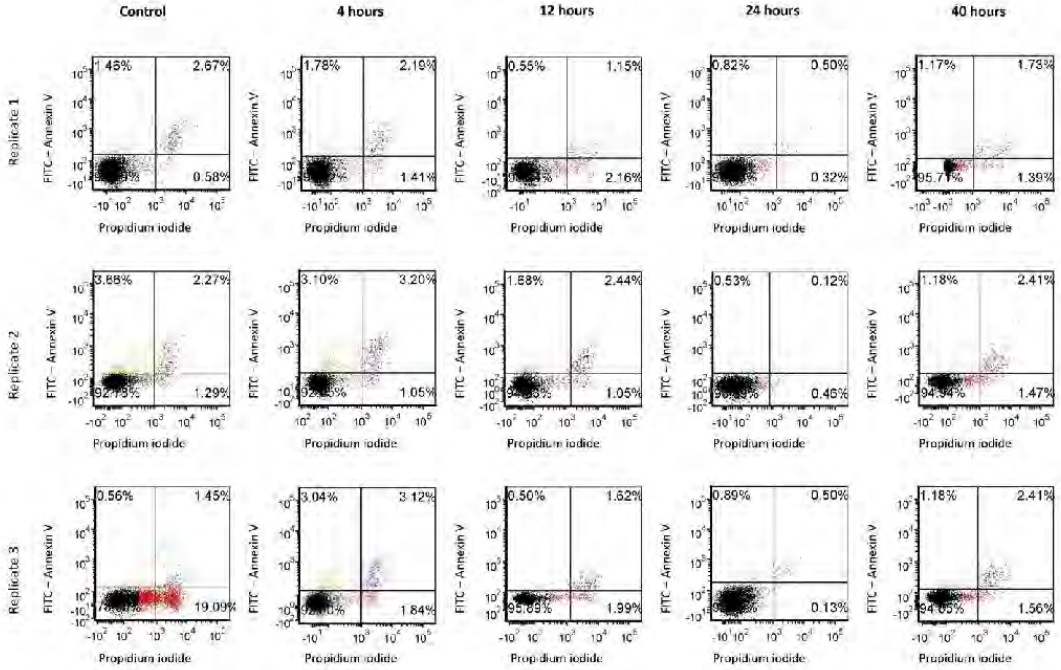

Fig. 4. Dot plots illustrating the amount of apoptotic and necrotic 143 B cells after different times of incubation. Flow cytometric analysis was performed on 143 B cells staining simultaneously with annexin-V-FITC and propidium iodide (PI). For each graph, the lower left quadrant represents viable cells, the upper left quadrant represents apoptotic cells, the upper right quadrant represents cells in late stage apoptosis, and the lower right quadrant represents necrotic cells. Every dot corresponds to a single cell. Replicates 1, 2 and 3 of the control column represent 143 B cells treated with 150, 300 and 600 mM of hydrogen peroxide, respectively.

before cfDNA extraction, and (4) growth medium was snap frozen and then thawed and treated with proteinase K prior to extraction.

3. Results and discussion

The origin of cfDNA is still elusive. There are several possible sources that may account for the occurrence of cfDNA, including apoptosis, necrosis, and active cellular release. In this study, the release of cfDNA from cultured cells was evaluated in order to gauge its potential use for elucidating the nature of cfDNA.

3.1. Kinetics of cfDNA release

Release of cfDNA was characterized over time (Fig. 1 A & B). After growth medium renewal, the amount of cfDNA increased incrementally, notably peaked after 24 h, and plateaued at a much lower level thereafter. Since the amount of cells increase over time, this is not surprising. However, when the values were normalized in terms of total cellular protein the tendency did not change, suggesting that more cfDNA is released per cell (Fig. 1 D & C). In the case where capillary electrophoresis was used to quantify cfDNA, the same pattern was obtained (Fig. 3). This phenomenon can be seen in at least three other reports in the literature [33–35]. Morozkin et al. assessed the kinetics and composition of cfDNA released by A431 (epidermoid carcinoma) and HeLa (cervical cancer) cells over 48 h. It was demonstrated that cfDNA concentration increases over time, and electrophoretic analysis indicated a molecular weight between 0.4 and 10 kilobase pairs. This suggested that the cfDNA is neither from apoptotic nor necrotic origin [34]. A few years later, they repeated the experiment but also assessed the DNA released by primary HUVEC (human umbilical vein endothelial) cells and mycoplasma infected

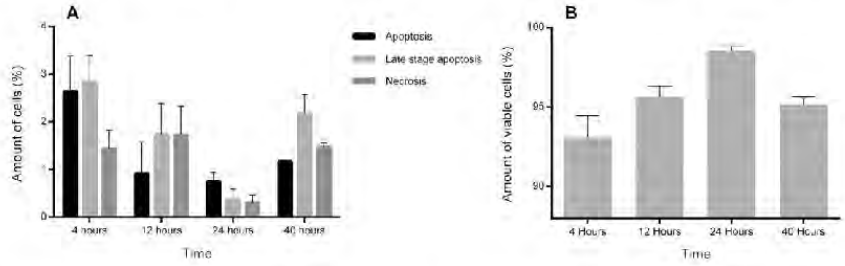

Fig. 5. Summary of the processed data obtained by the FITC Annexin V flow cytometric assay. A illustrates the percentage of apoptotic, necrotic, and late-stage apoptotic 143 B cells at the different time-points. B illustrates the percentage of viable 143 B cells at each time-point. These values represent the averages of the replicates showed in Fig. 3. Error bars indicate standard deviation.

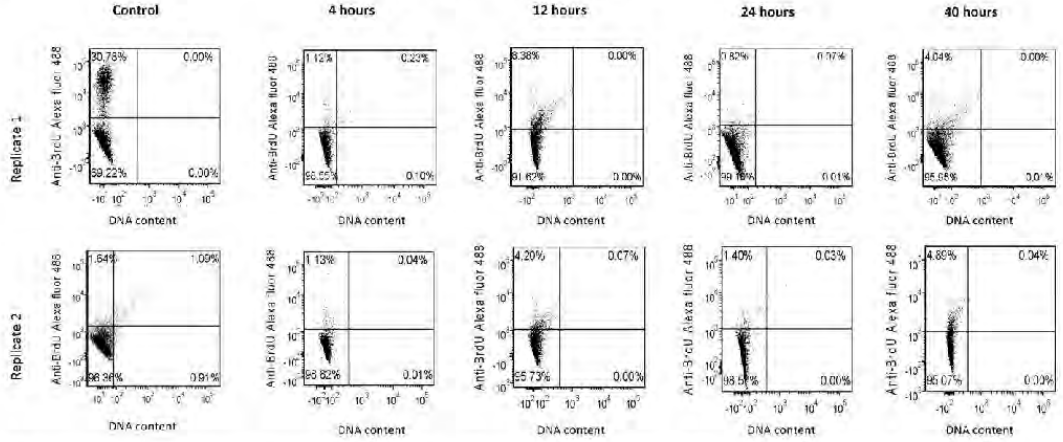

Fig. 6. Dot plots illustrating the detection of DNA fragmentation in 143B cells at different times as analyzed with the TUNEL assay by flow cytometry. The fluorescence of DNA content is plotted against Anti-BrdU alexa fluor 488 incorporation. Replicate 1 shows the positive control and replicate 2 shows the negative control. For each graph, the lower left quadrant represents viable cells, while the upper left quadrant represents apoptotic cells.

HeLa cells, showing that cfDNA concentration increased during the lag and beginning of the exponential growth phase. They considered this to be evidence for the active release of cfDNA [33]. Choi and coworkers designed a similar experiment for Jurkat (human T lymphocyte) and U937 (pleural effusion) cells. However, in addition to normal conditions, apoptosis and necrosis were induced in both cell lines. In the case of apoptosis, cfDNA concentration increased over time and also peaked rather dramatically after 24 to 48 h. CfDNA from untreated cells increased only slightly at these times. On the other hand, necrotic cells showed a decline of cfDNA over time. These results suggest that the release of cfDNA is mainly a consequence of apoptosis, but also suggests that cfDNA may be released by more than one mechanism [35].

3.2. Size of cfDNA

To examine the size of cfDNA isolated at the different time points, samples were subject to capillary electrophoresis (Fig. 2). After 4 h of incubation a prominent peak at 166 bp was observed, which is consistent with the literature and indicates an origin from apoptosis [32]. However, this peak diminished incrementally and disappeared after 24 h when a new peak of approximately 2000 bp dominated the scene. After 40 h of incubation, the cfDNA resembles multiples of nucleosomal repeats, with peaks forming at approximately 160, 340 and 540 bp, also suggesting an origin from apoptosis. These observations demonstrate a clear correlation between an increase in the release of cfDNA and the occurrence of higher molecular weight DNA. As far as we know, this distinct size of ~2000 bp has not yet been reported. This is peculiar, because its size suggests that it is neither from apoptotic nor necrotic origin.

3.3. Measurement of apoptosis and necrosis

To corroborate the observations made by electrophoretic analysis, and to help elucidate the origin of the cfDNA occurring at the different time points, the cells at each of the times correlating with the time-course study was analyzed for apoptosis, necrosis and cell cycle phase using three different flow cytometric assays. The FITC Annexin V assay

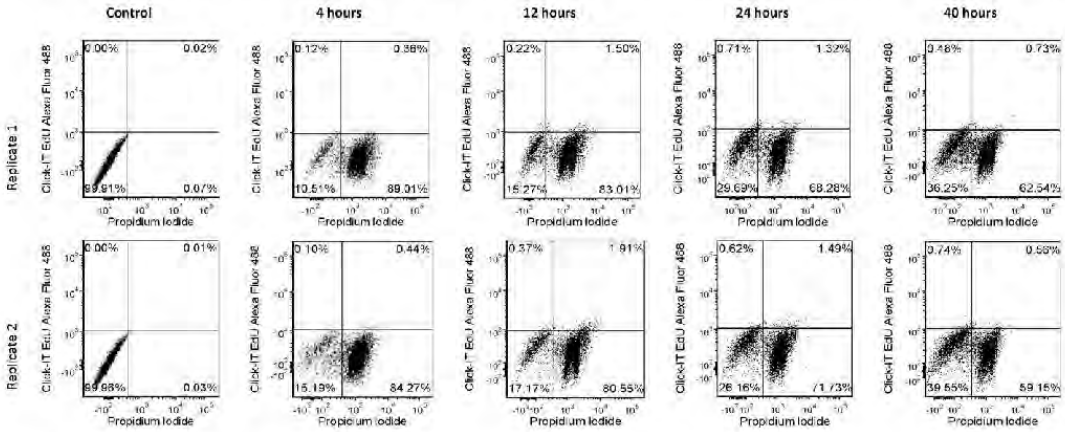

Fig. 7. Dot plots illustrating the proliferation of 143B cells at different times as analyzed with the Click-it EdU kit by flow cytometry. The DNA content (position in the cell cycle) is plotted against Edu alexa fluor 488 staining.

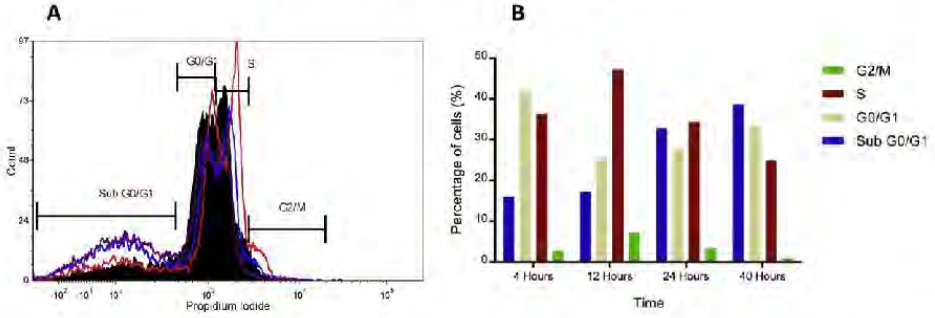

Fig. 8. Histogram and bar graphs showing the cell cycle distribution of 143 B cells at different points in time. In A, 4, 12, 24 and 40 h are represented by black, red, blue and purple, respectively. B shows the percentage cells in each phase as analyzed by FCS Express Multicycle. The high percentages of cells in the G0/G1 and S-phases are indicative of a fast growing cell population.

revealed that a fraction of cells are apoptotic after 4 h, whereas 24 h showed almost no apoptosis or necrosis (Figs. 4 & 5). Barring the time point of 4 h, these results were corroborated by the TUNEL assay (Fig. 6).

3.4. Cell cycle assessment

The assay used to measure cell proliferation reveals that, after 24 h of growth following medium renewal, there is a decline of cells in the S phase and significant increase of cells in the sub G0/G1 phase (Figs. 7 & 8). This suggests that the increase of cfDNA after 24 h of incubation is not associated with the process of DNA replication. The possible origin of cfDNA, in association with lipid-protein complexes (virtosomes), has been reviewed by Gahan where he reported literature showing that cfDNA in this form is released from both dividing and differentiated cells [26]. Since differentiated cells tend to be held in either the G0 or G1 phases of the cell cycle, Gahan suggested that this could be the realm in which cfDNA is synthesized, and that it is not likely related to mitotic DNA synthesis [26]. Our results support this notion.

3.5. Further characterization of cfDNA

To determine whether the cfDNA released from 143 B cells after 24 h of incubation could be similar to the virtosomes described by Gahan, growth medium was treated with denaturing agents prior to cfDNA extraction, and were compared to untreated samples (Fig. 9). In all cases, the yield of cfDNA was increased considerably by the addition of denaturing agents. Since it has been previously described that cfDNA is dissociated from the lipid-protein complex by freezing and thawing or by treatment with deoxycholate [36], the results of this experiment suggest that cfDNA is associated with proteins. Whether these proteins are simply nucleosomes, virtosomes, or extracellular vesicles remains unclear, and requires further experimentation.

4. Conclusion

Most *in vivo* studies report that the occurrence of cfDNA is associated with apoptosis or necrosis. However, the results obtained by this study suggest that the release of cfDNA from cultured 143 B cells after 24 h of incubation is not a consequence of apoptosis, necrosis, or a product of DNA replication, but primarily a result of actively released DNA, perhaps in association with a protein complex. Although other research groups have arrived at the same conclusion, very little attention has been drawn to this discovery.

The literature suggests that the purpose of actively released DNA is for the DNA to act as an intercellular messenger of sorts [37,38]. This is achieved by entering target cells and either integrating into the host genome, or to transiently elicit a biological effect [26]. Thus far, to mention the most prominent, the transfer of cfDNA between different cells has been implicated in the induction of tolerance against detrimental substances [40], immunomodulation [41], and the development of metastasis [42,43]. The latter has been demonstrated to occur *in vitro* [44] and *in vivo* [43]. In addition, results by Bergsmedh et al. suggest that the lateral transfer of cfDNA can not only mediate metastasis, but also generate the genetic instability necessary for malignancy [45]. On the other hand, Garcia-olmo and colleagues have recently shown that cfDNA derived from non-dividing healthy cells can halt tumor growth [46]. A recent review lends support to these findings and further illustrates the functional diversity of cfDNA [47]. It was found that apoptotic

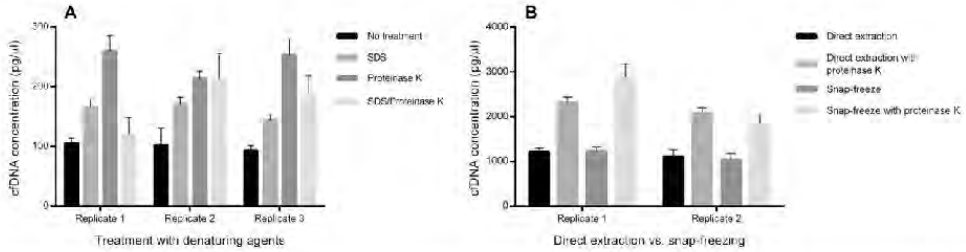

Fig. 9. The effect of treating growth medium with denaturing agents prior to extraction of cfDNA. A. The effect of the addition of denaturing agents. The use of SDS (0.05%), proteinase K (1.5 mg/mL), and a combination of the two were evaluated. In the cases where SDS was used, buffer NTB was used instead of buffer NTI. B. A comparison of cfDNA yield between direct extraction and snap-freezing, with and without the use of proteinase K. It is clear that, when the averages are calculated, snap-freezing does not increase the amount of cfDNA extracted and that the high yields can be ascribed solely to the addition of proteinase K.

death of stressed (irradiated) cells result in the release of oxidized cfDNA into circulation. This may serve as a biomarker of stress. In addition, it was demonstrated that these oxidized cfDNA molecules may confer survivability to other malignant cells by augmenting their resistance to radiation therapy. This is known as the bystander effect [39].

We further suggest, keeping in mind that cfDNA is readily transferred between cells, the possibility that cfDNA may play a role in the regulated generation of somatic genome variation. In addition, DNA has been detected in extracellular vesicles released by prostate cells that have been shown to interact with sperm cells [48,49]. This suggests that cfDNA may also be a potential bridge for information transfer between the soma and the germ line, which is still considered to be an unbridgeable chasm.

Although many aspects of the phenomenon of cfDNA messaging remain obscure, it is clear that the possibilities are intriguing. The observed messaging functions of cfDNA defy our traditional views of intercellular communication, especially relating to pathology. Furthermore, if cfDNA plays a role in somatic genome variation and transgenerational inheritance, it would strongly challenge the neo-Darwinian synthesis of evolution. Since cells in culture are isolated from the confusing variables encountered *in vivo*, *in vitro* characterization of the mechanisms that mediate the reactions of cfDNA should provide useful insight into its origin. This information is crucial for expediting the translation of *in vivo* cfDNA analyses to clinical practice, since it will provide novel markers for the diagnosis and therapy monitoring of several diseases, while ultimately leading to the treatment of certain diseases.

There are many ways in which cfDNA can be further characterized *in vitro*. For instance, the effect of cfDNA transfection on recipient cells can be evaluated by measuring cell viability, followed by metabolic and gene expression profiling. In addition, intracellular trafficking of these molecules can be traced by radioactive labeling, or alternatively by non-radioactive methods as described by Mansfield et al. [50]. Since cfDNA is often associated with extracellular vesicles such as exosomes, its cellular release and uptake could be visualized by live-cell microscopy using lipophilic dye and amino-reactive fluorophore labeling. This technique has been described by Tian et al. [51]. Arguably the best way to understand the molecular production of cfDNA is to examine its composition, in other words its sequence information. In the last couple of years, only a small number of papers have been published on the large scale sequencing of cfDNA. However, the objective of these studies was not to fully characterize cfDNA, but rather the sequence amplification of products to identify a small number of genes, while others have focused on methylation specific sequencing, or on the determination of molecular size [32,52–54]. Moreover, these studies were based on blood samples. The results of this study suggest that sequencing of cfDNA released by cultured cells may prove to be a useful way to circumvent the variables encountered in *in vivo* studies that may produce confusing sequencing results. In conclusion, the results of the current study demonstrate the potential of *in vitro* cfDNA analysis to aid in the elucidation of the nature of cfDNA.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Article VI

Kinetic analysis, size profiling and bioenergetic association of DNA released by selected cell lines in vitro

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ORIGINAL ARTICLE

Kinetic analysis, size profiling, and bioenergetic association of DNA released by selected cell lines in vitro

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Abstract Although circulating DNA (cirDNA) analysis shows great promise as a screening tool for a wide range of pathologies, numerous stumbling blocks hinder the rapid translation of research to clinical practice. This is related directly to the inherent complexity of the in vivo setting, wherein the influence of complex systems of interconnected cellular responses and putative DNA sources creates a seemingly arbitrary representation of the quantitative and qualitative properties of the cirDNA in the blood of any individual. Therefore, to evaluate the potential of in vitro cell cultures to circumvent the difficulties encountered in in vivo investigations, the purpose of this work was to elucidate the characteristics of the DNA released [cell-free DNA (cfDNA)] by eight different cell lines. This revealed three different forms of cfDNA release patterns and the presence of nucleosomal fragments as well as actively released forms of DNA, which are not only consistently observed in every tested cell line, but also in plasma samples. Correlations between cfDNA release and cellular origin, growth rate, and cancer status were also investigated by screening and comparing bioenergetics flux parameters. These results show statistically significant correlations between cfDNA levels and glycolysis, while no correlations between cfDNA levels and oxidative phosphorylation were observed. Furthermore, several correlations between growth rate, cancer status, and dependency on aerobic glycolysis were observed. Cell cultures can, therefore, successfully serve as closed-circuit models to either replace or be used in conjunction with biofluid samples, which will enable sharper focus on specific cell types or DNA origins.

Keywords Cell cultures · Circulating DNA · Cell-free DNA · Bioenergetics flux parameters · Aerobic glycolysis

Introduction

Since the discovery of circulating DNA (cirDNA) in human plasma [1], multiple studies have reported elevated levels of cirDNA in patients with various ailments, especially cancer (reviewed in [2]). In addition, the discovery of cell-free fetal DNA in maternal plasma has opened new avenues for the development of non-invasive prenatal screening methods [3]. However, despite more than 40 years of research, only one clinically validated application of cirDNA analysis as a disease screening marker is currently available, namely the Cobas® EGFR Mutation Test v2 (Roche Molecular Systems) for predicting the response of non-small cell lung cancers to treatment, particularly erlotinib (a tyrosine kinase inhibitor also known as Tarceva) [4, 5]. Although the development of clinical tests has been delayed by technological limitations and associated costs, this is becoming less of an excuse considering the continual and rapid improvements in sample handling products and high-throughput/ultra-sensitive techniques (e.g. NGS and ddPCR), which, in turn, reduce processing costs (discussed in [5]).

The lack of clinical applications for cirDNA analysis can be explained by the shortage of standard operating procedures and assay validation, which prevent the identification of normal reference values for correlating with diseases or the establishment of cut-off values for diagnosis and prognosis. This major drawback has been acknowledged by

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several researchers [5–12]. Furthermore, our knowledge regarding the biological properties, origin, and function of cirDNA is still lacking considerably.

Thierry et al. [13] has recently demonstrated the heterogeneity of the cirDNA population in plasma samples by highlighting various potential sources from which DNA could be released into circulation. It has also recently been demonstrated that the cirDNA derived from one cell or tissue type can be a product of different time dependent and environmentally modulated release mechanisms, which results in the presence of different amounts of cirDNA in a sample at a given time [14]. Research by Mittra et al. [15] showed potential damaging effects of apoptosis-derived cirDNA on cells, but also observed differences between the effects of cirDNA extracted from cancer patients and healthy controls, which may imply that the origin of the cirDNA may affect its function or effect on adjacent tissues once released into circulation. In addition, other researchers have demonstrated that the cellular release of mitochondrial DNA fragments can cause an inflammatory response [16–18]. However, analysis of these different effects in in vivo samples may prove to be difficult due to the presence of a vast range of putative cirDNA sources [19].

To overcome this problem, the implementation of in vitro cell culture models to study the biological properties of cirDNA has been proposed [14]. The purpose of this paper is to further encourage the utilization of cell cultures in elucidating the origin, composition, and function of cirDNA. Our previous studies have shown that the DNA released by cultured osteosarcoma cells into the growth medium [cell-free DNA (cfDNA)] consists of varying ratios of both apoptotic and actively released DNA fragments at various time intervals of incubation [14]. Furthermore, the DNA size profile observed in this study was shown to bear great similarity with the size profile of cirDNA derived from human blood, which exemplifies the potential of in vitro studies to aid in vivo research regarding the biological properties of cfDNA.

Under normal conditions, cells synthesize ATP most efficiently through glycolysis and oxidative phosphorylation (OXPHOS). Many cancers, however, undergo a metabolic conversion referred to as the 'glycolytic switch', after which glycolysis becomes uncoupled from respiration and lactic acid fermentation is used as the primary process for ATP production instead [20]. Such a switch occurs normally as a rescue mechanism for the production of energy during hypoxic conditions. In some cancer cells, however, the cells develop a particular glycolytic phenotype described by the controversial Warburg hypothesis as 'aerobic glycolysis'. At the first glance, it may seem like a highly inefficient path of energy production that converts far less ADP to ATP than OXPHOS. The benefits of this metabolic conversion, however, is that it potentially enables rapid ATP production and provides carbon intermediates allocable to branched biosynthetic pathways, which allows increased biomass expansion. Supporting this notion, the most common mutations in human tumors include mutations of the signalling pathways that regulate cellular biosynthesis and aerobic glycolysis [21]. Cancer cell metabolism is devoted primarily to consume glucose and glutamine, with very little glucose being used for OXPHOS [22–24] and correlations have been found between aerobic glycolysis and cancer cell proliferation [20]. It has also been determined that a switch of cancer cell metabolism from glycolysis to OXPHOS, upon inhibition of the pentose phosphate pathway, results in decreased proliferation [20]. The utilization of these unique metabolomic profiles of cancers as therapeutic targets may yield very promising new therapies [22, 25, 26]. Moreover, focusing on these characteristics may provide more insight into the elucidation of the biochemical characteristics and biological functions of cirDNA.

Therefore, in this study, we first compared the release patterns and fragment sizes of the cfDNA derived from various cell lines to determine whether variations in tissue origin, growth rate, and cancer status can influence cfDNA release characteristics. These cfDNA levels were then compared to the bioenergetics flux parameters, OXPHOS and glycolysis, of the respective cell lines to determine whether or not cfDNA release is affected by or dependent on cellular metabolic activity.

Materials and methods

Cell cultures and growth conditions

For the purpose of this study, the following seven cell lines with varying tissue origins, growth rate, and cancer status were identified: (1) rhabdomyosarcoma (RD) (ATCC® CCL-136™), large malignant tumor cells with a slow growth rate; (2) skin fibroblast (FIBRO) {also referred to as ZANLP in our previous study (Bronkhorst et al. [14])}, normal or healthy skin cells with a slow growth rate; (3) melanoma (A375) (ATCC® CRL-1619™), malignant skin cancer cells with a fast growth rate; (4) keratinocytes (HaCat) (AddexBio), spontaneously immortalized human skin cells with a relatively fast growth rate; (5) cervical adenocarcinoma (HeLa) (ATCC® CCL-2™), human papilloma virus (HPV)-induced cancer cervix epithelial cancer cells; (6) human embryonic kidney (HEK-293) (ATCC® CRL-1573™), normal or healthy adenovirus-transformed embryonal kidney cells; (7) hepatocellular carcinoma (HepG2) (ATCC® HB-8065™), cancerous liver epithelial cells with a significantly slow growth rate when cultured in Roswell Park Memorial Institute (RPMI) 1640 medium.

As an eighth cell line, data from our previous research regarding osteosarcoma (143B) (ATCC® CRL-8303™), bone cancer cells with a fast growth rate [14], were also added for comparison.

RD and FIBRO cells were grown in Dulbecco's modified Eagle's medium (Hyclone DMEM/high glucose), containing 4 mM L-glutamine, 4500 mg/L glucose, and 1 mM sodium pyruvate (Thermo Scientific), fortified with 10% fetal bovine serum (FBS) (Biochrom) and 1% penicillin streptomycin (Lonza). A375, HeLa, HaCaT, and HEK-293 cells were grown in the same growth medium with additional supplementation of 1% L-glutamine (Lonza), 1% non-essential amino acids (Lonza) and 1% amphotericin B (Biochrom). HepG2 was grown in RPMI 1640 (Hyclone), containing 2.05 mM L-glutamine (Thermo Scientific), fortified with 10% FBS, 1% penicillin streptomycin, and 1% amphotericin B. Initially, all of the cell lines were grown in 175 cm^2 flasks (Corning) to 90–100% confluence and incubated in a humidified atmosphere at 37 °C and 5% CO_2. Once the cell cultures reached the desired confluence, the cells were detached from the flasks via trypsinization and seeded into 75 cm^2 flasks (Corning) as follows: (1) the HaCaT and A375 cells at 30% confluence; (2) the RD, FIBRO, and HepG2 cells at 50% confluence; and (3) the, HeLa and HEK-293 cells at 25% confluence. Ten 75 cm^2 flasks were prepared for FIBRO cells, eighteen 75 cm^2 flasks for HaCaT cells, and twelve 75 cm^2 flasks for the remaining cell lines. All of the flasks contained a final volume of 12 mL. The 75 cm^2 flasks were incubated for 12 h, after which the growth medium was replenished. After this time, pairs of flasks were incubated for different time intervals for each cell line. The flask pairs of the A375 and RD cells were incubated for 4, 8, 12, 16, 20, and 24 h. The flask pairs of the HepG2, HeLa and HEK-293 cells were incubated for 4, 8, 12, 24, 48, and 72 h. The flask pairs of the HaCaT cells were incubated for 0, 2, 4, 6, 8, 12, 24, 36, and 48 h. The flask pairs of the FIBRO cells were incubated for 4, 18, 44, 72, and 98 h. The last time interval of each cell line serves as the time interval after which the cell lines reached confluence.

Sample collection and processing

At the end of incubation, the growth medium was collected in 15 mL tubes (SPL) and then centrifuged at 5000×*g* for 10 min and transferred to fresh 15 mL tubes. The samples were then stored at −80 °C until use. The cells were collected by trypsinization in 15 mL tubes, centrifuged at 5000×*g* for 5 min, rinsed with PBS, pelleted at 5000×*g* for 5 min, and stored at −80 °C for the extraction and determination of total cellular protein content.

Extraction and quantification of cellular protein

The frozen cell pellets were suspended in 3–4 mL cold PBS and 500 µL samples aliquots were sonicated with the Bioruptor UCD-200 (Diagenode). Before usage, the Bioruptor was cooled to 4 °C using distilled water and ice. Sonication settings were: power, H-position (high); sonication cycle, 30 s on/30 s off; total sonication time, 5–10 cycles. The total cellular protein content was quantified with the Qubit® Protein Assay kit and Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) according to the manufacturer's instructions.

Extraction and quantification of cell-free DNA

CfDNA was extracted directly from the growth medium using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's PCR clean-up instructions. Samples were thawed at 37 °C in a temperature controlled water bath, vortexed, and centrifuged briefly, and cfDNA was extracted in triplicate for each biological replicate. Extraction samples were prepared by mixing growth medium in a ratio of 1:2 with binding buffer NTI. The individual samples were vortexed, centrifuged briefly, the entire volume added to spin columns in three 600 µL regiments, and centrifuged at 11,000×*g* for 1 min at room temperature. The columns were then washed twice with wash buffer and the cfDNA eluted into 20 µL of elution buffer. To collect sufficient amounts of cfDNA for DNA fragment analysis, bulk extraction of the cfDNA of HEK-293 samples was performed using the NucleoSpin Gel and PCR Clean-up kit according to the manufacturer's PCR clean-up instructions, except binding buffer NTB was used instead of NTI. The cfDNA of each sample was quantified with the Qubit® dsDNA High Sensitivity Assay kit and Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) according to the manufacturer's instructions.

Fragment size evaluation of cell-free DNA

Capillary electrophoresis (CE) was performed to analyse the size distribution of the cfDNA that was extracted at the different time intervals for each cell line. The microchips and reagents of the High Sensitivity DNA kit were used according to the manufacturer's instructions and analyses performed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. Nucleic acids are separated analogously to CE, normalized to a ladder and two DNA markers, of which the sizes are calculated automatically by the software. The final results are displayed as electropherograms, where the two major peaks present at 35 and 10,380 bp in each electropherogram represent the two

size markers used to calculate the size of unknown samples and deviations from the baseline indicate the sizes of the cfDNA present in the samples.

Bioenergetic analyses

To assess the cellular metabolic activity for each of the eight cell lines, the Seahorse XFe96 Extracellular Flux analyser (Seahorse Biosciences, USA) was used. This instrument is capable of simultaneously measuring, in real-time, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells, representatives of the OXPHOS and glycolytic activity, respectively. Two different tests were carried out according to the manufacturer's instructions, namely the Seahorse XF Cell Mito Stress Test Kit and Seahorse XF Glycolysis Stress Test Kit (Seahorse Biosciences, USA) under similar conditions to that described by Zandberg et al. [27]. For both analyses, all cell lines were seeded at a range of cell seeding densities after which the final cell seeding density of 10,000 cells/well, with 5–6 replicate wells ($n = 5–6$), was selected, since all cell lines provided satisfactory results (cells were not over-confluent and provided OCR and ECAR readings within the acceptable ranges set out by the manufacturer) at this density. After seeding, all cells were incubated for 23 h under the conditions described in "Cell cultures and growth conditions". Thereafter, the growth media were replaced with assay media (modified DMEM supplemented with 2 mM L-glutamine, pH 7.4) for both tests (with the additional supplementation of 1 mM pyruvate and 5 mM glucose for the Mito Stress Test) and incubated for 1 h in a non-CO_2 incubator.

The instrument protocol consisted of three measurement cycles (3 min mix and 3 min measure per cycle) at the start of the analysis as well as following each of the three compound injections. For the Mito Stress Test, the following compounds were injected: 1 µM oligomycin (ATP synthase inhibitor), 0.5–0.75 µM FCCP (an uncoupler injected at the optimal concentration per cell line) and finally 0.5 µM rotenone (complex I inhibitor) and antimycin A (complex III inhibitor). The Glycolysis Stress Test included the following injections: 10 mM glucose, 1 µM oligomycin, and 50 mM 2-deoxyglucose (a glucose analogue). To account for differences in the proliferation rates of different cell lines and thus differences in cell seeding densities at the time of the XF analysis, all cells were normalized against total DNA content using the CyQUANT Cell Proliferation Assay Kit (Life Technologies, USA) according to the manufacturer's instructions. Version 2.2 of the Seahorse Wave software was used to process and analyse all results, while all outliers were removed using the Tukey method [28].

Statistics

All statistical analyses were performed and all graphs constructed using GraphPad Prism 7 (Version 7.0.2). The bioenergetic results were correlated to cfDNA release using Pearson's correlation coefficient (r), where a p value of less than 0.05 indicated a statistically significant result.

Results and discussion

In this study, the cfDNA release patterns and fragment size distributions of multiple cell lines were elucidated to determine whether or not variations in tissue origin, growth rate, and cancer status can influence cfDNA release characteristics. The bioenergetics flux parameters, OXPHOS and glycolysis, of each cell line were also determined and correlations between the metabolic activity and the cfDNA release levels of the cell lines evaluated.

Cell-free DNA release patterns

The release of cfDNA by eight cell lines after growth medium renewal was characterized over time. Figure 1 shows the cfDNA release levels quantified for each cell line, whereas Fig. 2 presents the cfDNA yields normalized to the total cellular protein of the cells collected from the flasks at each time interval. All of the cell lines were grown to confluence, after which the experimental sampling was terminated. Three distinct cfDNA release patterns were detected over time during the exponential growth phase of the cell lines (Fig. 2): (1) HepG2 and 143B cells showed a tendency of increasing cfDNA levels, (2) HeLa, RD and HEK-293 showed a tendency of decreasing cfDNA levels, and (3) the skin cells HaCaT, FIBRO, and A375 showed varied levels of increasing and decreasing cfDNA release with significant standard deviations.

HepG2 cells showed significantly high levels of cfDNA release [nearly 1.2 ng/µg protein (Fig. 2b)], with an increase in cfDNA release (ng per flask) over time, reaching its highest level at 48 h, followed by a significant decrease at 72 h (Fig. 1a). The large amount of cfDNA from the HepG2 cells is theorized to occur due to the prolonged exposure (48 h) of a relatively large amount of slow growing cells (seeded at 50% confluence). The cells also had a tendency to clump together rather than forming a monolayer, which may indicate that the cells could have reached confluency earlier than visually predicted. In a separate experiment, the growth medium of the HepG2 cells was changed from RPMI to high glucose DMEM fortified with non-essential amino acids and L-glutamine, resulting in a significant increase in cell growth rate (requires seeding at 15% confluence for cells to reach confluence within 72 h), no cell

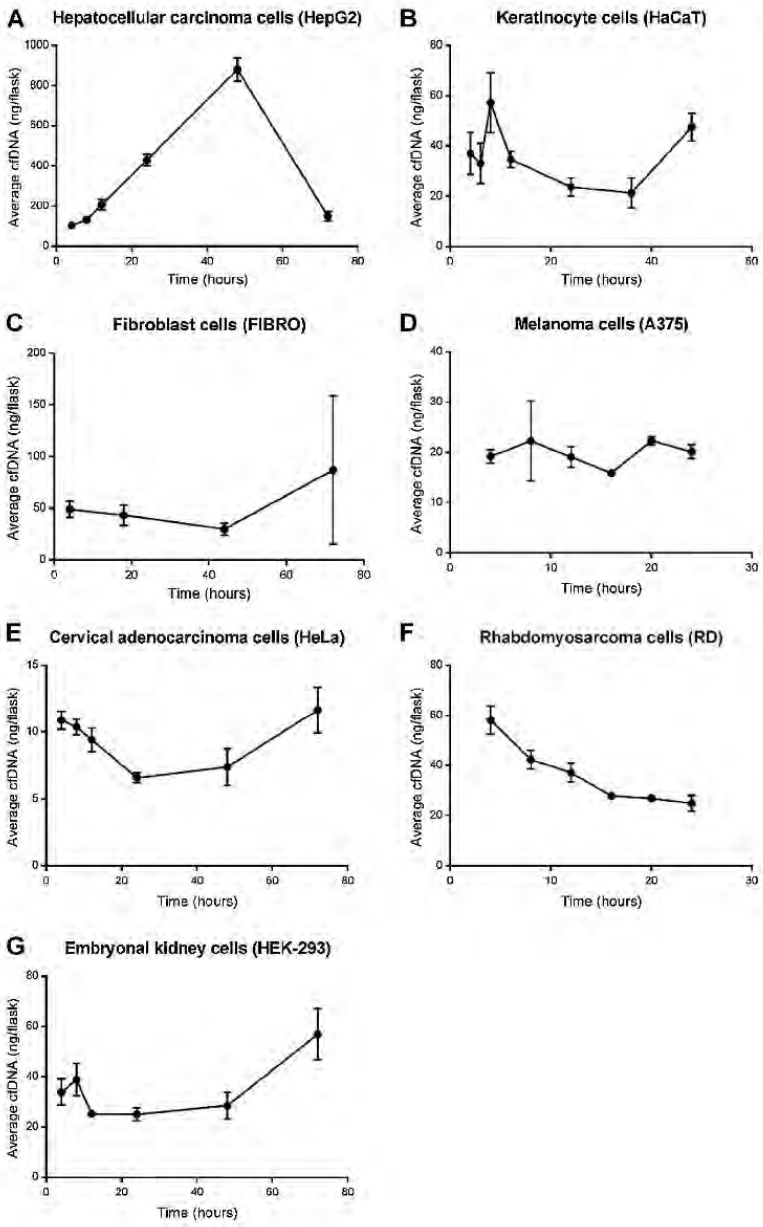

Fig. 1 CfDNA levels (ng/flask) of **a** HepG2, **b** HaCaT, **c**FIBRO, **d** A375, **e** HeLa, **f** RD, and **g** HEK-293 cell lines after several time intervals of incubation following medium renewal. The value of each bar represents the average (±SD, $n=6$) amount of cfDNA released in 12 mL of growth medium at each time interval

clumping, and a significant decrease in cfDNA release levels (30 pg/μg protein at 48 h).

Comparison of our previous results of 143B cells [14] (Fig. 2a) with HepG2 cells (Fig. 2b) revealed a similar cfDNA release pattern (pg/μg protein) with a gradual increase in cfDNA levels, followed by a significant decrease as the cell confluence increased. The significant decrease in cfDNA level of HepG2 and 143B cells at 72 and 28 h, respectively, was likely due to degradation via extracellular DNases. Studies have shown that healthy subjects present

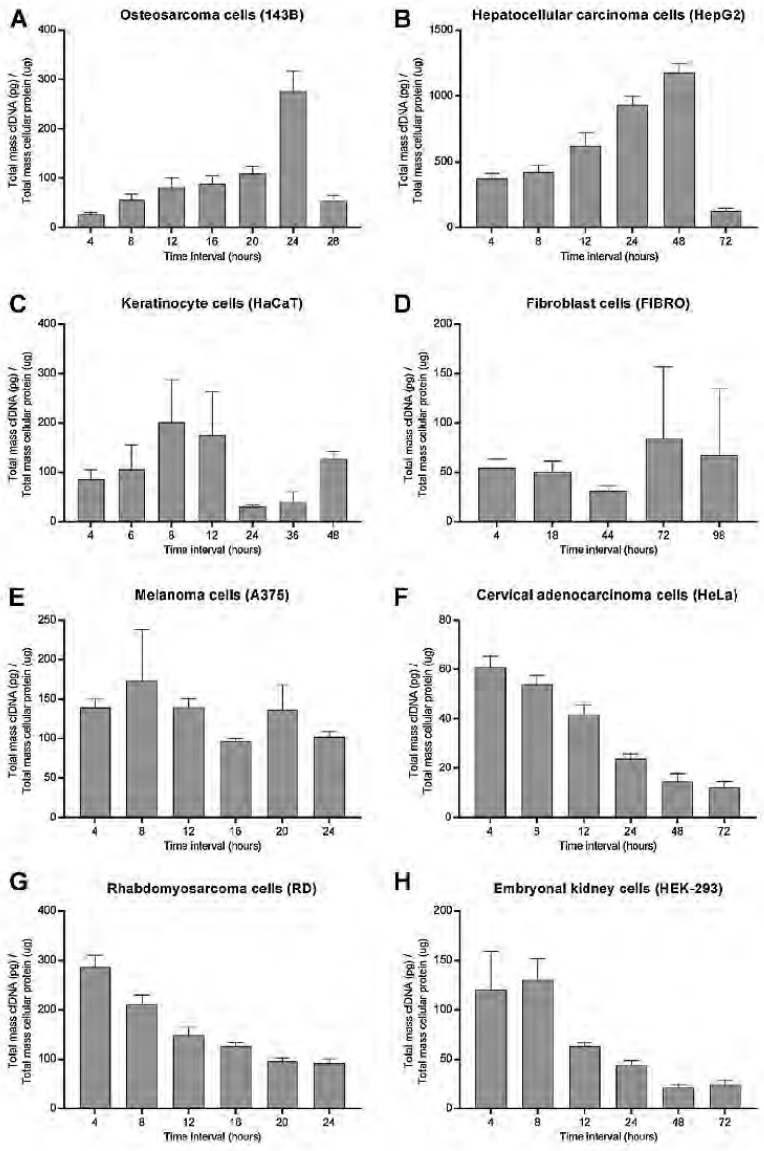

Fig. 2 CfDNA release patterns of **a** 143B quantified by qPCR using β-globin (from Bronkhorst et al. [14]), **b** HepG2, **c** HaCaT, **d** FIBRO, **e** A375, **f** HeLa, **g** RD, and **h** HEK-293 cell lines (quantified using the Qubit high sensitivity assay) after the indicated hours of incubation following medium renewal. The value of each bar represents the average (±SD, $n=6$) amount of cfDNA released in 12 mL of growth medium normalized in terms of the total cellular protein present in the culture flask at each time interval

with both low levels of cirDNA and high levels of DNase activity and that several cancers present with increased cirDNA levels and decreased DNase activity [29–31]. Significantly increased cirDNA levels in healthy trained subjects can also be reduced by increased endogenously expressed DNase activity [31]. It is, therefore, likely that a reduction in cfDNA levels present in the growth medium of these two and the other cell lines are also likely due, in part, to increased extracellular DNase activity.

HeLa, RD, and HEK-293 cells shared similar patterns of cfDNA release, where cfDNA levels decreased initially, followed by a significant peak and a subsequent decrease as the confluence increased (Fig. 2). HeLa cells released 60 pg/μg protein (Fig. 2f) and showed an initial

decrease in cfDNA levels (ng per flask), followed by a gradual increase (Fig. 1e). RD cells produced the second highest level of cfDNA at 286 pg/μg within 4 h of incubation, similar to the level of cfDNA release from 143B cells [14] (Fig. 2g), followed by a gradual decrease in cfDNA levels (ng per flask) as time progresses (Fig. 1f). HEK-293 released 130 pg/μg protein (Fig. 2h) with an initial increase in cfDNA levels (ng per flask) during the first two time intervals (4 and 8 h), followed by a slight decrease at 12 h, a slight increase at 24 and 48 h, and a more significant increase at 48 h (Fig. 1g).

The first two cfDNA release patterns (increased and decreased cfDNA release) did not appear to have correlations with tissue origins. The cfDNA release patterns was, on the other hand, less clearly increasing or decreasing for the cell lines, HaCaT, FIBRO, and A375 cells (Fig. 2), originating from skin cells. Since both the variable cfDNA release patterns and replicate inconsistencies occurred only in HaCaT, A375, and fibroblast cells, which all originate from skin cells, the results suggest that these characteristics can be related to the skin origin of the cells. Only the skin cell lines showed more prominent signs of replicate inconsistencies likely due to unstable growth rates resulting in significant differences in the amount of cfDNA and protein extracted in the duplicate flasks. HaCat released 170–200 pg/μg protein (Fig. 2c) with an initial increase in cfDNA levels (ng per flask) at 8 h, a gradual decrease at 12, 24, and 36 h, and a significant increase at 48 h (Fig. 1b). There are, however, significant replicate inconsistencies at 4–8 h. A375 also released between 170 and 200 pg/μg protein with less significant changes in cfDNA release patterns between time intervals (Fig. 2e), fluctuating cfDNA levels (ng per flask) (Fig. 1d) and significant inconsistencies between replicate flasks at 8 h. Visually, it appears that the following pattern of cfDNA release occurs: higher levels of cfDNA were initially detected, followed by a decrease in cfDNA release and a subsequent increase as the degree of confluence of the cell lines increased (Fig. 2e). A similar experiment screening for the 24 h cfDNA release patterns of A375 quantified via qPCR using β-globin revealed the same pattern of variable cfDNA release over time during the exponential growth phase (data not shown) with similar levels of replicate inconsistencies. FIBRO cells, on the other hand, released a constant cfDNA level of around 50 pg/μg protein during the first 24 h of incubation (Fig. 2d), showing a little variation in cfDNA release patterns (pg/ μg protein) between time intervals (data not shown) that only began to slightly change after 24 h, but resulted in significant standard deviations from 72 h. The cells showed a gradual decrease in cfDNA levels (ng per flask) at 4, 18, and 44 h, followed by a significant increase at 72 h with a significant standard deviation (Fig. 1c).

Cell-free DNA fragment size evaluation

To elucidate the fragment size distribution of cfDNA, four of the cell lines, HepG2, RD, HEK-293, and FIBRO, were selected and the cfDNA isolated at the different time intervals were used to perform microchip-based capillary electrophoresis. The results were then compared to that of 143B cells from Bronkhorst et al. [14].

The electropherograms of 143B (Fig. 3), HepG2 (Fig. 4), FIBRO (Fig. 5) and, to a lesser extent, RD (Fig. 6), and HEK-293 (Fig. 7) cells showed indications of the presence of small DNA fragments (<100 bp, particularly at 47–50 bp) at each time interval. CirDNA from tumor cells have been shown to consist of DNA fragments smaller than that of healthy subjects, particularly <100 bp, and these DNA fragments largely consist of the mutated DNA originating from the tumor [32, 33]. However, the normal, noncancerous cell lines, FIBRO and HEK-293, also present with small cfDNA fragments, an observation also observed in a recent study by Underhill et al. [34], where short DNA fragments of both rat and human were detected in controls. As such, the presence of smaller DNA fragments in noncancerous cfDNA makes it difficult to discern whether or not the small fragments of the cancerous cell lines are indeed mutated tumor DNA fragments, in the absence of DNA sequencing.

HepG2 cfDNA (Fig. 4) showed a typical ladder pattern characteristic of nucleosomal subunits of predictable sizes that are most prominent at around 150–200, 300–400, and 500–600 bp. Our previous study using 143B cells revealed that apoptosis occurs in samples where this typical ladder pattern is detected [14], indicating that apoptosis could be present in the HepG2 cell cfDNA at each time interval. These peaks decreased notably from 4 to 24 h, but increased again after 48 h of incubation. Concurrently, a peak at approximately 2000 bp formed at each time interval, which increases in intensity [fluorescent units (FU)] from 4 to 24 h as the ladder pattern fragment peaks decrease, followed by an increase in FU at 48 h when the ladder pattern fragment peaks start to increase. Interestingly, both the 2000 bp peak and apoptotic peaks of the HepG2 cells decrease in FU significantly at 72 h. The 143B electropherogram data [14] correlated well with that of HepG2 cells, showing a pattern of an apoptotic DNA fragment of 166 bp that decreases as a 2000 bp peak develops. At 24 h, the 2000 bp peak is at its largest and starts to decrease at further time intervals with a concomitant increase in the apoptotic DNA fragment peaks (Fig. 3). The 2000 bp peak was found to occur in the absence of apoptosis and necrosis, possibly indicating that this peak is the actively released form of cfDNA [14].

The FIBRO samples (Fig. 5) presented with apoptotic DNA fragments and a 2000 bp disturbance from 4 h.

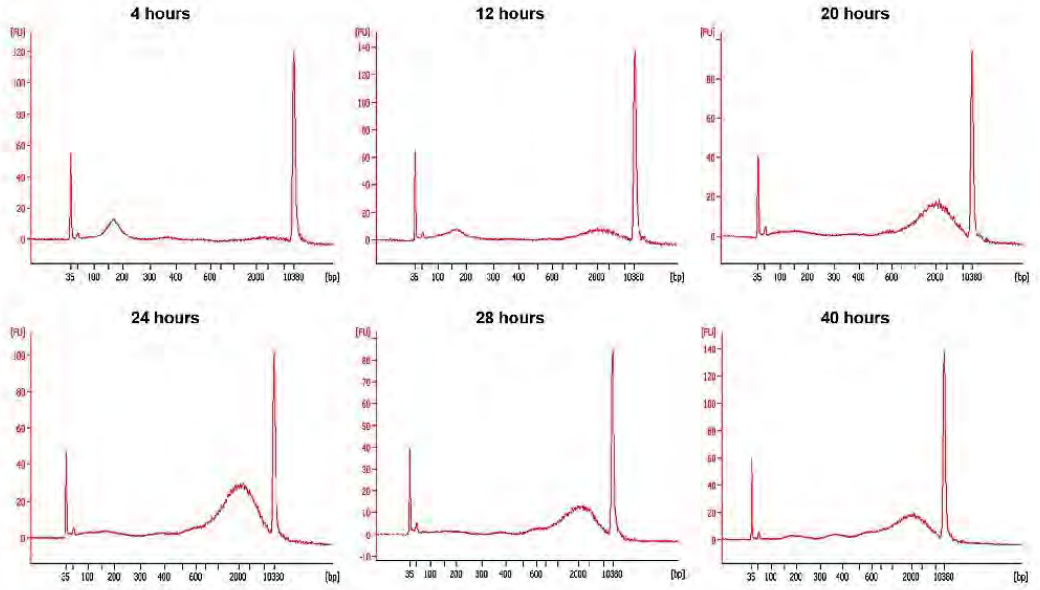

Fig. 3 Capillary electropherograms showing the fragment sizes of cfDNA isolated from 143B cells after 4–40 h of incubation following medium renewal [14]. The peak at 150–200 bp was shown to be representative of apoptosis, while larger peaks arising after 12 h of incubation and reaching a maximum at 24 h are not derived from either apoptotic or necrotic cfDNA release

Changes in the 2000 bp area are barely visible, but the nucleosomal ladder pattern peaks decrease in FU as time progresses and significantly increases at 72 h. RD cfDNA (Fig. 6) also showed a typical ladder pattern associable with nucleosomal subunits at each time interval. These peaks decreased to significantly low levels, becoming barely visible as time progressed. Concurrently, a peak at approximately 2000 bp is also present at each time interval, which increases in FU from 4 to 12 h as the apoptotic peaks decrease at 16–24 h. HEK-293 samples (Fig. 7) showed no indications of smaller DNA fragments. A peak forms at approximately 2000 bp at 4 h, which seems to gradually decrease in FU. At 72 h, however, there seems to be a more visible disturbance in the baseline from approximately 150 bp, which could indicate the initial formation of apoptotic DNA fragment peaks.

The patterns of the 2000 bp peaks, in particular, of the cell lines correlate well with the cfDNA release patterns demonstrated in Figs. 1 and 2. The 2000 bp peak of HepG2 and 143b (Figs. 3, 4) increases, followed by its decrease and concomitant increase in the other fragment peaks (less clearly visible for 143B in Fig. 2a), together forming a maximum level of cfDNA release. A later decline in all fragment peaks results in the significant drop in cfDNA levels in Fig. 2a, b. The 2000 bp peaks of RD and HEK-293 (Figs. 6, 7) and nucleosomal ladder fragment peaks of FIBRO (Fig. 5), on the other hand, decreased as time progressed, with an increase at 72 h for FIBROs, correlating with the cfDNA release patterns of Fig. 2d, f, and g. The different cfDNA release patterns are, in conclusion, not due to different ratios of apoptotic to actively released cfDNA, but primarily due to fluxes in actively released cfDNA. Electropherogram data did, therefore, not provide an explanation as to why there are different cfDNA release patterns.

Similar results have been observed in epidermoid carcinoma (A431), HeLa, human umbilical vein endothelial cells (HUVEC), human T lymphocytes (Jurkat cells), and pleural effusion cells (U937) [14, 35–37]. Morozkin et al. showed that cfDNA concentrations increase over time during the lag and beginning of the exponential growth phase of cell lines. They have also determined via electrophoretic analysis that the molecular weight of the obtained cfDNA was between 400 and 10,000 bp, suggesting that cfDNA is neither from apoptotic nor necrotic origin and is likely actively released from cells [36, 37]. Choi and colleagues, who studied their cell lines (Jurkat and U937) under normal, apoptotic, and necrotic conditions, have showed similar increases in cfDNA levels over time and that apoptosis results in a rather dramatic increases of cfDNA levels after 24–48 h of incubation in comparison with normal

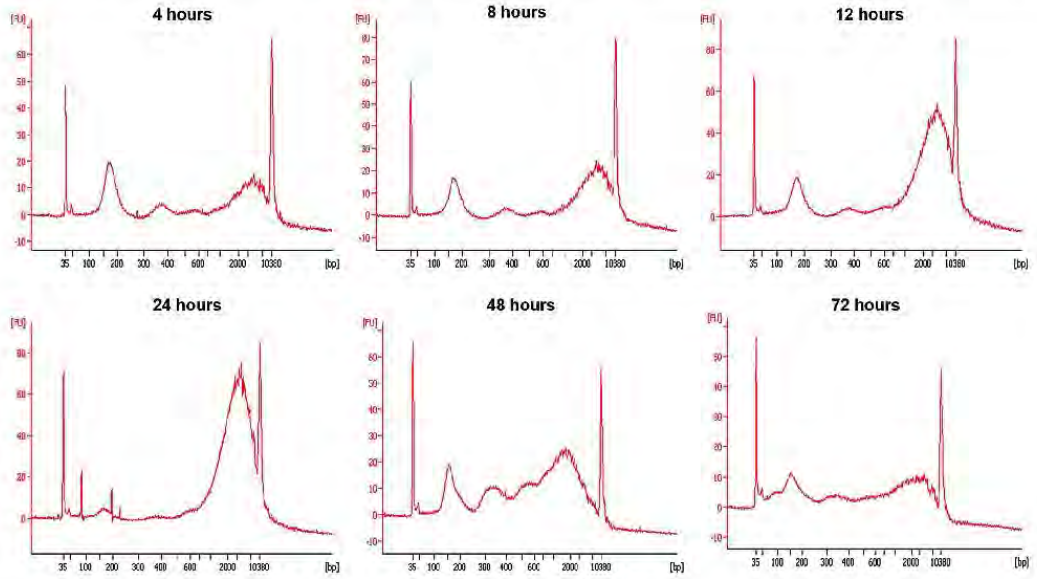

Fig. 4 Capillary electropherograms showing the fragment sizes of cfDNA isolated from HepG2 cells after 4–72 h of incubation following medium renewal. Each shows a typical ladder pattern associable with nucleosomal subunits of predictable sizes that are most prominent at around 150–200, 300–400, and 500–600 bp. After 12 h, a major peak is visible at around 2000 bp. This peak reaches a maximum of a little under 80 FU at 24 h, but then decreases, while the smaller peaks rise

or healthy cells. Necrosis, on the other hand, resulted in a decline of cfDNA levels over time, which indicated that cfDNA may be released by more than one mechanism, but that apoptosis can be primarily held responsible for high cfDNA levels [35].

The implications of apoptotic and actively released DNA fractions in circulating DNA

Little attention has been given to the impact that the different fragment sizes of cirDNA contents may have on studies in which recipient cells or animals are treated with cirDNA. For example, Mittra et al. [15] provided disconcerting evidence regarding the damaging effects cirDNA on recipient cells. Fragmented DNA and chromatin isolated from the blood of cancer patients and healthy volunteers were co-cultured with mouse fibroblasts, ovary, kidney and adipocyte cells, and HeLa cells, and were injected into mice, resulting in the induction of apoptosis. The fragmented DNA and chromatin of the healthy volunteers, however, resulted in a lower effect than that of the cancer patients. It is most likely that the DNA isolated from these subjects is of multiple intercellular origins, similar to that observed in cell lines. Studies have proposed that apoptosis serves as the source of cirDNA in both normal and diseased tissues [38]. Cells and whole organs in culture also spontaneously release nucleoprotein complexes in a homeostatic environment and newly synthesized DNA is preferentially released, indicating the presence of an active DNA release mechanism [39–42]. That being the case, the question becomes whether the apoptotic and actively released fractions of the DNA isolated will both result in damaging effects in vitro and/or in vivo or whether the damaging effects observed by Mittra et al. [15] were due to only one of the fractions.

Are both apoptotic and actively released DNA fractions responsible for damaging effects?

Although both healthy and tumor cells can undergo apoptosis, the levels of apoptosis in cancer patients will likely be higher than in healthy patients due to increased stress levels and/or decreased vascularization in both the tumors and surrounding tissues [43]. This could explain why the DNA isolated from the healthy volunteers had a less prominent effect on recipient cells and mice than that of the cancer patients. Bystander effect (the effect of information transfer from targeted cells exposed to damaging agents of physical nature or chemical nature to surrounding, non-irradiated cells) studies have shown similar damaging consequences when DNA released from irradiated cells are given to

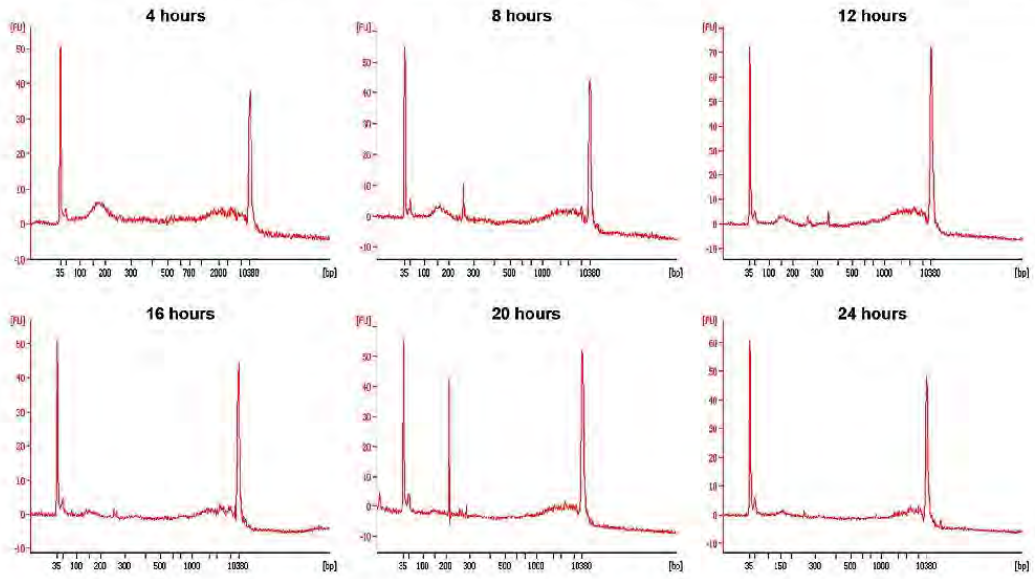

Fig. 6 Capillary electropherograms showing the fragment sizes of cfDNA isolated from RD cells after 4–24 h of incubation following medium renewal. A peak is prominent at 100–200 bp from 4 h of incubation that has been shown to be associable with apoptosis [14]. This peak can be seen declining over time, reaching a barely visible amplitude at 24 h of incubation. In these electropherograms, the baseline appears 'disturbed' throughout all time intervals. Also visible from the 4 h interval is a peak with a maximum amplitude at the 2000 bp range that has previously been shown to be unassociated with apoptotically and necrotically derived cfDNA. While the 2000 bp peak declines, peaks at lower increments of nucleosomal subunits again appear more prominent

in this study's eight cell lines [51]. These similarities in cfDNA and cirDNA contents are, however, dependent on the extraction methods used as the choice of extraction method may cause bias in blood-based studies. The Kingfisher method, for example, failed to extract cfDNA with a size of 2000 bp [51]. Furthermore, the origins of the cfDNA in the growth medium of 2D cultures are restricted to a particular tissue origin, cell morphology, and disease, thereby providing "more focused" sample contents that may simplify or aid the discovery of biological markers, the elucidation of biological functions, and the potential determination of the effects of cell morphology on cirDNA characteristics. Recent experiments screening the cfDNA characteristics of three-dimensional (3D) cell cultures have shown that the cfDNA of spheroids, developed with HepG2/C3A cells in microgravity bioreactors [52], effectively mirrors the brief and/or minor changes in the growth and glucose consumption during spheroid development and toxicological studies (unpublished data). Moreover, the fragment patterns of the spheroid cfDNA correlate with that of both 2D cell cultures and human plasma samples. An extension from 2D cell cultures to 3D in vitro models prior to in vivo research can, therefore, also be of great value to the cirDNA research field as one will be able to obtain more physiologically relevant samples while still having the benefit of a restricted environment of a specific cellular origin or disease.

Bioenergetic analyses

Mito stress test

Six parameters were calculated using the Mito stress test OCR results, namely: basal respiration (last OCR measurement prior to oligomycin injection), proton leak (the minimum OCR measurement following oligomycin injection), ATP production (proton leak subtracted from basal respiration), maximal respiration (the maximum OCR measurement following FCCP injection), and spare respiratory capacity (maximal respiration minus basal respiration). The sixth parameter, non-mitochondrial respiration (the minimum OCR measurement following rotenone/antimycin A injection), was an indication of all oxygen being consumed by processes independent of the OXPHOS system and was thus subtracted from the other five Mito stress test parameters. The OCR results for each of the OXPHOS parameters

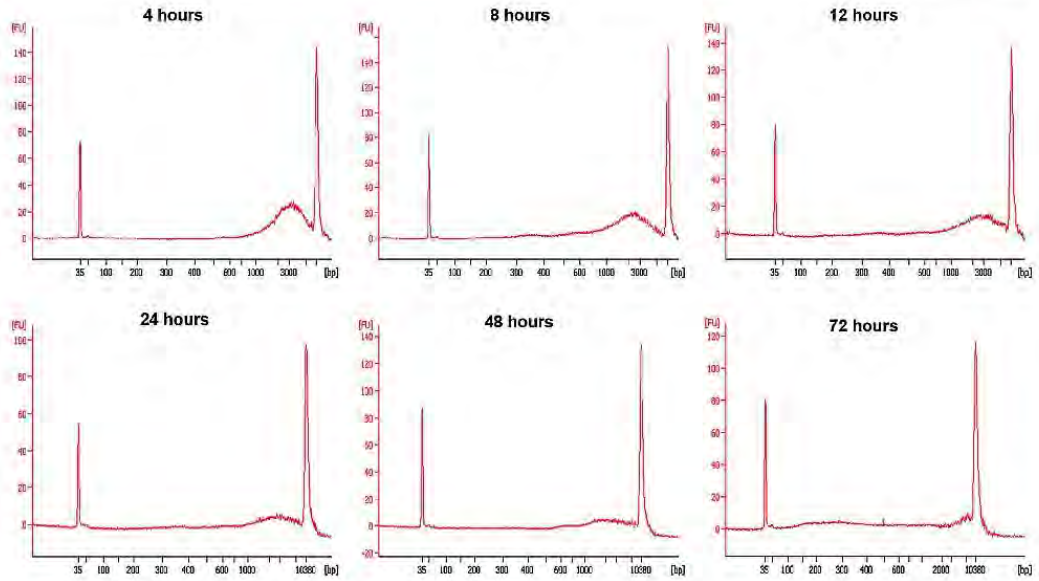

Fig. 7 Capillary electropherograms showing the sizes of cfDNA isolated from HEK-293 cells after 4–72 h of incubation following medium renewal. The electropherograms show no indications of smaller peaks. Instead, a peak with maximal fluorescence is visible at 2000–3000 bp. Once again, this peak diminishes over time, while slight disturbances in the baseline become visible after 8 h of incubation

for each cell line are illustrated in Fig. 8. HepG2, FIBRO, 143B, and RD cells were shown to have low OXPHOS activity, indicating that these cancer cells and fibroblasts may have a greater dependence for glycolysis as an energy source. HeLa and A375 showed slightly higher levels of OXPHOS activity, which may indicate that these cancer cell lines have a more moderate need for both OXPHOS and glycolysis for energy production. HaCaT and HEK-293 presented high OXPHOS activity, which is expected for normal or healthy cells. These results are discussed in "Correlation between bioenergetics results and cell-free DNA release".

Glycolysis stress test

Five parameters were calculated using the glycolysis stress test ECAR results, namely: glycolysis (maximum ECAR measurement following glucose injection), glycolytic capacity (maximum ECAR measurement following oligomycin injection), glycolytic reserve (glycolytic capacity minus glycolysis), and glycolytic reserve as a %. The non-glycolytic acidification (last measurement prior to glucose injection) was subtracted from all other glycolytic parameters. The ECAR results for each of the glycolytic parameters for each cell line are illustrated in Fig. 9.

HepG2, A375, and HEK-293 cells showed high levels of glycolysis and glycolytic capacity. The high glycolysis and significantly low OXPHOS activity of HepG2 cells, with a significantly slow growth rate, correlate well with the theory of cancer cell lines utilizing aerobic glycolysis as a predominant energy source. The moderate glycolysis and OXPHOS levels of RD are also indicative of this cancer cell line's slow growth rate and larger dependence on glycolysis. The high levels of glycolysis activity and moderate levels of OXPHOS activity in A375 show that higher levels of glycolysis are, indeed, required for these fast growing cancer cells, but that the glycolysis is not exclusive and that these cells can also use OXPHOS to a better extent than HepG2 cells. HeLa cells also showed moderate OXPHOS and glycolysis activity, supporting the proposal that this cell line does not necessarily have a predominant form of energy production as expected for cancer cells. 143B cells, with a growth rate similar to that of A375 cells, presented with slightly lower OXPHOS and considerably lower glycolysis levels compared to A375 cells. HaCaT cells showed moderately high levels of both OXPHOS and glycolysis activity, which is expected for normal cell lines. HEK-293 cells, however, shows significantly higher levels of both glycolysis and OXPHOS activity, which may indicate higher levels of anabolism in these cell lines compared to

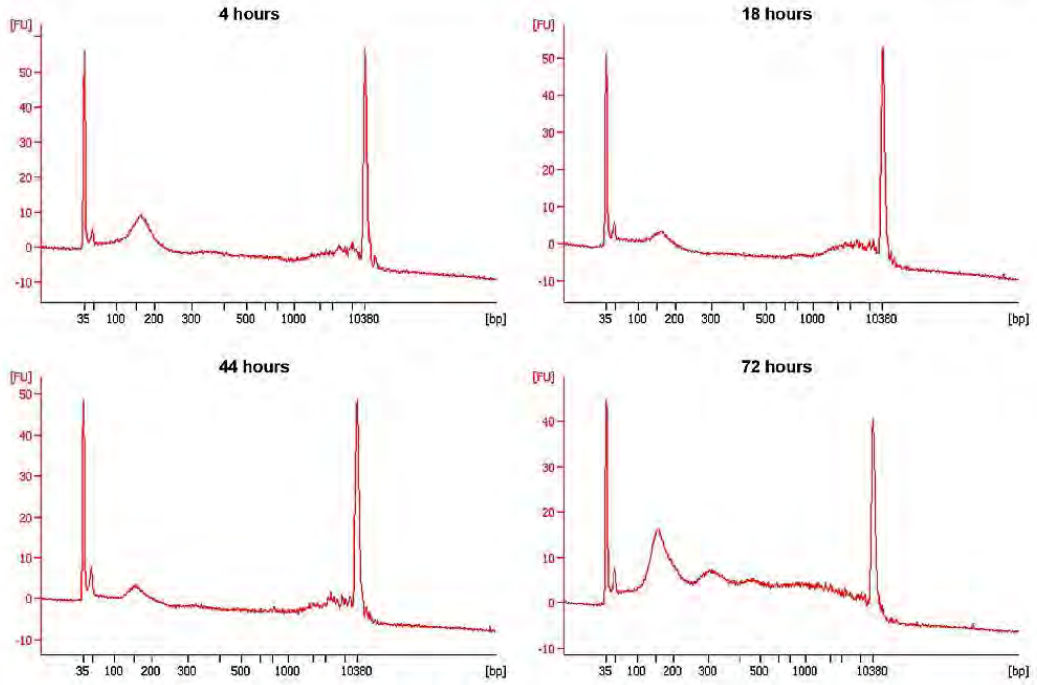

Fig. 5 Capillary electropherograms showing the sizes of cfDNA isolated from FIBRO cells after 4–72 h of incubation following medium renewal. Very prominent in each electropherogram is the peak at 100–200 bp. This peak is associable with apoptosis and becomes exceptionally prominent at 72 h of incubation. Also visible in each electropherogram is a typical ladder patter associable with multiple nucleosomal subunits. The entire baseline is elevated at 72 h of incubation as an indication of the presence of variable fragment sizes ranging from 35 to 10,000 bp

non-irradiated recipient cells [44–46]. Contrarily, Garcia-Olmo et al. [47] showed that newly synthesized and/or spontaneously released virtosomes from non-dividing cells reduced tumor growth and metastasis, but had little effect on normal dividing fibroblasts. This shows that actively released DNA may not have the damaging effects seen by Mittra et al. and during the bystander effect studies. It may also suggest that the effects of the different fractions of cfDNA may be dependent on the biology (type of cell) or status (healthy or diseased) of the recipient.

Puszyk et al. [48] and Bronkhorst et al. [49] showed that selectivity may be involved in the release of cfDNA, as there is an unequal representation of cfDNA sequences and genes in the blood samples of humans and culture growth medium, respectively. The transfer of cfDNA between different cells and recipients has been demonstrated both in vitro and in vivo on multiple occasions (refer to Bronkhorst et al. [14]), and the bystander effect studies and tumor growth inhibition effects of the cfDNA of non-dividing cells mentioned above further support the idea of cfDNA serving as an intercellular messenger of sorts, a concept that has been considered on occasion [11, 50] and that both actively released cfDNA and apoptosis-derived cfDNA may serve different purposes in a cellular environment. The elucidation of the separate effects of actively released and apoptosis-derived cfDNA fractions is, therefore, strongly encouraged to better understand the true biological role and clinical implications of cfDNA. For this reason, the utilization of cell cultures instead of (or in conjunction with) plasma or serum samples is strongly encouraged.

The utilization of in vitro cell cultures in circulating DNA research

Standard two-dimensional (2D) cell cultures have significant benefits for cirDNA research. It has been demonstrated that the contents of cell culture cfDNA and plasma sample cirDNA are very similar, as the electropherograms of plasma samples show the same patterns of nucleosomal ladder fragments and a prominent 2000 bp peak detected

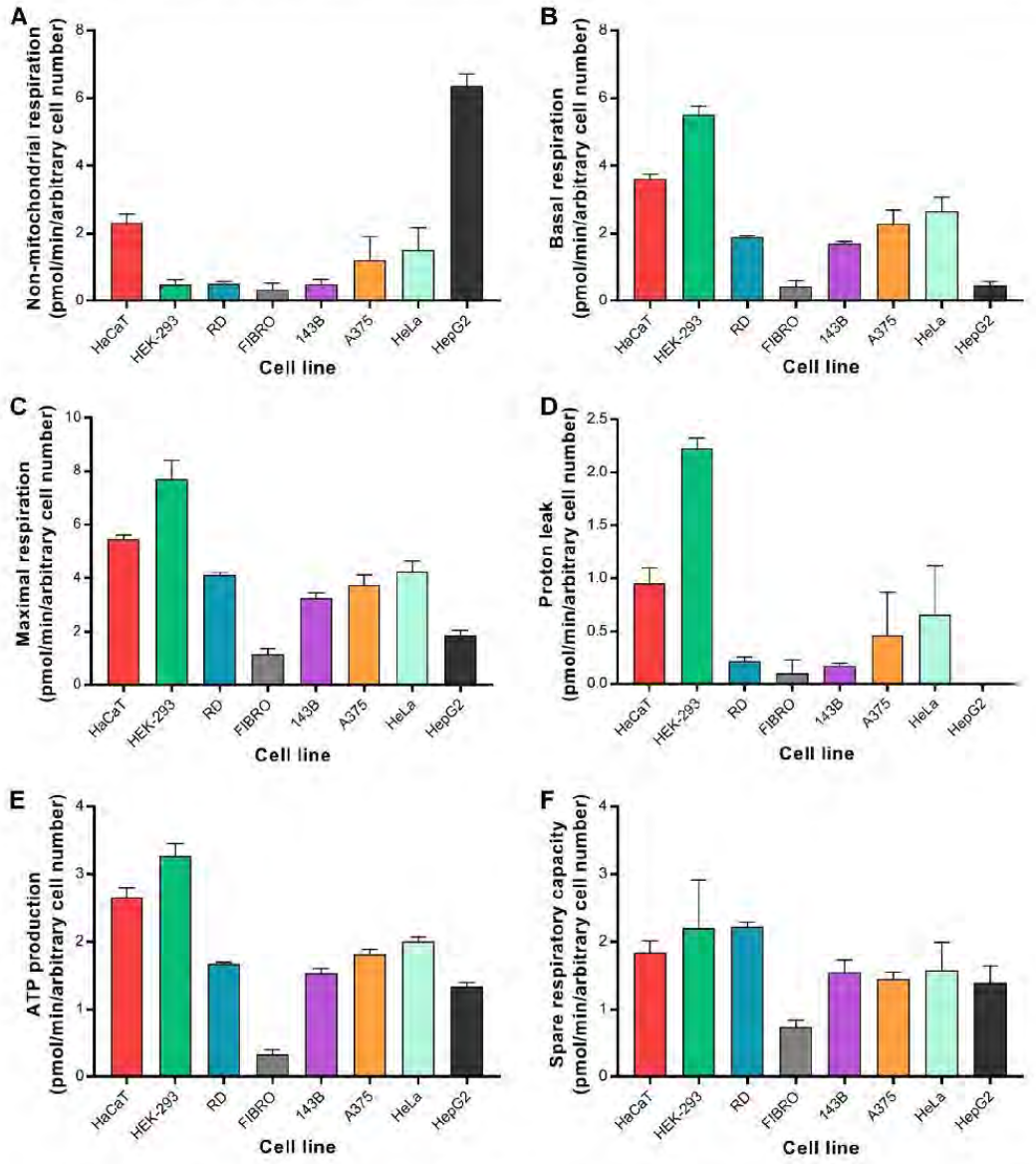

Fig. 8 Histograms depicting eight cell lines for each of the OXPHOS parameters determined using the Mito stress test (n=5–6). **a** Non-mitochondrial respiration, **b** basal respiration, **c** maximal respiration, **d** proton leak, **e** ATP production, and **f** spare respiratory capacity. *Error bars* indicate standard deviation

HaCaT cells. FIBRO cells presented with the lowest level of glycolysis than that of the other seven cell lines, most likely due to its slow growth rate. Combined with its low OXPHOS levels, the bioenergetics analysis indicates that this normal skin fibroblast cell line shares metabolic similarities to cancer cells, with a higher need for glycolysis.

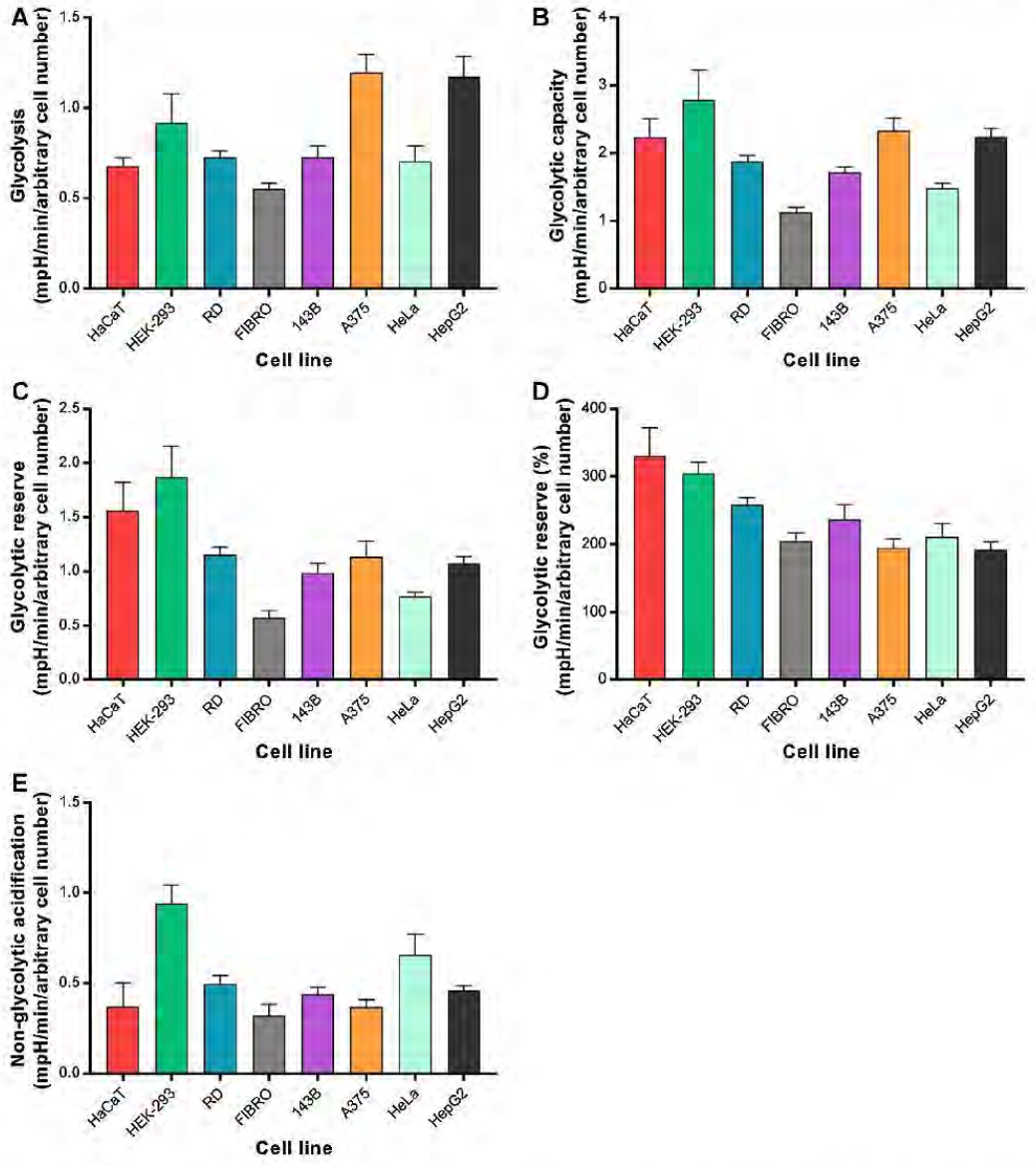

Fig. 9 Histograms depicting eight cell lines for each of the glycolytic parameters determined using the glycolysis stress test ($n = 5$–6). **a** Glycolysis, **b** glycolytic capacity, **c** glycolytic reserve, **d** glycolytic reserve (%), and **e** non-glycolytic acidification. *Error bars* indicate standard deviation

These results were used and further explained in the following section.

Correlation between bioenergetics results and cell-free DNA release

The bioenergetics results obtained in "Mito stress test" and "Glycolysis stress test" were correlated to the respective amounts of cfDNA released at 24 h of incubation (see "Cell-free DNA release patterns"), since the XF analysis was conducted 24 h after cell seeding, using Pearson's correlation coefficient, as shown in Table 1. The only parameter revealing a significant correlation was non-mitochondrial respiration, an indicator of other cellular processes outside of the mitochondrion which consume oxygen, and a scatter plot of this is given in Fig. 10. Scatter plots were constructed for each bioenergetic parameter against cfDNA yield (data not shown) and it was observed that while glycolysis did not show a significant correlation when using all eight cell lines, there did appear to be a trend when splitting the eight cell lines into two groups, namely group 1 (HEPG2, 143B, RD, and FIBRO) and group 2 (HEK-293, A375, HaCaT, and HeLa).

Since no significant correlations were seen between any of the OXPHOS parameters and cfDNA release, besides non-mitochondrial respiration, there does not appear to be a link between cfDNA release and the electron transport chain's capacity for substrate oxidation and meeting the energy demands of the cell. Non-mitochondrial oxygen consumption is believed to be due to certain detoxification, pro-inflammatory, and desaturase enzymes that consume oxygen outside of the OXPHOS system and tend to increase when subjected to stressors such as ROS [53]. It is generally a parameter that provides relatively low OCR values. As can be seen by Fig. 8a, the non-mitochondrial OCR values for the HEK-293, RD, FIBRO, and 143B cell lines are very low and similar, while the HaCaT, A375, and HeLa cells lie slightly higher. In Fig. 10, the HEPG2 cells can be seen to have much greater cfDNA levels and non-mitochondrial respiration compared to all the other cell lines. When removing the HEPG2 cell line from the correlation analysis, the significant correlation that was originally observed (Table 1) is then lost ($p = 0.379$). When studying Fig. 10b, one can also see that when looking at group 2 only (which excludes the HepG2 cell line), there does not appear to be a linear correlation and the HepG2

Table 1 Pearson's correlation coefficient and p values for eight cell lines testing the correlation between each bioenergetic parameter and the cfDNA release at 24 h[a]

OXPHOS parameters			Glycolytic parameters		
Statistic	r	p value	Statistic	r	p value
Non-mitochondrial respiration	0.8739	0.05	Glycolysis	0.5729	0.14
Basal respiration	−0.5124	0.19	Glycolytic capacity	0.1731	0.68
Maximal respiration	−0.4663	0.24	Glycolytic reserve	−0.1084	0.80
Proton leak	−0.6870	0.06	Glycolytic reserve (%)	−0.4237	0.30
ATP production	−0.2836	0.50	Non-glycolytic acidification	−0.1533	0.72
Spare respiratory capacity	−0.1987	0.64			

[a]The cfDNA data at 18 h was used for FIBRO, since data at 24 h was not available

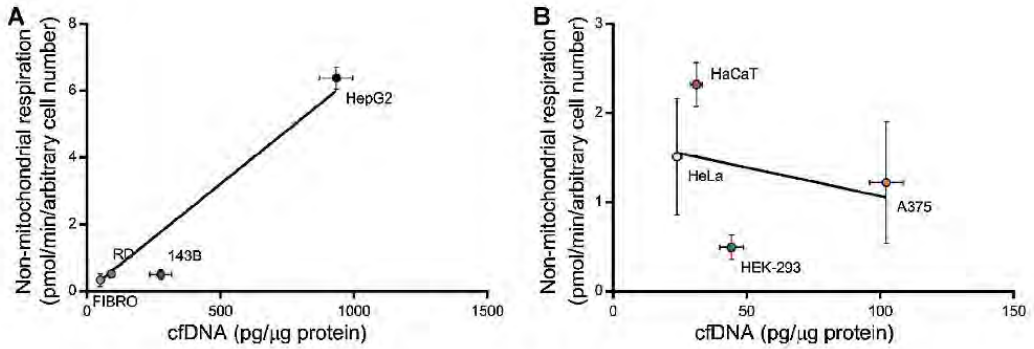

Fig. 10 Scatter plot depicting the correlation between non-mitochondrial respiration and cfDNA release for **a** group 1 (HEPG2, FIBRO, RD, and 143B) and **b** group 2 (HeLa, HaCaT, A375, and HEK-293). Values indicate the mean ± standard deviation

cell line was thus most likely skewing the data. Due to the markedly increased non-mitochondrial respiration and cfDNA levels of the HepG2 cells, there does appear to be a correlation, but this does not appear to be true for the other cell lines. Increased non-mitochondrial respiration is believed to negatively affect the bioenergetic status of cells, which is demonstrated in Fig. 8b, c, and e, where the basal respiration, maximal respiration, and ATP production were lower in the HepG2 cells compared to all the other cell lines (with the exception of FIBROs).

As shown in Table 1, a positive correlation could be seen for glycolysis and cfDNA release, but this correlation was, however, not statistically significant. As shown in Fig. 11a and b, when these two groups are separated, there is a statistically significant positive correlation with a very high effect size for both group 1 ($r=0.9703$, $p=0.030$) and group 2 ($r=0.9643$, $p=0.036$). When studying all of the other bioenergetics parameters relative to these two groups, no other statistically significant differences were observed. It would thus appear that greater cfDNA release occurred in cell lines with increased glycolytic rates. Glycolysis was determined by injecting a saturating concentration of glucose into the media (which did not initially contain glucose or pyruvate) and measuring the rate by which lactate production increased following the glucose injection (which was then converted to pyruvate and then to lactate).

It is interesting to note that the grouping of the cell lines into group 1 and 2 correlates not only with the bioenergetics analysis data obtained in "Mito stress test" and "Glycolysis stress test", but also with the cfDNA release patterns of "Cell-free DNA release patterns" and "Cell-free DNA fragment size evaluation". Group 1's HepG2, 143B, RD, and FIBRO cells presented with low OXPHOS activity and more predominant glycolysis dependence as energy source. Group 2's HEK-293, A375, HaCaT, and HeLa showed less dependence towards any specific energy source, with moderate-to-high levels of both OXPHOS and glycolysis. In addition, group 1 consists of the three cell lines (with the exception of RD) that presented with increasing levels of cfDNA release and group 2 consists of the cell lines (with the exception of HaCaT) that presented with decreasing levels of cfDNA release as the cells increases in confluence. The increasing or decreasing patterns of cfDNA release and active cfDNA release levels of cell lines, therefore, correlate with glycolysis activity. What is even more interesting to note is that the screening of potential housekeeping genes as reference genes for PCR-based quantification of cfDNA revealed that cell lines did not release SDHA, ATP5B, and CYC1 (housekeeping genes related to OXPHOS) into culture media, but were expressed by the cultured cells [49], which may explain or contribute to the lack of correlations found between cfDNA release and OXPHOS activity.

One of the limitations of this study was that bioenergetic analyses could only be performed after 24 h due to the specifications of the instrument. This correlation between glycolysis and cfDNA could be better confirmed if the bioenergetics of each cell line could be determined at the same time intervals as those used for cfDNA release and a consistent correlation can be seen.

Potential mechanisms for cell-free DNA release—glycolysis correlations

Aerobic glycolysis versus regular cellular metabolism To date, it is well known that aerobic glycolysis in cancer cells results in the increased consumption of glucose and glutamine, with very little glucose being used for OXPHOS [22–24]. This correlates well to the correlation found between glycolysis and cfDNA release of the cell lines in group 1 (Fig. 11) cell lines HepG2, 143B, and RD and, interestingly, normal fibroblasts. Proliferating fibroblasts rely on

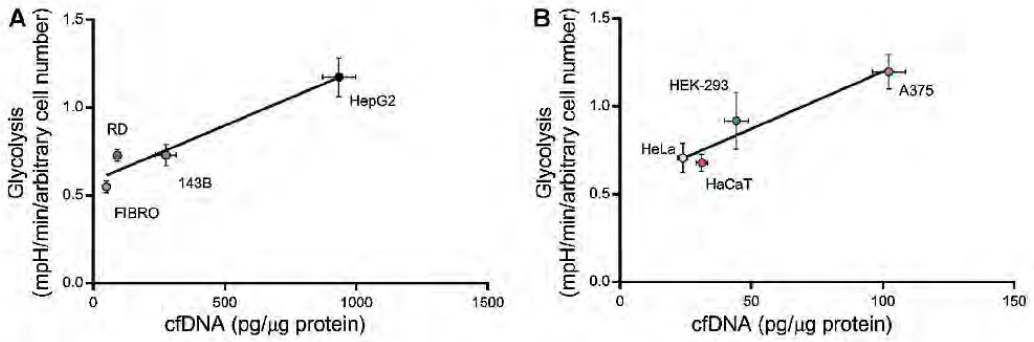

Fig. 11 Scatter plots depicting the correlation between glycolysis and cfDNA release for **a** group 1 (HEPG2, FIBRO, RD, and 143B) and **b** group 2 (HeLa, HaCaT, A375, and HEK-293). Values indicate the mean ± standard deviation

the pentose phosphate pathway to generate ribose for nucleotide synthesis [54]. The glucose and glutamine metabolism in the TCA cycle of proliferating fibroblasts is interrupted at the citrate step and glutamine is primarily used for anaplerosis (process that ensures the replenishment of TCA cycle intermediates) [55]. Fibroblasts also require higher glucose levels due to their high energy consuming function of producing large amounts of extracellular matrix components [54]. These factors may explain why FIBRO cfDNA levels correlated to glycolysis levels along with that of the cancer cell lines, whereas the normal cell lines, HaCaT and HEK-293, showed correlations in group 2 (Fig. 11) due to normal energy metabolism functions.

The cancer cell lines HeLa and A375, on the other hand, formed part of the correlation in group 2. HeLa and HaCaT cells have been shown to have similar central carbon metabolism enzyme activities, but HeLa cells showed an upregulation in almost all of the central carbon metabolism's enzymes using fructose 6-phosphate as a substrate [56]. It should, therefore, be more beneficial for HeLa cells to limit ATP production via the TCA cycle to maintain a more active phosphofructokinase. However, increased ATP utilization linked to cell proliferation results in increased ATP production rates via the pentose phosphate pathway. The similarities between enzyme activity of HaCaT and HeLa cells may indicate that the aerobic glycolysis dependence of HeLa cells may be less prominent than that of the other cancer cell lines, explaining or contributing to the lack of correlation between cfDNA release and glycolysis levels in HeLa cells. A375 cells may also serve as an example of cancer cells that do not primarily depend on aerobic glycolysis, as they presented with moderate levels of OXPHOS activity and their high glycolysis levels are likely due to their fast growth rate.

The involvement of other factors correlating to glycolytic activity Due to the complex nature of the cell and the numerous intra-, inter-, and extracellular factors that play a role, it is understandable that a correlation was only seen in one of the many bioenergetic parameters that were tested, and that other factors than cfDNA release that can correlate with glycolytic activity may have contributed to the observed glycolysis-cfDNA release correlation, e.g., apoptosis and proliferation capacity.

The electropherogram data of "Cell-free DNA fragment size evaluation" indicate that the ratio of apoptotic cfDNA fragments to the 2000 bp fragments (actively released cfDNA) is significantly low for the tested cell lines at 24 h. Apoptosis is, therefore, unlikely to serve as a contributing factor towards the observed correlations. While the underlying reason for this correlation is unclear, it would appear that the proliferating capacity of the cell lines may play a role. De Preter et al. [20] found that increased proliferation capacity (as measured by DNA synthesis) was significantly correlated to increased glycolysis but did not have a significant correlation to mitochondrial respiration. No analyses were performed to assess the proliferative capacity of each cell line, but this may be a useful approach to investigate in the future in conjunction with cfDNA release and glycolysis.

In vivo considerations that may affect the glycolysis—cell-free DNA release correlation

Whether cfDNA release will correlate with glycolytic activity in an in vivo setting requires investigation. There are several in vivo factors that can either mask or negate the observed correlations. First, whereas 2D cell cultures consists of specific cell morphologies or types, organ tissues and tumors consist of various cell types and morphologies, each likely to have its own level of contribution to the cfDNA sample and its own preference to glycolysis or OXPHOS activity. Second, the monolayer of cells in cultures cancels out the effects of both effective and ineffective circulation, respiration and clearance of nutrients, oxygen, and metabolites on cellular activity in healthy tissues and tumors, which will likely affect cirDNA release patterns. Finally, the utilization of adjacent, healthy cells as metabolic intermediate donors and self-digestion [13] to fuel tumor cells can also affect or alter the relationship between cellular metabolism and, in effect, cirDNA release. As discussed in "The utilization of in vitro cell cultures in circulating DNA research", there are 3D culture methods that can be used to effectively simulate these conditions whilst having a source of released DNA restricted to that of the targeted tissue or disease. The initial use of 3D cultures to study the relationship between cellular metabolism and cfDNA or cirDNA release before diverting to plasma samples may provide a necessary step-by-step progression in terms sample complexity required to finally elucidate the biological function of cirDNA.

Conclusions

To summarise, three distinct cfDNA release patterns were detected, namely (1) increased, (2) decreased, and (3) variable levels of cfDNA levels over time during the exponential growth phase of cell lines of different origins. The variable cfDNA release patterns occur in HaCaT, A375, and fibroblast cells, and are, therefore, theorized to be related to the skin origin of the cells. Patterns of apoptotic DNA laddering and a 2000 bp peak have been detected in all seven cell lines. As time progressed, increases in the 2000 bp peak are concomitantly followed by a decrease in nucleosomal ladder fragment peaks and vice versa. These

patterns of DNA fragment peak formations and losses correlate with the cfDNA release patterns presented for 143B cells [14] and correlate with the following observations made by Choi et al. [35–37]: (a) the cfDNA fragments of approximately 2000 bp in length are not of apoptotic or necrotic origin, (b) are likely released into the culture media via an active release mechanism, and (c) cfDNA is, therefore, released by more than one mechanism, primarily apoptosis and active DNA release. The strong similarities between cell culture cfDNA and plasma cirDNA, and the obvious implications of more than one source of cfDNA in samples, indicate that there should not be any concerns regarding the efficient translation of in vitro results into in vivo application and that cell culture models can be efficiently used instead of (or in conjunction with) biofluid samples for cirDNA research. Furthermore, we report that there were no statistically significant correlations between cfDNA release and OXPHOS, although there appears to be a slight tendency toward an inverse correlation between cfDNA release and ATP production. There is, however, a statistically significant correlation between glycolysis and the cfDNA release levels of HepG2, RD, 143B, and FIBRO cell lines that was attributed to aerobic glycolysis utilization of the cell lines, and between glycolysis and the cfDNA release levels of HaCaT, HEK-293, HeLa, and A375 cell lines, which is attributed to the normal energy metabolism activities of normal cells and lesser dependence of the cancer cells in question towards aerobic glycolysis. It is, therefore, concluded that a cell line's increasing or decreasing pattern of cfDNA release and its active cfDNA release levels correlate with the growth rate and cancer status of the cell line through its dependence on glycolytic activity.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Chapter 5: A provisional hypothesis for the origin and function of cfDNA in cancer

Article VII, submitted manuscript

Alpha-satellite DNA and active transposable elements are spontaneously released by bone osteosarcoma (143B) cells in vitro

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Abstract

We previously demonstrated that the cell-free DNA (cfDNA) present in the growth medium of cultured bone osteosarcoma (143B) cells is mainly a product of active cellular release. In this study, we investigated the origin and composition of this actively released cfDNA by using next-generation sequencing. Very interestingly, the cfDNA was comprised mainly of α-satellite DNA and transposable elements. Furthermore, we observed a strong correlation between the level of occurrence of an element in cfDNA and its current transposition activity, or capacity to become reactivated (by aberrant methylation, for example), in the human genome. For example, the L2 and L3/CR1 elements belonging to the long interspersed nuclear elements (LINE) family, which are currently not mobilizing in the human genome, are underrepresented in the cfDNA population. Conversely, the currently active L1 elements of the LINE family are significantly overrepresented. Furthermore, the results obtained in this study indicate that the presence of actively released cfDNA in the growth medium of cultured osteosarcoma cells is directly related to the demethylation of the pericentromeric regions of chromosomes 1 and 16. This provides compelling evidence for our hypothesis on the existence of a heretofore unknown mechanism by which eukaryotic cells actively release satellite DNA and active transposable elements to the extracellular environment. Whether these cfDNA fragments are deliberately released by 143B cells to perform a specific function remains an open question. However, we argue that the lateral transfer of tumor-derived transposable elements could lead to the oncogenic transformation of healthy target cells via the insertional activation of oncogenes, similar to retroviral mutagenesis. This hypothesis could provide a putative explanation for the phenomenon of genometastasis, which involves the induction of metastasis through the transfection of healthy cells with tumor-derived cfDNA.

Key words: cell-free DNA; biomarkers; liquid biopsy; transposons; satellite DNA; genometastasis.

Abbreviations:

SINE: Short interspersed nuclear elements

LINE: Long interspersed nuclear elements

ERV: Endogenous retrovirus

MaLR: Mammalian non-LTR retrotransposons

LTR: Long terminal repeat

TE: Transposable element

RE: Repetitive element

1. Introduction

Short fragments of cell-free DNA (cfDNA) were detected in blood for the first time in 1948 [1]. At first the phenomenon seemed trivial, but two decades later it provoked attention when clear differences were observed between the characteristics of cfDNA from healthy and diseased individuals [2-4]. Corroboration of these findings by independent groups prompted numerous researchers to investigate the correlation between the characteristics of cfDNA and a wide range of pathological data (reviewed in [5,6]). Although most studies obtained promising results, the development of clinical tests was deterred by the lack of appropriate molecular techniques. However, since the advent of ultra-sensitive technology (e.g., next generation sequencing & Droplet Digital PCR), cfDNA has been extensively scrutinized as a potential biomarker for the non-invasive screening of various pathologies [7]. For example, the characterization of cell-free fetal DNA isolated from maternal plasma now allows the detection of fetal genetic aberrations [8] and pregnancy complications [9]. In oncology, cancer patients generally present with elevated levels of cfDNA, which often correlate with progression, treatment and recovery. Additionally, these cfDNA fragments exhibit several tumor-associated mutations, which render them prime candidates for biomarkers [10]. Furthermore, the association between elevated levels of cfDNA and strenuous exercise, autoimmune disorders and traumatic injuries, etc., indicate that kinetic analysis of cfDNA may serve as a tool for predicting the severity and outcome of cellular damage and degeneration [11].

Apart from its development as a clinical tool, two other exciting research schemes are gradually emerging. First, cfDNA is being investigated as a possible mediator of intercellular communication. For example, several studies have implicated the lateral transfer (LT) of cancer cell-derived cfDNA as a causative agent in oncogenesis and the development of metastasis. Although the exact mechanisms are still unclear, it is suggested that the malignant phenotype of tumor cells are transferred to normal cells via the assimilation and transfection of genomic DNA contained in apoptotic bodies [12-15]. In addition, the LT of cfDNA has been implicated in the augmented resistance of cancer cells against radiation- and chemotherapy [16-18]. Conversely, it has also been demonstrated that the LT of cfDNA derived from healthy cells can halt the proliferation of cultured cancer cells [19]. Second, the involvement of the LT of cfDNA in somatic genome variation and trans-generational inheritance is becoming increasingly clear and is receiving more attention [20-23]. Here is also where some points of contact between cfDNA and extracellular vesicle research occur, in particular the regulatory functions associated with the genetic material transported between cells via exosomes [24-26]. Although these details are very intriguing, a more detailed discussion would take us too far afield. The important point is that further inquiry into the biological properties of cfDNA will certainly have a positive impact on clinical diagnostics, therapy development and our general understanding of molecular biology, pathology and conceivably, evolution. However, despite the effort afforded to the development of cfDNA analysis as a clinical tool, very few tests have been translated to practice, and routine application seems distant [27,28]. Indeed, barring non-invasive prenatal testing (NIPT), only one other clinically validated application of cfDNA analysis is currently available (refer to [7,29]). Furthermore, regarding the intercellular messaging capabilities of cfDNA, we have a very limited understanding of the cellular circuits that mediate its messaging functions, and the extent to which it affects biological function is unclear. Moreover, except that we know that cfDNA can be assimilated by cells [30-32] and be incorporated into the genome, the exact mechanisms involved remain unknown, and evaluation of its role in evolution is currently limited to theoretical deliberation [21-23,31,33,34].

We contend that the advancement of research in these areas is constrained by two substantial drawbacks. First, there is no analytical consensus, which leads to conflicting results between research groups. We have discussed this issue previously [28,35,36]. Second, we have inadequate knowledge regarding the origin and composition of cfDNA, while even the processes involved in its molecular generation remain obscure [37-40]. A useful way to investigate all of the latter is to analyze the nucleotide sequence information of cfDNA. In the last couple of years sequencing of cfDNA has increased considerably, but research has been mainly clinically motivated, focusing on size evaluation [41,42], ultra-deep amplicon [43] or exome sequencing [44], or methylation-specific sequencing [45-47], while very few studies attempt thorough cfDNA sequence characterization. However, one group sequenced the cfDNA obtained from the serum of healthy individuals. Although a large amount of sequences indicated an apoptotic origin, they observed an uneven distribution of apoptotic/necrotic DNA across the genome. Moreover, they demonstrated that nonspecific DNA release is not the sole origin of cfDNA [48]. This uneven representation is due to the inherent complexity of the *in vivo* setting, viz., the quantitative and qualitative characteristics of cfDNA in the blood of an individual at any given time are modulated by various internal processes and environmental factors, e.g., circadian oscillations, organ health and diet. In addition, since most cells release DNA (often cell-specific) the aggregate cfDNA profile comprises a muddled blend of mutated and non-mutated DNA released by various cells from different tissues and organs by different mechanisms. This makes it very difficult to surmise the properties of cfDNA *in vivo*.

Since cell cultures are insulated from most external elements, we argue that many of the difficulties encountered in *in vivo* experiments can be circumvented by *in vitro* models. For example, DNA is released from only a specific kind of cell in a typical cell culture experiment, rather than the hundreds of different kinds of cells in a complete organism. In a previous study [49,50], we characterized the DNA present in the culture medium of bone osteosarcoma (143B) cells. After 4 hours of incubation only a small number of 166 bp DNA fragments are present. However, after 24 hours there is a significant increase in the amount and size of DNA (~2000 bp). Typically, DNA with a size of 166 bp is a product of apoptotic fragmentation, while a size of 2000 bp can be explained by neither apoptosis nor necrosis. These results were confirmed by two independent flow cytometric assays. Furthermore, cell cycle analysis demonstrated a shift from the S to the sub G0/G1 phase after 24 hours of incubation, suggesting that the significant increase of cfDNA may not be associated with DNA replication. However, we cannot with complete confidence say that this is the case, since the timing associated with the intracellular trafficking of DNA before it is released as cfDNA was not investigated. Furthermore, we demonstrated that treatment of growth medium with denaturing agents (Proteinase K and SDS) prior to extraction significantly increases the amount of cfDNA, indicating an association with a protein complex. This finding is consistent with other reports in the literature [31]. Taken together, these observations suggest that the occurrence of cfDNA after 24 hours of incubation is primarily a result of an active release mechanism and not a consequence of apoptosis, necrosis, or the process of DNA replication (for cell division). Interestingly, this cfDNA size profile closely resembles the size profile of human plasma-derived cfDNA. However, the 2000 bp cfDNA fragments in human blood are generally dismissed as mere cellular DNA contamination [51].

As a continuation of our previous work, the purpose of this study was to thoroughly characterize the nucleotide sequence of the DNA that is actively released by cultured 143B cells in an attempt to infer a possible origin and/or function.

2. Results

2.1. The spread of cell-free DNA sequencing data

In order to investigate the composition of the DNA that is actively released by 143B osteosarcoma cells, the cfDNA present in the culture medium after 24 hours of incubation was isolated and sequenced. We have previously shown that much more DNA is released per 143B cell after 24 hours of incubation, which indicates an active release mechanism. However, a small fraction of this DNA may include DNA that is normally bound to the cell membrane. Before proceeding with subsequent analyses, the spread of the distribution of the cfDNA sequencing data was first evaluated. In order to eliminate potential sequencing artifacts, contigs with coverage less than 20 were excluded from analyses. Contig coverage was displayed as a box plot, with whiskers set at the 10^{th} and 90^{th} percentiles (Fig. 1a). This shows that the data is not distributed uniformly, and is skewed significantly to the right. Subsequently, the ROUT method was used to identify outliers, with Q set to 0.1% (the strictest threshold for defining outliers). Of the 4362 contigs that were analyzed 549 contigs (all with coverage greater than 108) were identified as outliers. Although the latter represents only ~12.5% of the sequences, it constitutes ~53.5% of the data in terms of coverage. Furthermore, in order to discern between the two populations more clearly, the contigs with coverage greater than 1000 (~16% of the data, which skewed the distribution significantly) was omitted and the box plot was redrawn (Fig. 1b). Alternatively, this data is presented as a scatter plot (Fig. 1c). This clearly shows the presence of at least two cfDNA populations, namely a large number of contigs with a relatively low coverage, and a small number of contigs with a very high coverage (the effects of potential sequencing bias is thoroughly assessed and discussed in section 2.7).

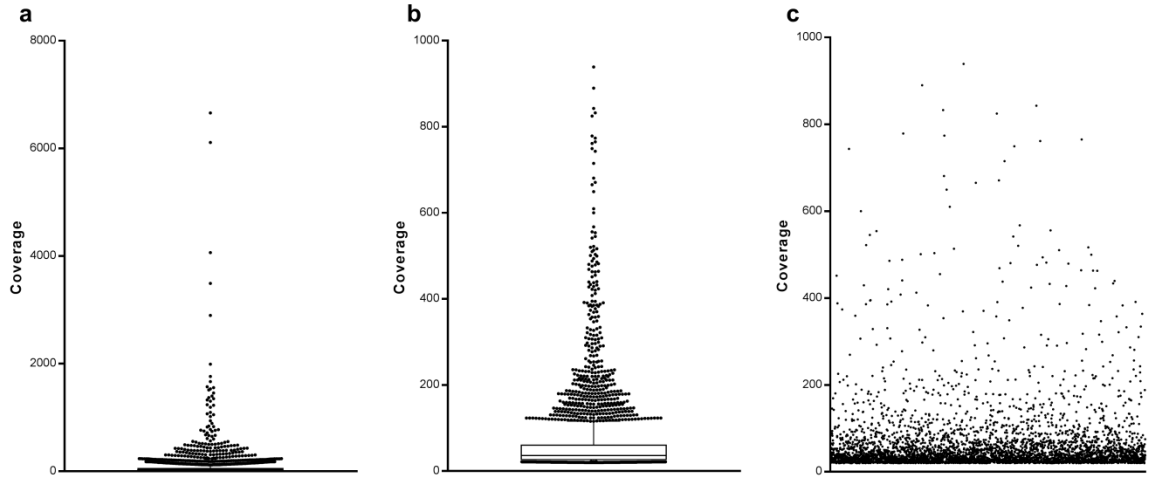

Fig. 1. Coverage distribution of cell-free DNA sequences. (**a**) Coverage distribution of all cfDNA contigs with a coverage greater than 20. (**b**) Box plot illustrating the coverage distribution of the data in (**a**) when contigs with coverage greater than 1000 are omitted. (**c**) A scatter plot of the data presented in (**b**).

2.2. Masking and representation of repetitive elements

To screen for repetitive elements (REs) and regions of low complexity in the cfDNA sequences, we used RepeatMasker Open software (4.0). To thoroughly characterize the REs of the entire cfDNA population and to account for the statistical effects of a non-normal distribution (as indicated in Fig. 1), three cfDNA data subsets were investigated, namely: (a) all contigs with coverage greater than 20, (b) contigs with coverage between 20 and 100, and (c) contigs with coverage greater than 100. To determine the representation of REs in each of these data sets, overlapping annotations were first omitted from the output files generated by RepeatMasker (~0.26% of the data). Subsequently, the number of bases masked by each RE in each query sequence was calculated (end position - begin position +1) and then multiplied by the coverage of the query sequence with which it aligned. The total number of bases masked by each RE was then added together. To determine the representation of each RE in the cfDNA population of each subset, the total number of bases masked by each RE was divided by the total number of bases in the entire cfDNA population. The total number of bases in the cfDNA population of each subset was calculated by first multiplying the length of each contig (that was submitted for RE screening) with its corresponding coverage value and then adding it together. The representation of REs was normalized in terms of contig coverage based on the direct relation between coverage and the number of bases (or copy number) as implied by the Lander/Waterman equation [52]. Furthermore, since the different REs are not equally represented in the human genome, RE representation was expressed as ratios of the percentage of cfDNA masked divided by the expected fraction of the human genome masked by the respective REs (masking values were obtained from RepeatMasker and a Cell SnapShot [53]).

In all subsets investigated only a very small portion of the cfDNA population consists of unique regions, while the amount of REs notably exceeds any value predicted for the human genome [54-56] (Fig. 2). Therefore, the DNA released by osteosarcoma cells after 24 hours of incubation is comprised mainly of REs. Furthermore, when taking into consideration the entire cfDNA population (Subset a), LINEs, SINEs and satellites, which include simple repeats, make up the majority of the cfDNA population, and is overrepresented compared to the human genome (Fig. 2a). Very interestingly, satellite DNA (satDNA) and simple repeats are significantly overrepresented, while LTR elements and DNA elements are underrepresented. As illustrated in Subset c (Fig. 2c), which depicts only the contigs with a coverage greater than 100, satDNA, simple repeats and LINE elements are significantly overrepresented in the cfDNA population as a result of a small number of sequences that have a very high coverage. Therefore, when these contigs (which significantly skew the data) are taken out of consideration (Subset b) (Fig. 2b), it becomes very clear that, regardless of the overall masking of each repeat class, specific elements in each class are significantly overrepresented, while others are significantly underrepresented or occur at levels comparable to the human genome: (i) regarding SINEs, *ALU*s are *2.7-fold* overrepresented, while MIRs are *7-fold* underrepresented, (ii) regarding LINEs, L1 is *1.5-fold* overrepresented, while L2 and L3/CR1 is *14.5-fold* and *28.5-fold* underrepresented, respectively, (iii) regarding LTR elements, ERV (K) class II and MaLR elements are *2.4-fold* and *1.8-fold* overrepresented, respectively, while ERV class I and ERV (L) class III elements occur at levels comparable to the human genome, (iv) regarding DNA elements, TcMar-Tigger elements are *1.7-fold* overrepresented, while hAT-Charlie elements are *3-fold* underrepresented.

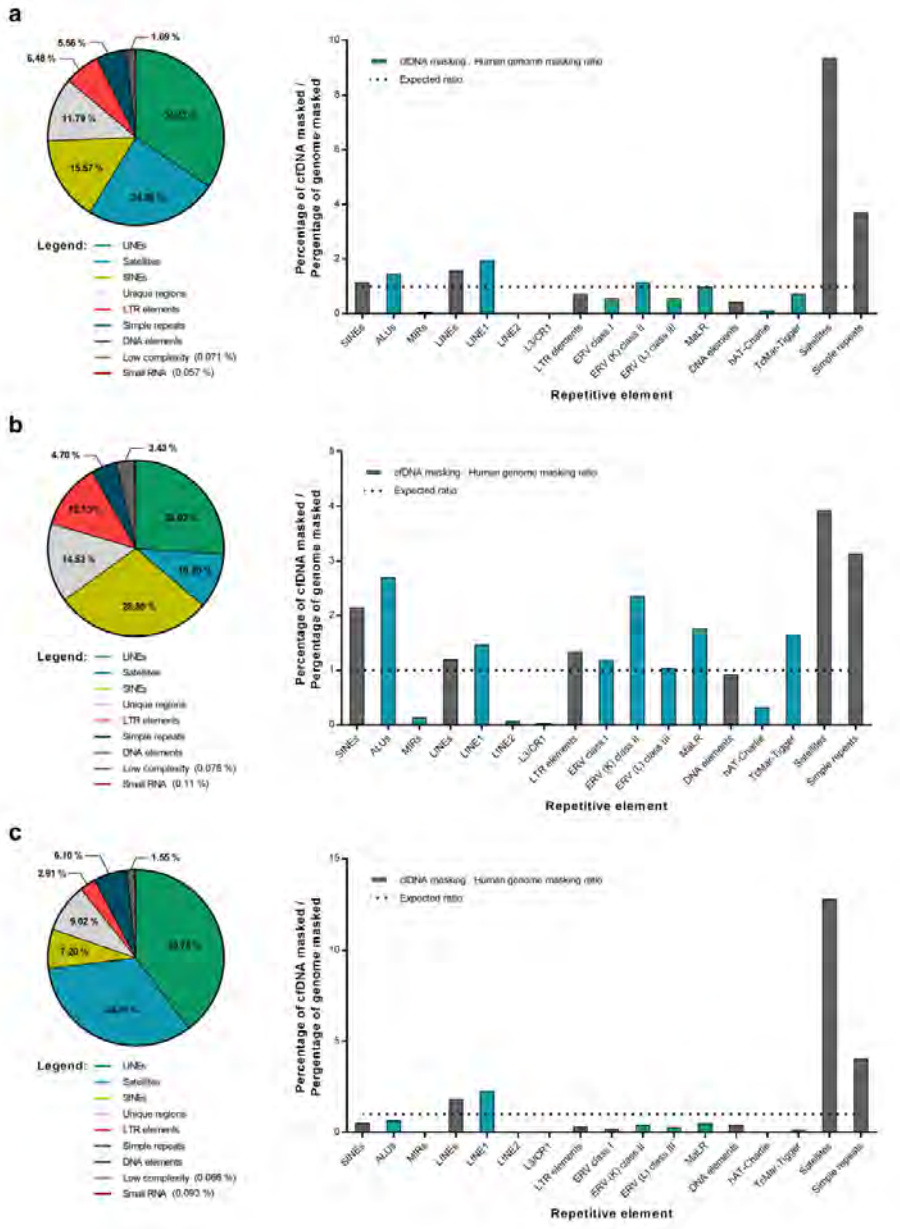

Fig. 2. Representation of repetitive elements in actively released cell-free DNA. Representation of the repeat elements in each of the cfDNA subsets that were analyzed (as described in section 2.2), including (**a**) all contigs with coverage greater than 20, (**b**) contigs with coverage between 20 and 100, and (**c**) contigs with coverage greater than 100, respectively. Pie graphs illustrate the representation of the different repetitive element classes in the cfDNA population of each subset. Each repeat class is denoted by a different color, as indicated by the legend. In addition, each chart shows the percentage of the cfDNA population that does not contain repetitive elements (denoted by ~~u~~nique regions"). Values were obtained by dividing the total number of bases masked by each repetitive element by the number of bases that constitute the entire corresponding subset. Bar graphs illustrate the representation of each repeat class, as well as the repetitive element types in each class, expressed as observed/expected ratios (i.e.., the percentage of cfDNA masked by each repetitive element was divided by the percentage of the human genome masked by the corresponding element). Grey bars illustrate the different repeat classes, while blue bars indicate the repetitive element types contained within the preceding class. The dashed line denotes the expected ratio of masking for all repeats. Thus, values that extend above this line indicate an overrepresentation of the repetitive element, while values below this line indicate an underrepresentation of the repetitive element.

2.3. Evaluation of individual repeat classes and associated subfamilies

Since the different RE families are not equally represented in the human genome, the coverage distribution of the contigs that comprise each RE type/family was evaluated. The purpose of this was to identify sequences that are significantly overrepresented within the context of each group. To do this, the coverage distribution of the contigs that comprise each RE population was displayed as a box plot, where data points above the 90^{th} percentile are considered to be significantly overrepresented. Fig. 3 illustrates the RE populations that were not overrepresented, including MIRs (Fig. 3a), L2 (Fig. 3b), ERV class I (Fig. 3c), ERV (L) class II (Fig. 3d), and hAT-Charlie (Fig. 3e). For reasons given below, simple repeats were also included in this figure (Fig. 3f). Fig. 4 illustrates the RE populations that were overrepresented, including ERV (K) class II (Fig. 4a), MaLR (Fig. 4b), TcMar-Tigger (Fig. 4c). For the same reasons as discussed in section 2.2, the box plots could not satisfactorily illustrate the spread of the data/identify outliers for ALU, L1 and satDNA elements. Alternatively, this data was displayed as scatter plots, wherein 5% of the contigs with the highest coverage (which are considered to be significantly overrepresented) was distinguished from the remaining 95% of the data (Fig. 5). Significantly overrepresented elements are summarized in Supplementary file S1. This includes the contig ID, the matching repeat, the length that it masked, the coverage of the sequence with which it aligned, as well as the total bases masked. Furthermore, the FASTA sequence of each of these overrepresented elements is indexed in Supplementary file S2, and can be located by its contig ID. To evaluate the composition of each RE type the representation of its corresponding subfamilies was determined (this was done in the same way as explained in section 2.2). This was done only for the RE types that were shown to be overrepresented in comparison with the human genome (see Fig. 2), namely ERV (K) class II (Fig. 4d), MaLR (Fig. 4e), TcMar-Tigger (Fig. 4f), ALUs (Fig. 5a), L1 (Fig. 5b), and satellites (Fig. 5c). Although simple repeats are also overrepresented, the sequences of this population are too diverse to be grouped and displayed. Furthermore, regarding the three most abundant REs in the cfDNA population, ALUs, L1 and satellites, the representation of their corresponding subfamilies within the top 5% and bottom 95% of the data was determined. This shows that there are individual subfamilies that contain contigs with an unusually high coverage.

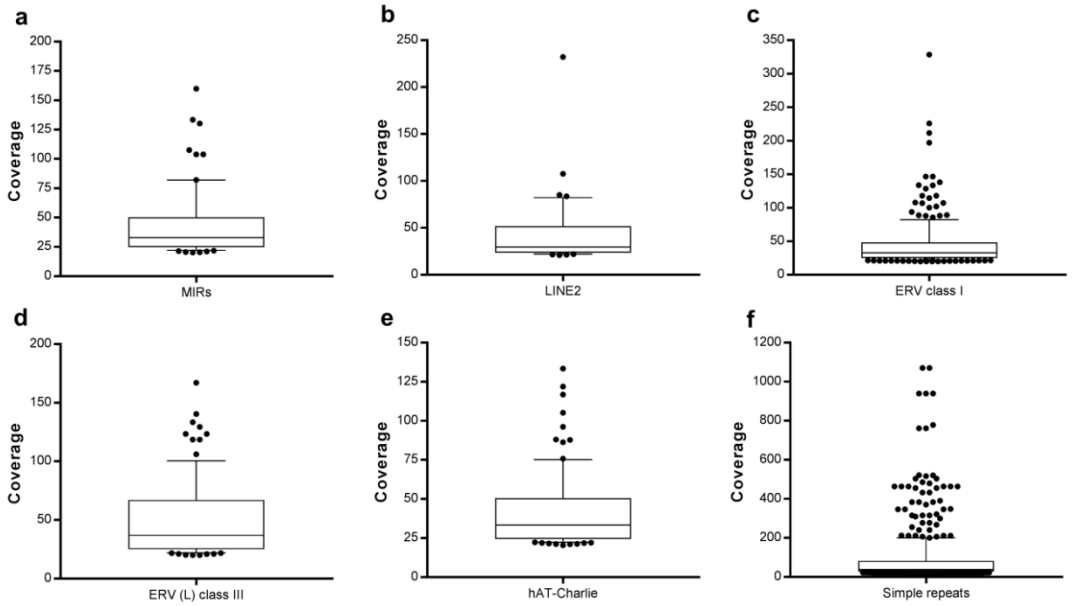

Fig. 3. Identification of significantly overrepresented repetitive elements. Coverage distribution of the contigs that constitute each of the different repeat element populations, including (**a**) MIRs, (**b**) LINE2, (**c**) ERV class I, (**d**) ERV (L) class III, (**e**) hAT-Charlie, and (**f**) simple repeats. Contigs with coverage values above the 90th percentile are considered to be significantly overrepresented.

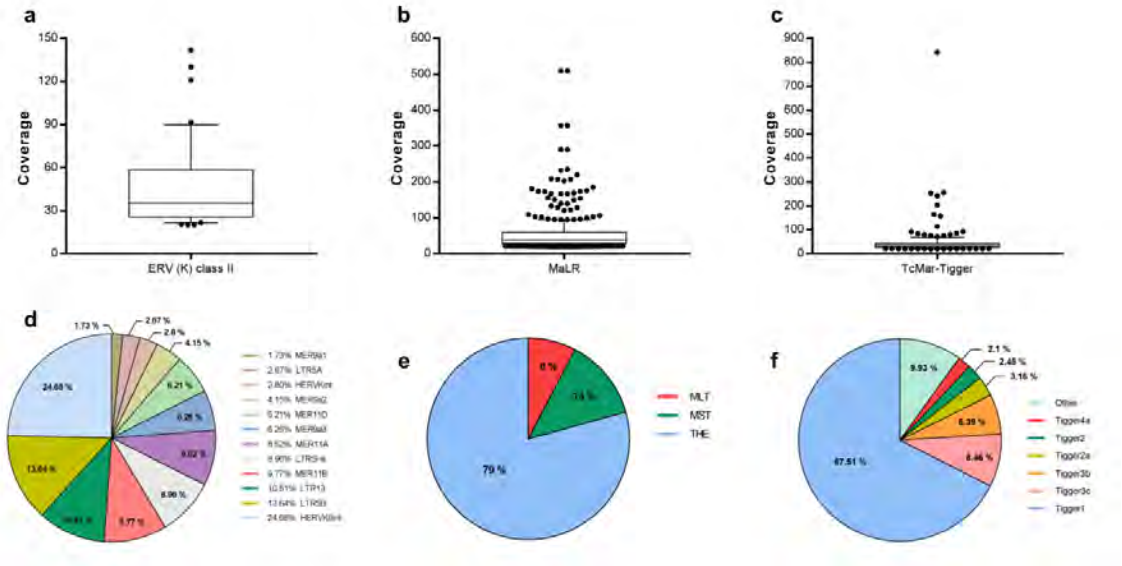

Fig. 4. Identification of significantly overrepresented repetitive elements. Coverage distribution of the contigs that constitute the repeat element populations that are significantly overrepresented (see Fig. 2), including (**a**) ERV (K) class II, (**b**) MaLR, and (**c**) TcMar-Tigger. Contigs with coverage values above the 90th percentile are considered to be significantly overrepresented. Furthermore, pie charts illustrate the representation of the subfamilies that comprise each of the aforementioned repeat element populations, including (**d**) ERV (K) class II, (**e**) MaLR, and (**f**) TcMar-Tigger. The representation of each subfamily was determined as described in section 2.3. Although simple repeats are also overrepresented, they were not included in this figure because the sequences of this population are tremendously diverse.

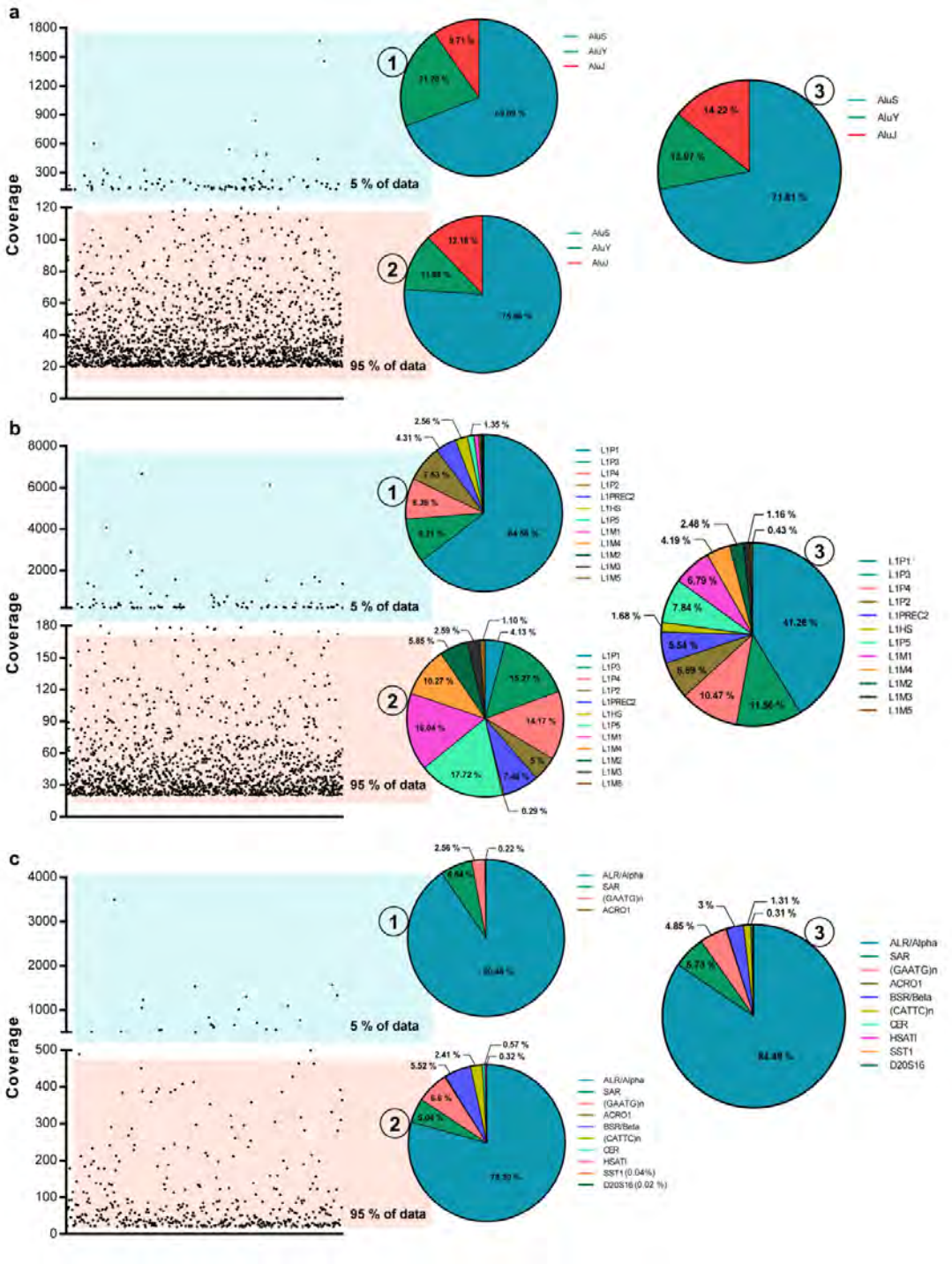

Fig. 5. Identification of significantly overrepresented repetitive elements. Scatter plots illustrating the coverage distribution of the contigs that constitute each of the repeat element populations of which significantly overrepresented elements could not be distinguished using box plots, including (**a**) ALUs, (**b**) LINE1, and (**c**) Satellites. Each dot represents a single contig. The superimposed blue box indicates the contigs that constitute the top 5% of the data when sorted according to increasing coverage, while the pink superimposed box indicates the contigs that constitute the bottom 95% of the data. Dots within the blue box, therefore, indicate significantly overrepresented contigs.. Pie charts illustrate the representation of the subfamilies that constitute each of the aforementioned repeat element populations, including (**1**) the top 5% of the data, (**2**) the bottom 95% of the data, and (**3**) the entire repeat element population.

2.4. Local alignment analyses and annotation

After repeat masking, 250 contigs were randomly selected for BLAST analyses. Since most of the sequences with a coverage greater than 20 were either completely masked or to a very small degree unmasked, the above selection ensured an accurate representation (Only 395 000 bases of the contigs were unmasked. Of the 250 contigs that were selected, 113 094 bases were unmasked, thus constituting ~30% of the unique bases). Of these sequences, only 20% did not align with the human genome, while 36% aligned with part of a gene. Interestingly, nearly one third of the sequences originate from the centromeres, notwithstanding the already established overrepresentation of satDNA (as illustrated in Fig. 2). In addition, 11% of these sequences aligned with one, but unidentified, position in the genome (Fig. 4a). Furthermore, annotation of the cfDNA sequences that aligned with part of one gene revealed that less than 10% originate from protein coding regions, while more than 90% aligned with non-coding regions (Fig. 4b). We can therefore conclude that the cfDNA that is actively released by 143B cells into the culture medium after 24 hours of incubation is primarily composed of non-coding DNA (which does not necessarily mean non-functional, as will be explained in the Discussion).

2.5. Chromosomal distribution

To further investigate the origin of the masked cfDNA sequences, chromosomal distribution was evaluated. Regarding the sequences originating from the centromeres, chromosomes 1, 2, 7, 16 and 21 are overrepresented overall, while many chromosomes were not represented at all (Fig. 5a). When the number of sequences is normalized in terms of chromosome size, chromosomes 1, 16 and 21 are overrepresented, and when normalized in terms of gene density only chromosomes 1, 2 and 16 are significantly overrepresented (Fig. 5b). Regarding the sequences originating from one gene, chromosomes 1 and 5 are overrepresented overall (Fig. 5c). This is perhaps more clearly demonstrated by the chromosome ideogram map (Fig. 5e). However, when normalized in terms of chromosome size, chromosomes 1, 5, 19 and 21 are significantly overrepresented. When normalized in terms of gene density only chromosomes 1 and 5 is significantly overrepresented (Fig. 5d). Normalized values were obtained by dividing the number of sequences originating from each chromosome by the corresponding chromosome length and gene density, respectively.

2.6. Identification of sequences that did not align with the human genome

To investigate the origin of the sequences that did not align with the human genome, a second search against the National Center for Biotechnology Information (NCBI) nucleotide collection (nr/nt) database was performed using the Megablast algorithm. For the sequences that again returned no results, a third search was performed using the Blastn algorithm. The top 10 scoring hits of each BLAST query were indexed, after which the hits with the highest maximum score and ID percentage of each query were tabulated. In addition, all binomial names were converted to their non-scientific counterparts (e.g., *Wuchereria bancrofti*/parasitic roundworm). Taken together, the majority of sequences appear to originate either from domesticated cattle, sheep and parasitic roundworms (that typically infect cattle). Therefore, we can argue that the presence of these sequences can be ascribed mainly to presence of these DNA sequences in the fetal bovine serum that was used to fortify the growth medium.

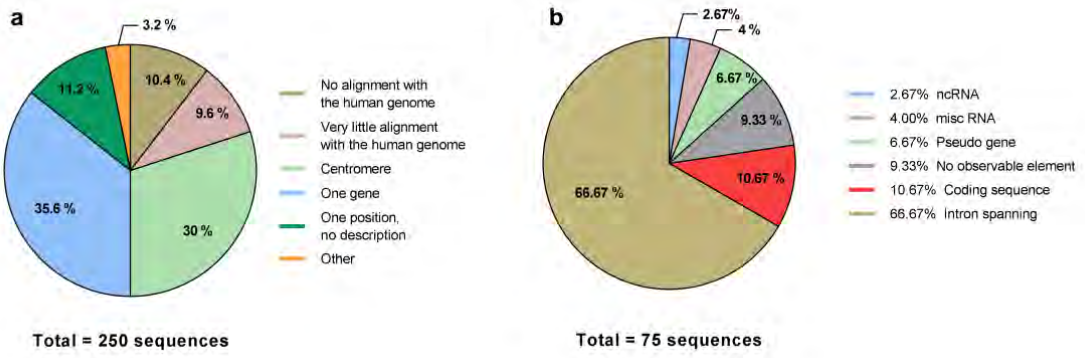

Fig. 6. Local alignment analysis and annotation. (**a**) Position of masked cfDNA sequences in the human genome inferred from local alignment analyses. (**b**) Annotation of masked cfDNA sequences originating from one gene.

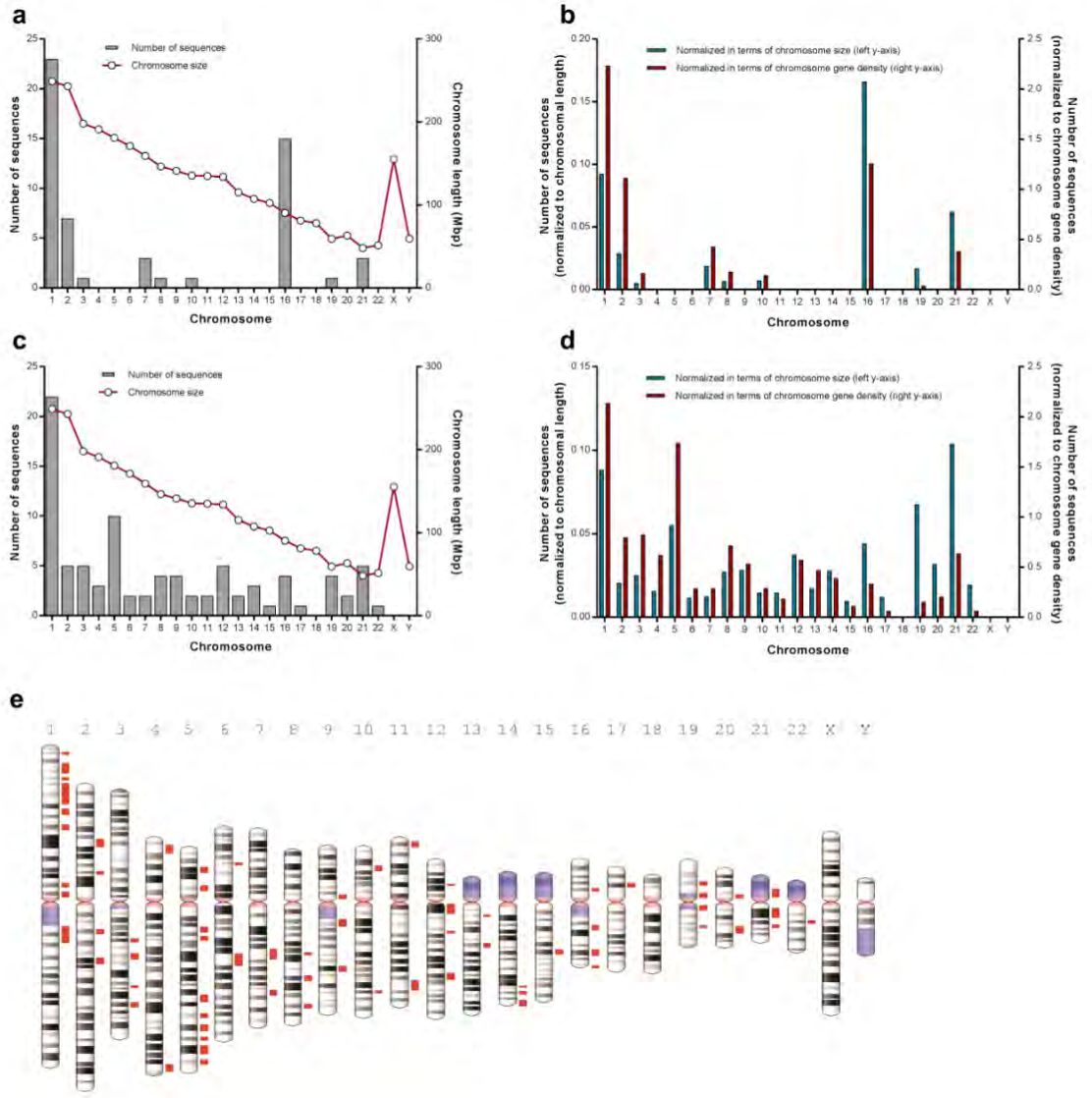

Fig. 7. Chromosomal distribution of cfDNA. (**a**) Chromosomal distribution of sequences originating from the centromere, with a secondary axis for chromosome length. (**b**) Chromosomal distribution of sequences originating from the centromere normalized in terms of chromosome size (left y-axis) and chromosome gene density (right y-axis). (**c**) Chromosomal distribution of sequences originating from one gene, with a secondary axis for chromosome length. (**d**) Chromosomal distribution of sequences originating from one gene normalized in terms of chromosome size (left y-axis) and chromosome gene density (right y-axis). Normalized values were determined as described in section 2.5. (**e**) Chromosome ideograms showing the positions of cfDNA sequences originating from one gene.

2.7. Assessing potential sequencing bias and procedural errors

Since REs are typically longer than the reads produced by next-generation sequencing, they generally have substantially deeper coverage than unique regions [57]. Thus, it is imperative to evaluate potential coverage bias (deviation from the uniform distribution of reads across the genome). To do this, contigs were first grouped into different coverage ranges at increasing increments in the CLC genomics workbench, after which the FASTA sequences were exported. Each of these datasets was then subject to repeat analysis and the level of RE masking was plotted against its corresponding coverage. This revealed that there is no correlation between coverage and the percentage of sequences occupied with repeats (R^2 = 0.01; p = 0.72) (Fig. 6a).

Another potential cause of coverage bias to consider is GC-content, since GC-rich and GC-poor regions are typically prone to low coverage [58]. As discussed in section 2.2, there is considerable variation in total coverage and masking between the different RE populations. In addition, as discussed in section 2.3, there is considerable variation in the coverage and masking of the contigs that constitute each of the different RE populations. Therefore, the correlation between GC-content and the level of masking by each of the different RE populations was evaluated. To do this, the average GC-content of the contigs that constitute each RE population was plotted against its observed masking value vs. expected masking ratio (as determined in section 2.2), in ascending order. This showed a weak negative correlation (R^2 = 0.43; p = 0.022) (Fig. 6b). However, when excluding satDNA (which has the highest coverage and lowest GC-content), there is no longer a statistically significant correlation (R^2 = 0.18; p = 0.20) (Fig. 6c). Furthermore, when examining the average GC-content of each of the different RE populations (Fig. 6d), it is clear that none of these values are considered to be GC-rich or GC-poor, and should not cause coverage bias. Indeed, the observed GC-values of each RE population correlate with its expected value in the human genome [59]. Interestingly, it has been demonstrated that younger ALU elements are preferentially inserted into GC-rich areas, while younger L1 elements typically cluster in AT-rich DNA [60]. In addition, similar to L1 elements, satDNA is composed mainly of AT-rich DNA [61]. Our data also correlates perfectly with these values. Lastly, to account for structural differences between contigs with high- and low coverage, the average GC-content of the overrepresented RE subfamilies (as determined in section 2.3) was compared to the average GC-content of its corresponding family, which showed that there is no significant differences between any of these groups (Fig. 6e). Therefore, the overrepresentation of these elements is not a result of sequencing bias.

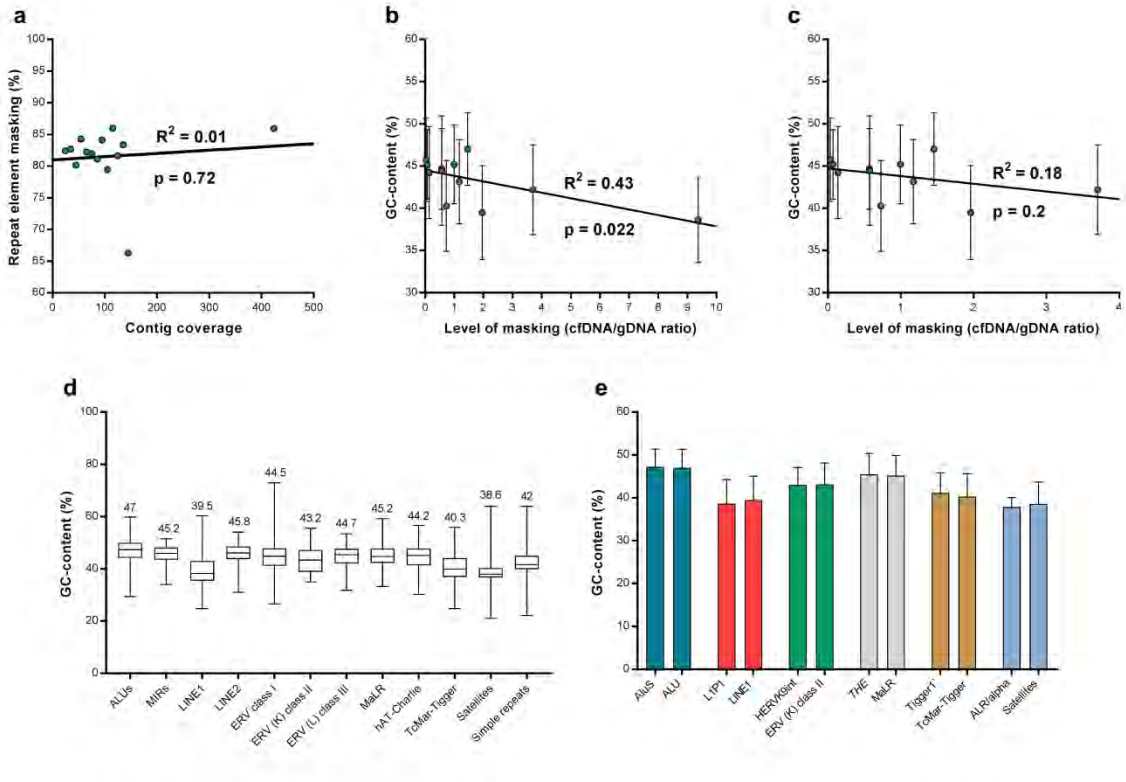

Fig. 8. Evaluation of potential coverage bias. (**a**) Correlation between the level of repeat masking and contig coverage. All contigs with coverage >20 were divided into a sliding window of coverage size with a 10 unit step up until 150 coverage, after which all contigs were grouped. The average coverage of each group was then plotted against its corresponding level of repeat masking. (**b**) Scatterplot showing the GC-content of each RE population against its level of masking. Elements are sorted from lowest to highest cfDNA/gDNA masking ratios (as determined in section 2.2). (**c**) Scatterplot showing the GC-Content of each RE population against its level of masking when the satDNA group is omitted. In each graph, the solid black line indicates the linear regression line and the (R^2) values indicate the regression coefficient, where values close to 1 denote a perfect correlation and values close to 0 denote no correlation. Furthermore, statistical significance is indicated by (p), where a p-value of less than 0.05 indicates a statistically significant result. (**d**) Box plots (min/max) illustrating the GC-content distribution of the contigs that comprise each RE population. The average GC content of each RE population is indicated above the maximum whisker. (**e**) Comparison of the GC-content of overrepresented RE subfamilies with is corresponding RE class. Error bars indicate standard deviation.

3. Discussion

In previous studies we have demonstrated that the cfDNA present in the growth medium of cultured cancer cells is mainly a product of an active release mechanism [50], and appears to vary somewhat from expressed genes (i.e., there are cfDNA sequences present in the extracellular environment that are not present as mRNA in the cell) [62,63]. Other research groups have made similar observations [64-66], but further investigation concerning the biological properties of these peculiar cfDNA molecules is lacking [37]. Therefore, in this follow-up study we sequenced the DNA actively released by cultured osteosarcoma (143B) cells. The cfDNA sequences were re-assembled and then screened for REs, followed by local alignment analyses and annotation.

Initial RE screening results showed that most of the cfDNA consists of repetitive DNA (88%) (Fig. 2), which exceeds any value predicted for the human genome (typical estimates range between 50-66%). Local alignment revealed that nearly half of the remaining non-repetitive DNA sequences originate from the centromeres, while the other half is comprised mainly of introns (Fig. 6a&b). Furthermore, it was determined that the centromeric DNA sequences originate mainly from chromosomes 1 and 16 (Fig. 7a&b), while the coding region derived DNA originates from several chromosomes, although chromosomes 1, 5, 19 and 21 are overrepresented (Fig. 7c&d). Further evaluation of the RE screening data showed that specific RE types are overrepresented, while others are underrepresented or occur at levels comparable to the human genome (Fig. 2). In addition, it was demonstrated that different subfamilies within each RE type is notably overrepresented, while others are underrepresented (Figs. 3-5). In the following section, we discuss the likely reasons why specific chromosomes and each of the REs are over- or underrepresented and elaborate on the potential implications of these findings.

3.1 The representation of class I transposons correlate with transposition activity

3.1.1 Short interspersed nuclear elements (SINEs)

As shown in Fig. 2b, *Alu* elements are clearly overrepresented, but something more interesting is observed when the different *Alu* subfamilies are scrutinized: the cfDNA population contains nearly ten times more elements originating collectively from the *AluS* and *AluY* subfamilies than from the *AluJ* family (Fig. 5a). The former subfamilies harbor more functionally intact elements and are, therefore, more likely to move (transpose) along the genome, whereas the *AluJ* lineage is the least active and is considered to be functionally extinct [67]. Like *AluJ*, MIR elements are also underrepresented in the cfDNA population (Fig. 2b) and no longer possess any transposition activity in the human genome [68]. A few *in vitro* studies indicate that *Alu* elements are capable of retrotransposing in cells that are somewhat differentiated (refer to [69,70]) and several brain regions [71], which gives reason to believe that *Alu* elements may also be active in somatic tissues.

3.1.2 Long interspersed nuclear elements (LINEs)

The underrepresentation of L2 and L3/CR1 elements also correlates with its inactivity [73], while the overrepresentation of L1 elements correlates with its active transposition status. Although there are more than 500 000 L1 copies in the human genome, most of these are considered to be molecular fossils [74]. It has been estimated that an average human genome contains only 80-100 retrotransposition-competent copies, which all belong to the human-specific L1 (L1HS) subfamily. L1HS elements are then further stratified into the L1-Ta subfamily (pre-Ta, Ta-0, Ta-1, Ta1-d, Ta1-nd) [75], which is estimated to account for ~31.5% of all L1HS elements [76], and the older L1P1 subfamily (L1PA2 and L1PA3) that comprises the remainder of the L1HS subfamily [77]. As illustrated by Fig. 5b (3), the L1P1 subfamily constitutes nearly half of the L1 population. When taking into consideration the copy number of each of the different L1 subfamilies in the human genome [83], it becomes clear that the overrepresentation of the L1P1 elements cannot be ascribed to copy number differences. Therefore, we can conclude that the representation of the different L1 elements is directly related to its transposition activity. Furthermore, there is a relatively small number of these L1P1 elements that have a very high coverage (Fig. 5b (2)), which may be indicative of a regulated process, or relate to a specific function.

3.1.3 Long terminal repeats (LTRs)

LTRs are thought to possess little to no transposition activity, and it has been estimated that most LTRs have entered the early vertebrate genome more than 25-100 million years ago [85]. However, there are a number of reports that highlight cases in which specific ERVs can become reactivated. For example, the deletion of DNA methyltransferase 3-like (Dnmt3L), a gene involved in the *de novo* methylation of retrotransposons during a brief perinatal period, from the germ cells of adult male mice, prevents the methylation of both LTR and non-LTR retrotransposons, which results in the reactivation of transposition activity [89]. Similar results have been obtained by the deletion of other genes [90]. Apart from transposition activity, there are other reasons why ERVs may be overrepresented. For example, it has been demonstrated that aberrant activation of the *THE1* subfamily of the MaLR elements contribute to lineage-inappropriate expression of genes in B cell–derived Hodgkin‘s lymphoma cells, which typically confers considerable survivability to tumor cells [93]. Interestingly, ~80% of the MaLR elements detected in the cfDNA population consist of the *THE* subfamily (Fig. 4e). Furthermore, a few studies have demonstrated the up-regulation of MaLR-containing genes in cancer [94,95].

3.2 The representation of class II transposons correlate with evolutionary age

As illustrated by Fig. 2, the TcMar-Tigger elements are overrepresented in the cfDNA population, while hAT-Charlie elements are underrepresented. Unlike the class I retrotransposons, there is currently no evidence for the activity of DNA transposons in the human genome [96]. However, we can suggest one plausible explanation for the varying representation of these elements in the cfDNA. The TcMar-Tigger subfamily is comprised of several more subfamilies (Tigger1, Tigger2, Tigger2a, Tigger3, etc.), where the numerical suffix loosely correlates with the age of the element, which refers to the point in time when it diverged structurally from older subfamilies. This divergence then correlates with transposition activity [97]. Or in other words, the youngest elements are those that have transposed the most recently. Thus, when the different TcMar-Tigger subfamilies were further scrutinized, it was revealed that two-thirds consist of Tigger1 (Fig. 4f), the youngest member of TcMar-Tigger family. In addition, the representation of the

remaining Tigger elements also correlates with age [97]. Therefore, the representation of DNA elements appears to correlate with its capacity to transpose.

Although there is currently no evidence for the movement of DNA transposons in the human genome, there is a possibility that it has been occurring unnoticed. The following points illustrate that the movement of DNA transposons in the human genome, within and between cells, is very possible: (i) Several cases of their horizontal transfer (HT) among insect species have been documented, which suggest that DNA elements rely heavily on HT for their evolutionary conservation [98,99], (ii) DNA transposons are particularly well adapted for HT, as several *in vitro* studies have shown that transposase is the only protein required for transposition (reviewed in [100]), (iii) TcMar-Tigger elements are non-autonomous, i.e., they themselves encode for the transposase protein that is necessary for their mobilization, (iv) Our results show that TcMar-Tigger elements are overrepresented in the DNA that is actively released by cells, (v) cfDNA fragments have been shown to be readily assimilated by most cells and can be integrated into the genome [30].

3.3 The overrepresentation of satellite DNA in cfDNA is a common occurrence

As shown in Fig. 2, the DNA actively released by 143B cells contains a very large number of α-satDNA. This finding is corroborated by several similar observations. First, peri-centromeric simple repeats $(CCATT)_N$ and α-satDNA was shown to be enriched in cytoplasmic membrane-associated DNA (cmDNA), which is considered to be a source of cfDNA when dissociated. Furthermore, cmDNA was found to co-localize with DNA dependent RNA polymerase II, which suggests that cmDNA can be transcribed into RNA [101]. Interestingly, actively released cfDNA has also been shown to be associated with this transcription system, which indicates that actively released cfDNA and cmDNA could associated [31] (Several biological roles of satDNA transcripts, such as gene regulation and chromatin modulation, have been considered in a recent publication [102]). Second, it has been demonstrated that both nucleosome DNA and CENP-B (as well as CENP-A and CENP-C) is present in the blood of patients with Systemic Lupus Erythematosus (reviewed in [103]), which also suggests that heterochromatin may be a component of cfDNA. Last, the cellular uptake of fragmented DNA and chromatin (to simulate cfDNA) was recently investigated and FISH analysis using whole genome and pan-centromeric probes showed signal co-localization in chromosome arms and sub-telomeric regions, which indicated that these DNA fragments are inserted as concatamers that often harbored centromeric sequences [30].

3.4 A hypothesis for the origin of actively released cell-free DNA

3.4.1 The association between the hypomethylation of repetitive DNA and carcinogenesis

There is accumulating evidence for the causal involvement of global DNA hypomethylation and demethylation in human carcinogenesis. Although hypomethylation of gene regions has been implicated in carcinogenesis, it is becoming increasingly clear that this epigenetic abnormality is most frequently associated with repetitive DNA elements, including tandem centromeric α-satDNA, juxtacentromeric satDNA, Alu and LINE-1 repeats. The hypomethylation of these

repeats can contribute to tumour formation and progression in various ways. First, transposable elements, which include endogenous retroviruses, are normally repressed by methylation, but can become reactivated by demethylation. Subsequently, aberrant mobilization and insertion of transposable elements can disrupt coding regions, splice signals, and activate oncogenes [72]. For example, it has recently been demonstrated that the insertion of an L1 element in the *APC* tumour suppressor gene can initiate tumour formation in human colorectal cancer [84], and reactivated ERVs have also been shown to promote tumor development in mice [91]. Similarly, since many transcription regulators bind selectively to Alu and L1 sequences, methylation changes in these regions could alter the sequestration of transcription control proteins and affect the expression of cancer-related genes. Second, increased transposon-induced rearrangements in these pericentromeric regions can result in unbalanced translocations, which can (a) affect the copy number of genes relevant to cancer, and (b) lead to chromosomal instability [87,88]. For example, by monitoring Epstein-Barr virus transformed human lymphoblastoid cell lines *in vitro* for more than two years, researchers were able to show that the hypomethylation of satDNA at the peri-centromeric regions of only chromosomes 1 and 16 leads to rearrangements and decondensation. Similar observations have been made in other human cell lines and in the cells from patients with immunodeficiency and facial anomalies syndrome. Very interestingly, we have demonstrated that a large fraction of the DNA actively released by osteosarcoma cells is comprised of satDNA derived from chromosomes 1 and 16 (Fig. 6a&b). Furthermore, in Section 3.1 we have shown that all of the SINE and LINE transposons that are currently active in the human genome are significantly overrepresented in the cfDNA, while the inactive transposons are underrepresented or occur at expected levels. We have also demonstrated that these differences are not due to varying copy numbers. Furthermore, it was demonstrated that the overrepresentation of specific LTR elements may be linked to reactivation via demethylation. In Section 3.2 it was shown that DNA transposons of a young evolutionary age are notably overrepresented in cfDNA, while older elements are underrepresented. These observations strongly indicate that the presence of actively released cfDNA in the culture medium of osteosarcoma cells is linked to the demethylation of pericentromeric repetitive DNA. However, in order to better understand the association between the demethylation of the pericentromeric regions of chromosomes 1 and 16, the resulting chromosomal instability, and the presence of DNA originating from these regions in the extracellular environment, we have to consider the structural and functional relationship between satDNA and transposable elements. We also have to understand the interaction between these sequences and centromeric proteins.

3.4.2 Structural and functional similarities between satellite DNA and transposable elements

It has been demonstrated that L1 elements are typically abundant in peri-centromeric (A+T)-rich alpha satDNA, possibly due to their preference for inserting into 5'-TTAAAA-3' sites [104,115]. Furthermore, it was shown that L1HS elements are present only in the homogenized DXZ1 alpha satDNA fraction of the X-chromosome, which relates to the active centromere. This may have contributed to alpha satellite evolution (or still does) and it has also been suggested that L1 elements may be actively involved in centromeric chromatin formation and function. Conversely, the older (inactive) primate-specific elements were shown to be enriched in the flanking centromere-incompetent satDNA [116]. Furthermore, sequence homologies between satDNA and TEs have been identified in several species. This can be ascribed to the formation of satDNA arrays from the tandem amplification of specific parts of TEs. A genome wide

survey of REs in the human genome revealed that 25% of all satDNA sequences and simple repeats may be derived from TEs. This study also showed that the tandem repeats that form structural components of TEs (e.g. 3' untranslated regions (UTRs), TIRs, and long terminal repeats (LTRs)) could also present as "classical" satDNA [117].

3.4.3 Structural and functional similarities between centromeric proteins and transposases

There is extensive sequence similarity between centromere-protein B (CENP-B), a protein associated with the centromeres of most human chromosomes, and the transposase encoded by the human Tigger DNA transposon. In addition, the terminal inverted repeats (TIRs) of the Tigger2 elements contain a near perfect match to the CENP-B binding site (or CENP-B box) [105], which consist of a 17-bp alpha satDNA motif [106]. These structural similarities suggest two intriguing possibilities. First, CENP-B, akin to transposases, may also possess the ability to cause single-stranded breaks (SSBs) [105]. Second, transposases could induce SSBs adjacent to the CENP-B box. There is strong evidence that both of these phenomena occur in higher organisms [113, 114].

3.4.4 CENPB and transposase-mediated excision of satellite DNA and transposable elements

The formation of both CENP-B and transposase induced SSBs adjacent to CENP-B boxes could have various effects and/or functions. For instance, it may allow the movement of satDNA monomers along the genome [107,108]. This is supported by evidence of their insertion into various sequence structures (reviewed in [104]). This may facilitate the evolution and maintenance of satDNA sequences, and is possibly involved in the higher order organization of the centromere and/or kinetochore. However, the mechanisms by which alpha satDNA facilitate centromere formation is a topic that is still under investigation [109]. It is also possible that larger DNA fragments could be excised by this mechanism. It has been suggested that DNA elimination through this process is involved in the reduction of repeat expansion in somatic cells [110]. In line with this, it was shown that more than 70% of the DNA eliminated in nematodes consists of short satDNA repeats [111]. Except for the reduction of repeat-expansion, several hypotheses have been proposed to explain the potential biological significance of DNA elimination [112]. Furthermore, a recent review described cases in which transposases encoded by active TEs are used to facilitate the elimination of DNA from the genome [113]. Through the same process *Oxyticha triffalax* eliminates 95% of its germline genome during development, which includes all transposon DNA. This process was then showed to be mediated by the expression of germline-limited transposase genes during germline-soma differentiation, which suggests that transposases also function in larger eukaryotic genomes that contain large numbers of active TEs [114]. If Tigger transposases are capable of facilitating the excision of satDNA stretches from the centromere, as suggested above, this may explain why both centromere-derived satDNA (mainly alpha) and Tigger elements are overrepresented in the cfDNA. In addition, keeping in mind the structure of repetitive DNA, the excision of large stretches of satDNA could explain why active L1 and Alu elements are significantly overrepresented. Another possibility is that transposable elements are actively released by cells by a different pathway, which may relate to other intermingling functions and/or structures of repetitive elements [118] or the spatial organization of chromatin in the nucleus [119], for example. This hypothesis is summarized in Fig. 9.

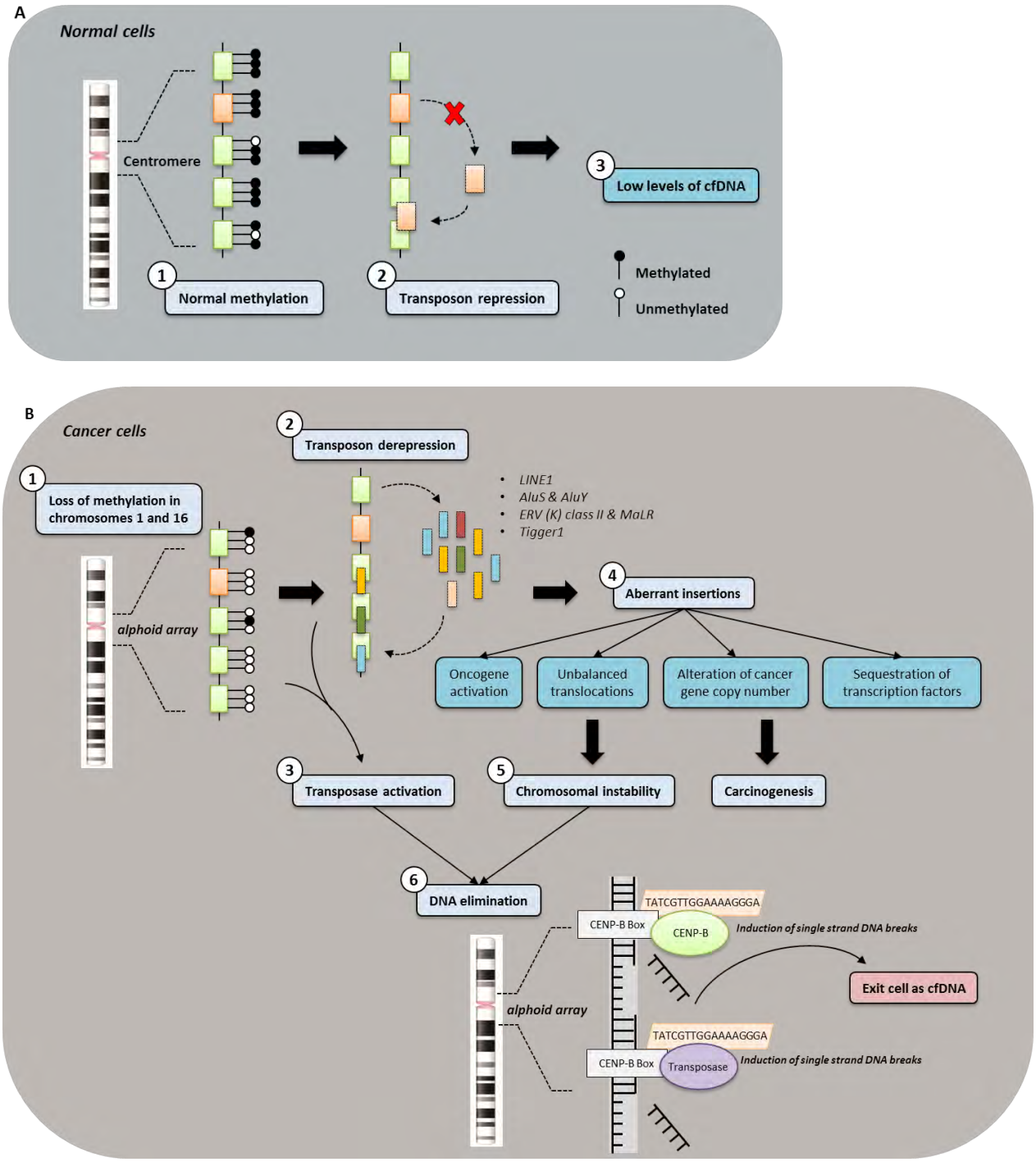

Fig. 9. A provisional hypothesis for the origin of actively released cell-free DNA. (**A**) In normal cells, the peri-centromeric regions of chromosomes 1 and 16 are normally methylated (**1**), which represses the mobilization of transposons (**2**), and results in low levels of cfDNA (**3**). In cancer cells (**B**), the demethylation of the peri-centromeric regions of chromosomes 1 and 16 (**1**) leads to derepression (**2**) and mobilization of transposable elements (**3**). Subsequently, aberrant translocation (**4**) of these elements causes chromosomal instability (**5**). Bearing in mind the structural similarity between CENP-B, and transposases, which are activated by demethylation, both CENP-B and transposases may facilitate the excision of large stretches of satDNAs. Again keeping in mind the inextricably laced sequences of satDNA and transposable elements, it seems plausible that the presence of overrepresented TEs could be a result of programmed DNA elimination (6).

3.5 Implications of these results

Throughout the Discussion we have highlighted numerous cases that demonstrate how the activation and transposition of TEs may contribute to genome instability and mutagenesis in cancer. In keeping with this, several reports have described how cfDNA can be transported throughout the body, while other studies have demonstrated their capacity to enter target cells and alter their biology, either transiently or by genomic incorporation [30,31,34,120,121]. This phenomenon has been implicated in the oncogenic transformation of normal cells both *in vitro* [13,122] and *in vivo* [14], the induction of cancer-cell resistance against radiation- and chemo therapy [16-18], as well as other mutagenic effects [123]. Since it is not clear how cfDNA elicits these effects, satDNA and TEs may yet prove to be among the key effector molecules. This could offer an entirely new paradigm to both cfDNA and cancer research. In addition, the presence of these elements may be useful for diagnostic and prognostic purposes.

Moreover, when viewed in light of the central theorem of the extended phenotype [124], in which the malignant behavior of cancer cells should maximize the survival of genetic instructions that allow malignant behavior, it makes perfect sense that osteosarcoma cells would up-regulate and laterally transfer TEs to neighboring cells with the purpose of transforming them. In line with this argument, we suspect that the composition and function of the TEs released by normal cell lines will differ from cancer cells to a large extent, and will probably differ between different cancer cells. The nature of these differences may be very interesting, and warrants further investigation.

Aside from detrimental effects, the transposition or LT of satDNA and TEs may also have a tremendously diverse impact and crucial role in biological innovation and evolution. Since satDNA and TEs play major roles in gene regulation [102,125,126], and rearrangement and duplication of DNA [127], respectively, researchers have argued that the introduction of satDNA and TEs into eukaryotic genomes by HT is an operative factor that drives somatic genome variation and biological innovation. Similar to the benefits conferred to bacteria by HGT [128,129] and HT of non-coding DNA [130], the LT of satDNA and TEs between different cells within one organism should create new coding and regulatory sequences, which may offer many new capabilities to pre-existing frameworks. This is possible due to localized structural diversification of satDNA and TEs, and the use of mechanisms that allow the targeting of preferential insertion sites in various parts of the genome. Furthermore, although the excision of TEs and subsequent integration into host cell genomes is clearly facilitated by its inherent mobility and capacity for replication, the precise mechanisms that allow the transfer of TEs between organisms remain largely obscure.

Thus, our results not only provide a potential mechanism for the transfer of specific TEs between organisms, but also indicate the possibility that both TEs and satDNA can be transferred between different cells within one organism. To our knowledge, such a phenomenon has not yet been described. If this turns out to be an active force in higher organisms, we would expect this to be of most value to learning (neurons), immunity (lymphocytes) and detoxification (hepatocytes). Here we would also like to speculate that, similar to bacteria, the prospect of the LT of satDNA and TEs between different cells/tissues should make the different mutants receptive to intra-organismal Darwinian selection, which should further allow diversification and evolutionary conservation.

4. Conclusions

The DNA that is actively released by cultured bone osteosarcoma cells is comprised mainly of α-satellite DNA and transposable elements. In addition, there is a strong correlation between the activity of each of the different transposable elements (or its capacity to become reactivated, for example by aberrant methylation changes) and its level of occurrence in cfDNA. This provides compelling evidence for the existence of a mechanism by which active TEs and satDNA sequences are actively released by eukaryotic cells into the extracellular environment. This finding leads to three hypotheses: (i) TEs and satDNAs are actively and deliberately released by osteosarcoma cells to perform a specific function in the extracellular environment, (ii) The TEs and satDNAs that are present in the extracellular environment are the by-products of a cellular process (e.g. DNA elimination) and are incidentally biologically active, or (iii) They are biologically-inert by-products. Bearing in mind the correlation between TE representation in cfDNA and its current transposition activity status in the human genome, the phenomenon of aberrant TE activation in cancer cells, as well as the inherent mobility of TEs and their capacity for replication, we are inclined to maintain the position that these molecules are indeed functional. Whether the biological effect is intentional or accidental remains an open question, but we suspect that the release of specific TEs and satDNAs may be a regulated, perhaps hijacked, cellular process. We also believe that the non-cancer cells should present with a very different cfDNA profile and different functionality. This is a very provocative idea and indicates that further investigations are warranted.

Further investigation of the molecular characteristics of actively released DNA, and inquiry into the mechanisms involved in its release, may provide deeper insight into the correlations observed between the properties of cfDNA and clinicopathological data, and may also expedite the search for appropriate diagnostic, prognostic and theranostic cfDNA biomarkers. For example, since cfDNA has yet to be found absent in any bio-fluids [34], satDNA and TEs could be exchanged between different cells within a body via the lymphatic-, circulatory-, or recently suggested primo-vascular-system [131-133]. In addition, cfDNA can presumably be exchanged between mother and fetus via the placenta, or neonatally through breastfeeding, and between different individuals via blood transfusions (as evidenced by numerous studies on animals [21,134]). Bearing in mind the mutagenic capacity of TEs, the LT of TEs may prove to have various harmful ramifications. Since actively released DNA is presumably present at much lower levels in human blood in comparison with the circulating DNA derived from cellular destruction processes (e.g., apoptosis and necrosis), this may be one of the reasons why the active release of DNA has not yet been observed *in vivo*. Moreover, even though the active release and uptake of DNA (*in vitro*) has been demonstrated by several researchers, the potential biological significance of actively released DNA *in vivo* is tarnished by the disproportionate amount of attention given to apoptotic/necrotic derived DNA.

However, in our previous *in vitro* studies we have determined that actively released DNA fragments have a size of 2000-3000 bp [50], as distinct from the cfDNA derived from apoptosis (166 bp) and necrosis (~10 000 bp). It has recently been demonstrated that the automated KingFisher cfDNA isolation systems, which are used the most often in clinical investigations, are tailored for short fragments and typically fail to isolate DNA fragments with a size of 2000-3000 bp [51]. There are other methods that can isolate this cfDNA population and, indeed, it has been reported to be present in

the blood of humans [51]. However, this cfDNA with a size of 2000-3000 bp is generally regarded to be mere cellular DNA contamination. Here we again provide compelling evidence that this may be an erroneous assumption. Furthermore, the information revealed in this study should contribute to our understanding of the origin of cfDNA and the extent of its biological significance. It may also serve as an entry point for bridging *in vitro* and *in vivo* studies.

5. Methods

5.1. Cell culturing and growth medium processing

The human bone cancer (osteosarcoma) cell line 143B was obtained from the American Type Culture Collection (ATCC® CRL-8303™), and was cultured in accordance with ATCC protocols. Cells were grown in Dulbecco's modified Eagle's medium (Hyclone DMEM/high glucose) (Thermo Scientific; #SH30243.01) containing 4 mM L-Glutamine, 4500 mg/L glucose, and sodium pyruvate, and fortified with 10% fetal bovine serum (FBS) (Biochrom; #S0615) and 1% penicillin/streptomycin (Lonza; #DE17-602E). Cells were incubated in humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded (at ~50% confluency) and cultured in 75 cm^2 cell culture flasks (Nunc) and grown for 12 hours in 12 mL growth medium. After this time, the growth medium was renewed and cells were further grown for 24 hours. This was done in duplicate. At the end of incubation, the growth medium was collected in 15 mL nuclease-free tubes (Ambion; #3108090) and then centrifuged at 10 000 x g for 10 minutes and transferred to fresh 15 mL tubes. Samples were then stored at –80 °C.

5.2. Extraction and quantification of cell-free DNA

cfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the manufacturer's PCR clean-up instructions, with slight modifications. Briefly, samples were thawed at 37 °C in a temperature controlled water bath. After incubation, the samples were vortexed and centrifuged briefly. For each biological replicate, 12 mL of growth medium was mixed with 24 mL of binding buffer NTI. Samples were then vortexed, the entire volume of growth media added to the spin column in small regiments, and moved through the filter using a vacuum manifold set-up and pump. Hereafter, the columns were washed twice, followed by the elution of cfDNA into 50 µL of elution buffer. The duplicate samples were then pooled and quantified using the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) and Qubit dsDNA HS Assay kit, and then stored at 4 °C.

5.3. DNA library generation and sequencing

Extracted cfDNA (~100 ng) was sheared into 100-700bp fragments by sonication with the Bioruptor UCD-200 (Diagenode), by applying three 5 minute cycles of 30s on/30s off, on the medium setting. After shearing, the fragment ends were end-repaired and polished with the Ion Plus Fragment Library kit. Ion A and P1 adaptors were then ligated onto the polished fragments, and 330bp fragments were size-selected using 2% agarose gels on the Egel® system. These

libraries were then amplified using the Ion Plus Fragment Library kit and quantified using the Qubit dsDNA HS Assay kit. The libraries were diluted to 100 pM and a sequencing template for each library was prepared on the Ion OneTouch2 system using the Ion PGM Template OT2 200 kit. Each sample‘s templated ISPs (Ion Sphere® Particles) were manually loaded onto an Ion 318v2 chip and sequenced on the Ion PGM using the Ion PGM Sequencing 200 kit v2. Except where indicated, all kits and reagents were obtained from Thermo Fisher Scientific. Default parameters on the Ion Torrent Suite (v4.4) were used to perform base calling, trimming and filtering, and alignment to hg19. A good quality run was ensured, with higher than 85% ISP loading density and approximately 6 million reads, and approximately 1.365 Gb of data (6184354 reads) was generated.

5.4. Sequence-analysis pipeline

Binary alignment matrix (BAM) files, containing raw sequencing reads, were used to perform a strict de-novo assembly in the CLC genomics workbench (Version 7.0), followed by read-mapping. Selected contigs were converted into a FASTA format, after which the files were exported and converted to text-file format for further analysis. To screen for repetitive elements and regions of low complexity, we used RepeatMasker, a program utilizing RepBase (a service of the Institute for Systems Biology). Hereafter, when applicable, three consecutive local alignment-analyses were conducted using the BLAST (Basic local alignment search tool) search engine to compare the generated array of cfDNA sequences with known sequences, including the human genome. The first search was against the ENSEMBL human GRCh38.p5 genome database. For the queries that did not return any hits, a second search against the National Center for Biotechnology Information (NCBI) nucleotide (nr/nt) database was performed using the megablast algorithm. Then again, for the queries that returned no results, a third search was performed using the blastn algorithm. In all cases, default search parameters were used. This procedure was repeated for the hits that covered less than 50% of the query sequence length in the ENSEMBL BLAST search. For each sequence, the highest-scoring BLAST hit, its physical location, overlapping gene (where relevant), and genomic location (e.g., in the centromere, in telomeres, in one gene, etc.) was recorded. This information was used to categorize the cfDNA sequences. For mapping, gene symbols were converted to cytogenetic locations using the NCBI bulk conversion page, followed by ideogram map generation using the NCBI genome decoration tool (http://www.ncbi.nlm.nih.gov/genome/tools/gdp). Masked sequences were annotated using the NCBI genome annotation pipeline.

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Chapter 6: Summary, conclusions, and future prospects

6.1 The importance of investigating the characteristics of cfDNA in human biology (aim I)

In the last 40 years, many proof-of-principle studies have intermittently demonstrated the translational potential of cfDNA as a versatile non-invasive screening tool for a wide range of diseases, physiological conditions and other clinical scenarios. However, abstracting data in a research setting and applying it in medical practice proved to be much more complicated than expected. Except for the implementation of a small number of prenatal tests (NIPT), only one other clinically validated and FDA approved application of cfDNA is currently available (Brown, 2016; Lowes et al., 2016). The most commonly held assumption is that the development of comprehensive clinical assays, coupled with the scope of the utility of cfDNA, is constrained mainly by a lack of an analytical consensus between research groups, and by the limits of current technologies. However, a growing number of reports are beginning to concede that a lack of knowledge on the biological properties of cfDNA may be another substantial obstacle in way of the rapid translation of research to medical practice (Thierry et al, 2016; Jiang & Lo, 2016).

Accordingly, the first major premise that was argued in this thesis, is that the difficulties imposed on translational cfDNA research by analytical discrepancies and technological limitations is exacerbated by a lack of knowledge on the biological properties and function of cfDNA, and by the indifference towards the consideration thereof in the design of clinical validation experiments. This state of affairs can be attributed to the prevalence of a biased mindset in the cfDNA community that favoured the inflation of clinically motivated studies, a trajectory followed since the discovery of cfDNA in 1948. For example, although the function of cfDNA as an intercellular messenger was conceptualized in the 1960's (Gahan & Chayen, 1965; Stroun et al, 1966; Bell, 1969), it has to this day not received adequate attention.

Therefore, the first aim of this study was to develop a deeper understanding of the origin, structure, fluctuation and function of cfDNA in human biology. To achieve this, the available literature on the topic was critically assessed, which culminated in the writing of two review articles (Articles **I** and **II**).

In Article **I** it was argued that many of the theories and experiments surrounding the discovery and conceptualization of cfDNA as an intercellular messenger were abandoned because they emerged either in the era that preceded the structural elucidation of DNA, or a time shortly after this when ideas that challenged the "central dogma" were easily dismissed. Thus, by reconsidering these neglected works in the new light of contemporary biology, it was recognized that cfDNA has the innate tendency to be readily transferred between different cells from all kingdoms of life, with concomitant biological effects. In human biology, this phenomenon could be implicated in diverse detrimental effects ranging from mutagenesis and oncogenesis to chemo-resistance and metastasis. Conversely, cfDNA transfer may also be involved in many potentially positive biological phenomena, including the blocking of tumour growth, generation of somatic genome variation, cellular adaptation and transgenerational inheritance.

In Article **II** it was not only demonstrated that a myriad of potential sources and causes can result in the presence of cfDNA in the extracellular environment, but that many of these sources and causes may be inextricably linked by a complex interplay of cellular and physiological interactions. Furthermore, it was recognized that these interactions are highly sensitive to environmental perturbations and can produce seemingly erratic fluctuations of both the quantitative and qualitative characteristics of cfDNA in the blood of an individual at any instance, the nature of which can differ considerably between individuals. As an example of this, the relationship between the rate of cfDNA release and its clearance from blood is dependent on the rate of cell death, inflammation and nuclease activity, which in turn is subject to influence by age, weight, gender, fitness, organ health, diet, smoking, circadian oscillations, oxidative and medicinal status, etc. (van der Vaart & Pretorius, 2008).

Two other very important conclusions arrived at in both Articles **I** and **II** was that, (i) the cfDNA present in the blood of both healthy and diseased individuals is not only derived from malignant cells, and is not only the product of aberrant processes or cellular demise, but can also result from active DNA release, and (ii) both healthy and malignant cells can release both wild-type and mutated DNA.

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Taken together, Articles **I** and **II** emphasize the reality that there is a notable dearth of knowledge about the properties and significance of cfDNA in human biology. However, although the true nature of most of the biological activities of cfDNA remain uncertain, it could be conjectured that cfDNA, along with the structures with which it is associated, has both a fundamental and diverse role in both normal biology and pathology. This seems to suggest that further enquiry into the biological properties and function of cfDNA will not only benefit applied or translational cfDNA research, but could also provide a new framework for a deeper scientific understanding of molecular biology, pathology and the process of evolution. Therefore, the need for elucidating the biological properties of cfDNA is fully rationalized. However, another crucial problem highlighted by Articles **I** and **II** is that the inherent complexity of the *in vivo* setting presents a major obstacle to the characterization of cfDNA in biological samples. From the arguments presented in Articles **I** and **II**, the following solutions can be inferred:

- There is a substantial amount of meaningful knowledge and ideas relating to the biological properties and function of cfDNA that populate the trash bin of scientific literature, which may be very useful when re-evaluated in the light of contemporary biology.

- There is currently a chasm between applied and basic cfDNA research, and a consolidation thereof will be very beneficial to the research field.

- Alternative *in vitro* strategies for the characterization of cfDNA should be developed and used more frequently, and in conjunction with current *in vivo* methods.

6.2 Development of robust methodology for *in vitro* cfDNA analysis (aim II)

In order to better understand the origin and function of cfDNA in human biology, it is important to have knowledge of its structure, fluctuation and cellular conditions under which it is released. However, in Chapter 2 it was shown that this is largely lacking, but it was also demonstrated that the elucidation of both the quantitative and qualitative characteristics of cfDNA in blood samples is severely complicated by the inherent complexity of the *in vivo* setting. Therefore, since cell cultures are insulated from most of the confounding elements that the human body is faced with, the second major premise that was argued in this thesis, is that many of the difficulties encountered in *in vivo* experiments can to a certain extent be circumvented by *in vitro* cell culture models. Accordingly, in order to evaluate the potential of *in vitro* cell culture models to study cfDNA, fortify *in vivo* studies, and contribute to our understanding of the phenomenon of cfDNA, the most important aim of this study was to investigate the origin, structure, fluctuation and function of the DNA present in the growth medium of cultured cells (see Section 6.3).

However, before this could be done it was necessary to establish a robust preanalytical workflow. As discussed in Chapter 3, many experimental variables were evaluated in order to do this. The standard method that was developed is reported in the Methods and Materials sections of Articles **V**, **VI**, and **VII**. The most important aspects of the methods were: (i) cells needed to be seeded at the correct density and allowed to settle for 12 hours before medium is replenished, after which new medium could be collected for analyses, (ii) the protocol for cell culturing and culture conditions should not change between experiments, (iii) collected growth medium should be stored at the same temperature during all experiments (-80 °C), and (iv) the same DNA extraction method should be used throughout the study. Furthermore, the results reported in Articles **III** and **IV** demonstrated that *in vitro* cell cultures could be used to facilitate the optimization, standardization and development of robust preanalytical workflows for *in vivo* bio-fluid samples.

6.3 Investigating the characteristics of cfDNA present in the growth medium of cultured cells (aim III)

It is generally assumed that the cfDNA in the blood of healthy and diseased individuals is mainly a result of apoptosis. However, some studies suggest that cfDNA may also be a product of necrosis or an active DNA release mechanism (Bronkhorst et al., 2016). Thus, the origin of cfDNA is poorly understood. To investigate the origin of the cfDNA present in the growth medium of bone osteosarcoma (143B) cells, the release pattern of cfDNA was characterized over time, and the sizes of the cfDNA fragments at each of the respective time intervals were evaluated. This data was then compared with corresponding apoptosis, necrosis, and cell cycle profiles as determined by flow cytometry (Article **V**).

As described in Article **V**, after 4 hours of incubation only a small number of 166 bp DNA fragments are present in the growth medium of 143B cells. However, after 24 hours there is a significant increase in the amount and size of DNA (~2000 bp). Typically, DNA with a size of 166 bp is a product of apoptotic fragmentation, while a size of 2000 bp can be explained by neither apoptosis nor necrosis. These results were confirmed by two different flow cytometric assays. Furthermore, cell cycle analysis demonstrated a shift from the S to the sub G0/G1 phase after 24 hours of incubation, suggesting that the significant increase of cfDNA may not be associated with DNA replication. However, we cannot with complete confidence say that this is the case, since the timing associated with the intracellular trafficking of DNA before it is released as cfDNA was not investigated. Furthermore, it was demonstrated that treatment of growth medium with denaturing agents, Proteinase K and SDS, prior to DNA extraction significantly increases the yield of cfDNA, indicating an association with a protein complex. This finding is consistent with other reports in the literature (Gahan et al., 2010). Taken together, the results reported in this article suggest that the occurrence of cfDNA in cell culture medium is a result of different mechanisms, including apoptosis, necrosis and active DNA release. However, the majority of the DNA released by 143B cells appear to be the product of some unknown active release mechanism.

These results begged the questions: is this a trait common to all cell types? And do all cells release equal amounts of DNA and in the same way? This prompted further investigation and expansion of the study. In order to do this, the characteristics of the cfDNA from seven different cell lines, including "healthy" and malignant cells, were assessed. These results are reported in Article **VI**. First, the observation made in the previous work (Article **V**) - that one cell type can produce different types of DNA at different stages - was corroborated. Secondly, it was demonstrated that most cell lines release DNA with a size of approximately 2000 bp, and that this occurs at a time of both steady growth and decline of apoptotic cfDNA levels. Thus, Articles **V** and **VI** in concurrence show that the cfDNA with a size of ~2000 bp is the product of some unknown active release mechanism, and does not seem to be of apoptotic or necrotic origin. Very interestingly, the cfDNA size profile obtained in these studies, when superimposed, closely resembles the size profile of human plasma-derived cfDNA.

Another aspect revealed by the experiments described in Article **VI**, is that different cell types release different amounts of DNA at different time points. In addition, bioenergetics analyses showed that cfDNA levels (the 2000 bp fraction) correlate with glycolysis metabolism only in cancer cells, which rely heavily on aerobic glycolysis consumption. Since cfDNA levels were normalized in terms of total cellular protein content, this suggests that the active release of DNA is associated with the growth rate and malignancy of cells. This indicates that different cells of the human body could be predisposed to release different amounts of DNA at different stages, depending on the nature of their basal metabolic rate, and changes thereof upon environmental stimulus (As discussed in Chapter 2). In addition, as reported in Article **IV**, many of these cells not only release different amounts of DNA, but they also release different DNA fragments, and there seems to be some selectivity involved (i.e., in comparison with cellular mRNA, there are cfDNA sequences absent in the extracellular environment).

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To recapitulate, Articles **V** and **VI** have demonstrated that DNA fragments with a size of ~2000 bp are actively released by cultured 143B cells into the extracellular environment after 24 hours of incubation, and that this is the result of some regulated energy-dependent process. Moreover, results described in Article **IV** suggest the intriguing possibility that there could be some intent and selectivity involved in the release of cfDNA. To investigate this, the nucleotide sequence of the actively released cfDNA was determined by massively parallel semiconductor sequencing (Article **VII)**. After sequencing, the cfDNA sequences were re-assembled and then screened for repetitive elements, followed by local alignment analyses and annotation. Four very significant observations were made:

1) The majority of cfDNA actively released by 143B cells consist of repetitive DNA (88 %), which exceeds any value predicted for the human genome (50-66%). Sequence analysis of genomic DNA from 143B cells confirmed that this is not a result of sequencing or statistical bias. Indeed, other studies have made similar observations (Mittra et al., 2015; Podgornaya et al., 2016).

2) Further statistical analyses showed that Alu, LINE1, ERV (K) class II, MaLR and TcMar-Tigger elements as well as α-satellites and mini-satellites are significantly overrepresented when compared to its expected amount in the human genome. Moreover, specific subfamilies of each of these repetitive element families were significantly overrepresented. Conversely, MIR, LINE2, LINE3/CR1, ERV class I, ERV (L) class III and hAT-Charlie elements are notably underrepresented or occur at levels comparable to the human genome.

3) Based on a critical review of the literature, it was concluded that the representation of the different repetitive elements correlates strongly either with its current transposition activity, or with its ability to become reactivated. This appears to be a new discovery.

4) Finally, local alignment analyses revealed that the majority of these sequences originate from the centromeres of chromosomes 1 and 16.

6.4 The origin and function of actively released cfDNA in cancer

Studies have demonstrated that the hypomethylation of satellite DNA at the peri-centromeric regions of chromosomes 1 and 16 leads to rearrangements, decondensation, and finally chromosomal instability (Vilain et al., 2000). Therefore, keeping in mind that DNA hypomethylation is a hallmark of cancer cells (Ehrlich et al., 2009), and that transposons can become reactivated by DNA demethylation (Howard et al., 2008; Bourc'his et al., 2004), it was hypothesized that the demethylation of these regions in 143B cells leads to the derepression and mobilization of transposons, followed by aberrant translocations and chromosomal instability (Wong et al., 2001; Ehrlich et al., 2002). There is extensive sequence similarity between centromere-protein B (CENP-B), a protein (that can induce DNA breaks) associated with the centromeres of most human chromosomes, and the transposase encoded by the human Tigger DNA transposon. In addition, the terminal inverted repeats of the Tigger2 elements contain a near perfect match to the CENP-B binding site (Kipling & Warburton, 1997). This suggests that both CENP-B and transposases, which are liable to activation by demethylation, may facilitate the excision of satellite DNA. Furthermore, considering the structural and functional similarities between satellite DNA and transposons (Schueler et al., 2005; Ahmed et al., 2012), it is likely that the presence of overrepresented transposons is a result of programmed DNA elimination. Although there are limited reports of this phenomenon in humans, there is strong evidence that this occurs in other higher organisms (Vogt et al., 2013; Nowacki et al., 2009) . A detailed description of this hypothesis is given in Section 3 of Article **VII**.

Questions raised by these observations were whether satellite DNA and transposons are (i) deliberately released by cancer cells to perform specific functions in the extracellular environment, (ii) by-products of a normal cellular process and are incidentally biologically active, or (iii) biologically-inert by-products. In this study it was not only demonstrated that certain repetitive element families are significantly overrepresented in the cfDNA released by 143B cells, but that specific members of each family are

overrepresented, such as the L1P1 subfamily of LINE1, and the HERVK9int subfamily of ERV (K) class II (human endogenous retroviruses). Interestingly, it has recently been demonstrated that the insertion of a single LINE1 element in the *APC* tumour suppressor gene can initiate tumour formation in human colorectal cancer (Scott et al., 2016), and the role of endogenous retroviruses in cancer is well documented (Kassiotis et al., 2014; Gonzalez-Cao et al., 2016) . In keeping with this, many reports have described how cfDNA can be transported throughout the body, while other studies have demonstrated their capacity to enter target cells and alter their biology, a phenomenon implicated in the oncogenic transformation of normal cells and the development of metastases (Bendich et al., 1965; Garcia-Olmo et al., 2010; Trejo-Becerril et al., 2012), the induction of cancer-cell resistance against radiation- and chemo therapy (Kostyuk et al., 2012; Glebova et al., 2015; Ermakov et al., 2011), and other mutagenic effects (Basak et al., 2016). Since the mechanisms involved in these phenomena are unknown, this study indicates that satellite DNA and transposons may yet prove to be among the key effector molecules.

A related phenomenon that is not well understood, is that cancer patients generally present with elevated levels of cfDNA (Fleischhacker & Scmidt, 2007). A partial explanation for this can be derived from the observation made in this study that cultured cancer cells release notably more DNA than normal cells, and that this is related to their unique metabolism. This, together with the correlative relationship between the malignancy of cancer cells and degree of demethylation, suggests that the level of cfDNA release increases concomitantly with malignancy. Viewed in light of the central theorem of the extended phenotype (Dawkins, 1999), in which the malignancy of cancer cells should maximize the survival of genetic instructions that promote malignant behavior, it stands to reason that cancer cells would up-regulate the mobilization and lateral transfer of transposons to neighboring cells with the purpose of transforming them. In line with this premise, it can be argued that the composition and function of the DNA released by normal cells will differ from cancer cells on a fundamental level, and it is also likely that the cfDNA from different cancer cells differ.

In this regard, further investigation of the molecular characteristics of cfDNA, and elucidation of the mechanisms involved in its release and function, could provide a deeper understanding of the tumour microenvironment, clonal evolution, the expansion of therapy-resistant variants, and how metastasis occurs. Consequently, this could aid the development of alternative and more effective therapies, and the identification of more appropriate biomarkers for diagnosing cancer, estimating tumour burden, and monitoring response to therapy. In theory, it can facilitate the development of comprehensive clinical assays based on personalized treatment regimens and patient follow-up.

Furthermore, as it was demonstrated that normal cells also release cfDNA, these results not only implicate satellite DNA and transposons in detrimental effects, but also provide a potential mechanism for the transfer of satellite DNA and transposons between otherwise healthy somatic cells. Since it is well established that satellite DNA and transposons play integral roles in gene regulation, and the rearrangement and duplication of DNA, it is likely that the transfer of satellite DNA and transposons between different cells within one organism is an operative factor that drives somatic genome variation and biological innovation. To our knowledge, such a phenomenon has not yet been described. If this turns out to be an active force in higher organisms, we would expect this to be of most value to learning (neurons), immunity (lymphocytes) and detoxification (hepatocytes). Furthermore, it seems conceivable that, similar to bacteria, the prospect of the lateral transfer of satellite DNA and transposons between different cells/tissues should make different mutants receptive to intra-organismal Darwinian selection, which should allow diversification and evolutionary conservation.

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6.5 Concluding remarks and directions for future research

The aims of this study were largely fulfilled by writing the seven core scientific articles presented in Chapters 2, 3, 4 and 5 of this thesis. As outlined in Chapter 1.4, the aims of this study were to: (i) develop a better understanding of the various origins, structures and functions of cfDNA by conducting an extensive literature review, (ii) establish both a robust cell culture model and reliable preanalytical workflow for the *in vitro* characterization of cfDNA, and (iii) investigate the origin, fluctuation, structure and function of the cfDNA present in the growth medium of cultured cells. Detailed suggestions for future studies were made in the concluding sections of Articles **I**, **II**, **III**, **V**, **VI** and **VII**.

The most important question raised by this thesis is whether the inference can be drawn that the observations made *in vitro* is also reflective of the *in vivo* setting. DNA fragments with a size of ~2000 bp have in fact been detected in human blood. However, it is readily dismissed as mere cellular DNA contamination (Application note: A complete next-generation sequencing workflow for circulating cell-free DNA isolation and analysis). Therefore, this study provides compelling evidence that this may be an erroneous assumption. Interestingly, it has recently been demonstrated that automated cfDNA isolation systems (e.g., KingFisher), which are the most commonly used in clinical assays, are tailored for short fragments and typically fail to isolate DNA fragments in the range of ~2000 bp (refer to Figures 5 & 6 of aforementioned application note). Since the majority of cfDNA research is clinically motivated, it explains why this cfDNA population is not often encountered. In this regard, the results and arguments presented in this thesis suggest that the commonly held assumption that apoptosis is the main origin, and most relevant fraction, of cfDNA in human blood may be incorrect, restrictive, and should be reconsidered. It may be a major breakthrough in the field of clinical diagnostics if the DNA actively released by cultured cells bears any resemblance to the DNA originating from its *in vivo* counterpart. Therefore, this warrants the implementation of comparative studies.

Further inquiry into the biological properties of actively released DNA will not only benefit applied research, but could also provide a new framework for a deeper understanding of molecular biology, pathology and the process of evolution. Furthermore, this study demonstrates the utility of *in vitro* cell culture models for studying the phenomenon of cfDNA, and as such also emphasizes the importance of consolidating basic and applied cfDNA research.

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Appendix I: Conference outputs and published proceedings

Article VIII

A Historical and Evolutionary Perspective on Circulating Nucleic Acids and Extracellular Vesicles: Circulating Nucleic Acids as Homeostatic Genetic Entities

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A Historical and Evolutionary Perspective on Circulating Nucleic Acids and Extracellular Vesicles: Circulating Nucleic Acids as Homeostatic Genetic Entities

17

Janine Aucamp, Abel Jacobus Bronkhorst, and Piet J. Pretorius

Abstract

The quantitative and qualitative differences of circulating nucleic acids (cirNAs) between healthy and diseased individuals have motivated researchers to utilize these differences in the diagnosis and prognosis of various pathologies. The position maintained here is that reviewing the rather neglected early work associated with cirNAs and extracellular vesicles (EVs) is required to fully describe the nature of cirNAs. This review consists of an empirically up-to-date schematic summary of the major events that developed and integrated the concepts of heredity, genetic information and cirNAs. This reveals a clear pattern implicating cirNA as a homeostatic entity or messenger of genetic information. The schematic summary paints a picture of how cirNAs may serve as homeostatic genetic entities that promote synchrony of both adaptation and damage in tissues and organs depending on the source of the message.

Keywords

Circulating nucleic acids • Extracellular vesicles • Genetic homeostasis • Metabolic DNA • Bystander effect • Genometastasis

Introduction

Since the discovery of cirNAs in human plasma in 1948, there has been considerable amount of research regarding their diagnostic applications (Fleischhacker and Schmidt 2007). Despite the progress made, there are still inconsistencies that bolster clinical application and this is mainly due to a lack of standard operating procedures in the storage, extraction and processing of cirNAs. However, our lack of knowledge regarding the origin and purpose of cirNAs is an equal culprit. The aim of this review is to illustrate the development of cirNA research in order to elucidate cirNA as homeostatic entities or messengers of genetic information. The

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position maintained here is that reviewing the rather neglected early work associated with cirNAs and extracellular vesicles (EVs) is required to fully describe the nature of cirNAs. The review consists of an empirically up-to-date schematic summary of a few of the major events that developed and integrated the concepts of heredity, genetic information and cirNAs. This reveals a clear pattern implicating cirNA as a homeostatic entity or messenger of genetic information.

Homeostasis and DNA

DNA is vulnerable to change or damage, especially during transcription and replication. These processes are highly regulated to prevent errors from occurring, but changes can still occur and our living environment provides additional challenges to genome integrity. If all other body functions utilize homeostatic functions to maintain stability and balance, is it possible that there can be some form of homeostatic regulation for genetic information?

In 1954 Michael Lerner coined the term "genetic homeostasis", referring to the ability of a population of organisms to equilibrate its genetic composition and to resist sudden changes (Hall 2005). As the term stands, it is not really applicable to the article's aim, unless one refers to different organs, tissues and cell types as populations within the body of an organism. As with populations of organisms, genetic changes do not always occur uniformly throughout the organs and tissues of the body, but in most cases begin as isolated incidences. These changes, however, can spread to and affect nearby cells and tissues. Take the bystander effect, for example, which refers to the effect of information transfer from targeted cells exposed to damaging agents of a physical or chemical nature to adjacent cells (Ermakov et al. 2013). Targeted UV irradiation results in the release of clastogenic factors by irradiated cells that can induce apoptosis and necrosis in adjacent non-irradiated cells. These clastogenic factors have been identified as extracellular DNA (Ermakov et al. 2011) and their effects have also been found to persist in the progeny of irradiated cells that survived irradiation (Seymour and Mothersill 2000).

The Four Paradigms of the History and Development of cirNAs and EVs

The question now is whether cirNAs can serve as homeostatic entities or messengers to promote stability and equilibrium of genetic data among a population of tissues/organs. We believe this is the case and there are several instances in the discovery and development of cirNAs and extracellular vesicles (EVs) that strongly indicate this. The history and development of cirNAs and EVs consist of four main topics or paradigms, namely heredity, DNA, messengers and the cirNAs and EVs (see Fig. 17.1).

Conclusions

To conclude we ask again, can cirNAs serve as homeostatic entities or messengers to promote stability and equilibrium of genetic data among a population of tissues or organs? The answer is yes. According to our empirically up-to-date schematic summary of the history and development of cirNA research and the contributions of EV research:

(i) Darwin coined the idea of free moving updated data particles originating from all tissues;
(ii) Griffith and Avery showed us that these particles are nucleic acids and can transfer beneficial information from one place to another and can be inherited;
(iii) De Vries, Bell, Stroun, Pelc, Anker and Gahan showed us that these particles are not necessarily for heredity, but to convey messages;
(iv) Pelc showed us that metabolic DNA, and therefore spontaneously released DNA or virtosomes (if metabolic DNA serves as

Fig. 17.1 Empirically up-to-date schematic summary of a few of the major events that developed and integrated the concepts of heredity, genetic information, cirNAs and EVs. The relationships of the four paradigms of cirNA research are illustrated: Heredity (*black* with *white font*), DNA research (*white*), messengers (*dark grey* with *black font*) and cirNA and EV research (*dash lines*). Areas with other combinations of *dash lines* and *fonts* represent data that fall under more than one of the four categories

their precursors), are separate entities from our genetic DNA;

(v) The transcession studies and subsequent genometastasis and exosome research showed us that cirNA release becomes prominent when change occurs (e.g. bacterial exposure leading to transcription of bacterial DNA into recipient cells, cancer mutations, epigenetic changes, damage and/or repair due to stressors such as irradiation exposure);

(vi) CirNAs contain the changes or mutations of the parent tissue;

(vii) Garcia-Olmo's patent showed us that it is not only cirNAs related to diseases, disorders and/or damage that can induce change;

(viii) Genometastasis and the bystander effect showed us that these cirNAs can transfer change from one place to another and can become persistent in following generations. CirNas could, therefore via horizontal gene transfer, serve as homeostatic entities or messengers of genetic information.

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Article IX

Methodological Variables in the Analysis of Cell-Free DNA

Abel Jacobus Bronkhorst, Janine Aucamp, Piet J. Pretorius

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Methodological Variables in the Analysis of Cell-Free DNA

29

Abel Jacobus Bronkhorst, Janine Aucamp, and Piet J. Pretorius

Abstract

In recent years, cell-free DNA (cfDNA) analysis has received increasing amounts of attention as a potential non-invasive screening tool for the early detection of genetic aberrations and a wide variety of diseases, especially cancer. However, except for some prenatal tests and BEAMing, a technique used to detect mutations in various genes of cancer patients, cfDNA analysis is not yet routinely applied in clinical practice. Although some confusing biological factors inherent to the *in vivo* setting play a key part, it is becoming increasingly clear that this struggle is mainly due to the lack of an analytical consensus, especially as regards quantitative analyses of cfDNA. In order to use quantitative analysis of cfDNA with confidence, process optimization and standardization are crucial. In this work we aim to elucidate the most confounding variables of each preanalytical step that must be considered for process optimization and equivalence of procedures.

Keywords

Cell-free DNA • Cancer • Preanalytical factors • Prenatal diagnostics • Quantitative analysis

Introduction

Since the discovery of cfDNA in human blood, the focal point of most cfDNA studies has been to scrutinize it as a potential non-invasive diagnostic and prognostic marker for solid tumours. Although considerable progress has been made in this regard, it is as yet a partial victory. Except for some prenatal tests (Chiu et al. 2013) and BEAMing (Lawrie et al. 2008), cfDNA analysis

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has not really been translated to clinical practice and routine application seems distant. This can be ascribed to three factors that overlap: (i) a lack of knowledge regarding the origin and function of cfDNA, (ii) insufficient molecular characterization, and (iii) the absence of an analytical consensus. Although it is generally accepted that the last mentioned is a major obstacle, very few have addressed it (El Messaoudi et al. 2013). As a result, most research groups have, by their own discretion, developed in-house procedures that are prejudicial to the smooth translation of cfDNA analysis to clinical practice (El Messaoudi and Thierry 2015). In a recent review we have addressed this issue, and focussed specifically on quantitative analysis of cfDNA (Bronkhorst et al. 2015). In this, we have reached two prominent conclusions. Firstly, the lack of head-to-head comparative studies between the different techniques utilized has led to conflicting results between research groups. Secondly, although it is clear from the literature that a single quantitative assessment is of very limited value, it is discernible that an analysis of the kinetics of cfDNA concentration will be a strong auxiliary component to qualitative characterization. However, in order to confidently use quantitative analysis for this purpose a major proviso becomes crucial, i.e., the optimization and standardization of procedures (van der Vaart and Pretorius 2010).

Therefore, in this work we have aimed to elucidate the most confounding variables at each preanalytical step that need to be considered in this endeavour. Using the growth medium of cultured cells as a source of cfDNA, we assessed the influence of variations to the centrifugation regime, storage temperature, thawing temperature, and storage tube type on the yield of cfDNA. Moreover, regarding the isolation of cfDNA, we assessed the effect of tube type, type of denaturing agent, binding buffer, elution buffer volume, elution regime, and the tube in which isolated cfDNA is stored. Although growth medium was used instead of blood, there are many points of contact between the methods used. The results will thus be relevant to both *in vitro* and *in vivo* studies.

Materials and Methods

Below is described the standard method we used for quantitative analysis of cfDNA. The details of the changes to this basic protocol is described in the results and discussion, but more detailed descriptions of these changes can be found in Bronkhorst et al. (2015).

Cell Culturing

Culture medium of the human bone cancer (osteosarcoma) cell line 143B (ATCC® CRL-8303TM) was used as a source of cfDNA. Cells were cultured in T75 flasks in Dulbecco's Modified Eagle's medium (DMEM) (HyClone; #SH30243.01) fortified with 10 % fetal bovine serum (Biochrom; #S0615) and 1 % penicillin/streptomycin (Lonza; #DE17-602E) at 37 °C in humidified air and 5 % CO_2.

Extraction of cfDNA

CfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the instructions described by the PCR clean-up user manual. For each biological replicate, cfDNA was extracted in triplicate. For every sample, 600 µL of growth medium was mixed with 1200 µL of binding buffer. Samples were then vortexed, the entire volume of growth media was added to the spin column in small regiments, and centrifuged

at 11,000 g for 1 min at room temperature. The columns were then washed twice, followed by the elution of cfDNA into 20 μL of elution buffer.

Quantification of Cell-Free DNA

PCR amplification of cfDNA was measured using a real-time quantitative assay for the β-globin gene. All assays were performed on a Rotor-Gene Q detection system (Qiagen) using a 72 well ring-setup. The reaction mixture consisted of 2 μL DNA and 23 μL master mix, which was composed of 8.1 μL H_2O, 12.5 μL TaqMan Universal MasterMix (Life technologies; #1502032), 0.4 μL of 10 μM dual fluorescent probe 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3′, and 1 μL of 10 μM forward and reverse primers, respectively. The primers used were: F1, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, and R1, 5′-CCT TGA TAC CAA CCT GCC CAG-3′. These primers and the probe were synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific). PCR conditions were set to: 95 °C for 10 min, followed by 45 cycles of 15 s denaturation at 95 °C, 1 min annealing at 60 °C, followed by 30 s extension at 72 °C.

Results and Discussion

Using the literature as a guideline, several changes to the standard cfDNA handling protocol were investigated. The details of these modifications are described in (Bronkhorst et al. 2015). Results are summarized in Figs. 29.1, 29.2, 29.3 and 29,4 and are discussed in the figure captions.

Conclusion

By conducting a literature survey, we have previously identified and discussed several methodological variables that could potentially influence quantitative measurements of cfDNA (Bronkhorst et al. 2015). From this, we have selected the most confounding variables to evaluate further experimentally. Using the growth medium of cultured cells as a source of cfDNA, we found variations to the centrifugation regime, storage temperature, thawing temperature, and storage tube type to affect the yield of cfDNA considerably. Furthermore, regarding the isolation of cfDNA, we found variations to the type of denaturing agent, binding buffer, elution buffer volume, elution regime, and the tube in which isolated cfDNA is stored to also greatly affect yield. Indeed, some changes resulted in a significant increase of cfDNA yield. A combination of certain changes should appease cfDNA extraction which may appease quantification, gene expression profiling and sequencing. However, it is evident that too many changes work against each other, and establishing the optimal combination of changes requires further experimentation. In conclusion, we propose that many of these variations have not yet been considered, and that it should be useful to consider them when optimizing protocols and setting up a standard operating procedure.

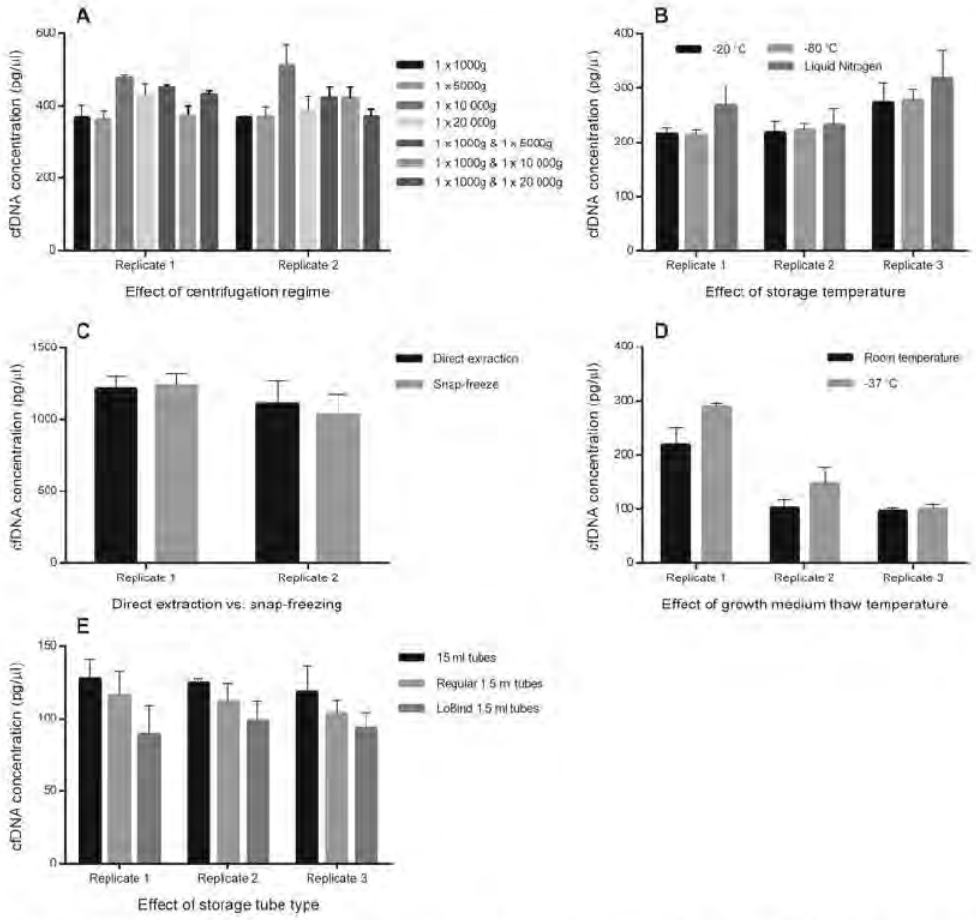

Fig. 29.1 The effect of sample handling on quantitative measurements of cfDNA. (**a**). The effect of varying centrifugal forces and repetition. Samples were centrifuged for 10 min at the different forces. In both experiments, one centrifugation at 10,000 g delivered the highest yield. (**b**). Changes in cfDNA yield during storage. After the medium was centrifuged, it was transferred to fresh tubes and stored until cfDNA was extracted. In all three experiments, storage at −20 °C and −80 °C delivered comparable results, while snap-freezing the samples in liquid nitrogen before storage at −80 °C resulted in an increase of cfDNA yield, although only replicate one is statistically significant. It could be argued that this extra step dissociates the DNA from the protein complexes with which it is associated. However, in (**c**) (results taken from Fig. 29.2b.) we see that the amount of cfDNA extracted after snap-freezing correlates with the amount of cfDNA that is extracted from medium directly after collection. It can thus be argued that in the case of storage at −20 °C and −80 °C a fraction of cfDNA is lost. (**d**). The effect of the medium thawing temperature on the amount of cfDNA extracted. In all experiments, the cfDNA yield was higher when the medium was thawed at 37 °C as opposed to room temperature. This is convenient, given that thawing at 37 °C takes only about 3 min, whereas thawing at room temperature takes at least 10 times longer. (**e**). The loss of cfDNA due to tube type. Storage of medium in three different tubes resulted in three different amounts of cfDNA extracted. Storage in nuclease-free 15 mL tubes resulted in the least amount of DNA loss, followed by regular 1.5 mL Eppendorf tubes, with DNA LoBind Eppendorf tubes resulting in the most loss of cfDNA. In the case of the 15 mL tubes, it may be argued that less cfDNA is lost due to a smaller tube area-to-sample ratio than the 1.5 mL tubes

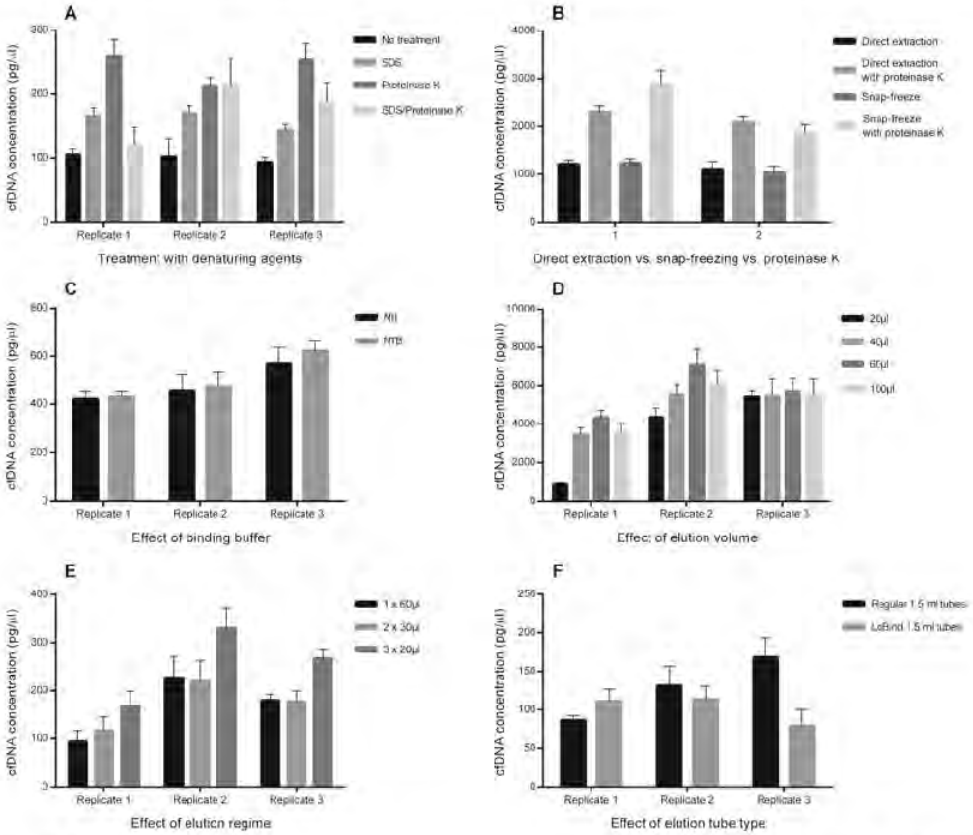

Fig. 29.2 The effect of modifications to the extraction protocol on quantitative measurements of cfDNA. (**a**). The effect of the addition of denaturing agents. The use of SDS increased the yield of cfDNA by approximately 50 % while proteinase K increased the yield by more than 100 %, whereas a combination of the two delivered confusing results. (**b**). A comparison of cfDNA yield between direct extraction and snap-freezing, with and without the use of proteinase K. Since the medium of all the samples in the previous experiment was snap-frozen, this experiment was performed to determine whether the high yields of cfDNA are wholly or only partly due to the addition of proteinase K and not partly due to snap-freezing. It is clear that snap-freezing does not increase the amount of cfDNA extracted and that the high yields can be ascribed solely to the addition of proteinase K. (**c**). The effect of binding buffer type. In the case of buffer NTB, the ratio of sample to buffer is 1:5. In the case of extractions where buffer NTI is used, the sample to buffer ratio is only 1:2. Furthermore, we wanted to compare these two buffers in the absence of SDS. The use of buffer NTB resulted in only a very slightly higher yield of cfDNA than that obtained with buffer NTI. Since the difference is not statistically significant and since the use of NTB is more arduous, this convention is not advised. (**d**). The effect of elution volume. CfDNA was extracted and eluted into 20 µL, 40 µL, 60 µL and 100 µL of elution buffer, respectively. Except for replicate three where no statistically significant difference was observed, DNA yield increased as the elution volume increased up to 60 µL where it reached its peak, and declined rather dramatically after that. (**e**). The effect of elution regime. CfDNA was extracted and eluted into 20 µL of elution buffer and repeated twice more to have a final volume of 60 µL. This was followed by the elution of DNA into 30 µl of elution buffer and repeated once more to achieve a final volume of 60 µL. The former was compared to DNA elution directly into 60 µl of elution buffer. In each experiment, the yield of DNA was significantly higher when eluted in three steps of 20 µL at a time. (**f**). The effect of tube type on the loss of extracted cfDNA. To examine the loss of eluted DNA due to adsorption to the tube walls, we compared regular 1.5 mL Eppendorf tubes with 1.5 mL Eppendorf DNA LoBind tubes. In two of the experiments, the concentration of cfDNA stored in the LoBind tubes was much lower than that of DNA stored in standard tubes. These results agree with the results obtained in Fig. 29.2e

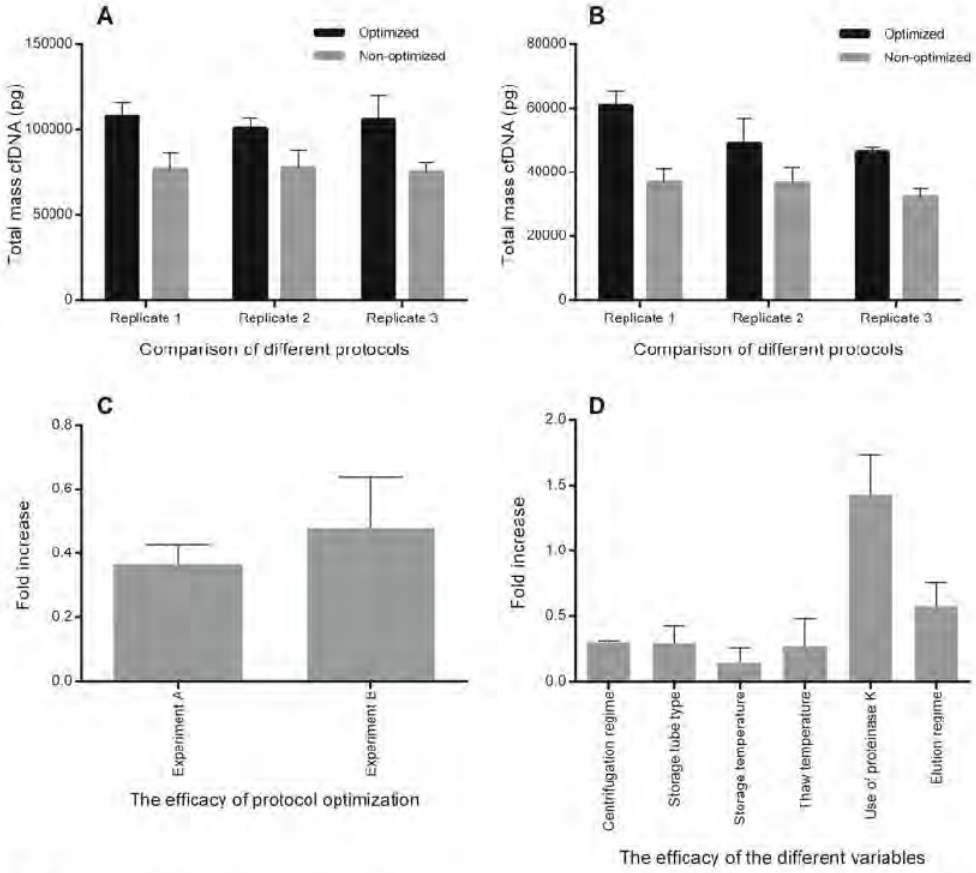

Fig. 29.3 Comparing different extraction protocols. (**a**). The variables that produced the highest increase in cfDNA yield were incorporated into the extraction protocol and compared to a protocol consisting of its less effective counterparts. In the optimized protocol, media were collected and centrifuged at 10,000 g and transferred to fresh 15 mL tubes. The media were then snap-frozen in liquid nitrogen and stored at −80 °C. The samples were then thawed at 37 °C and incubated with proteinase K (1.5 mg/mL) for 30 min at 37 °C. CfDNA was extracted and eluted into 60 μL of elution buffer in three steps (3×20 μL) into regular 1.5 mL Eppendorf tubes. In the non-optimized protocol, media w collected and centrifuged at 1000 g and transferred to fresh 1.5 mL Eppendorf DNA LoBind tubes and stored at −20 °C. Before extraction, the medium was thawed at room temperature, and no denaturing agent was added thereafter. CfDNA was then extracted and eluted into 20 μL of elution buffer in one step. Samples were stored in 1.5 mL Eppendorf DNA LoBind tubes. In (**b**) the former experiment was repeated with three new replicates. In (**c**), the average efficacy of the optimized protocols is shown and compared to (**d**), which shows the average efficacy of all variables as determined in the separate experiments. Presumably, the optimized protocol should deliver a value close to this sum total (2.99), but that is not the case. Clearly, some of these changes are working against each other

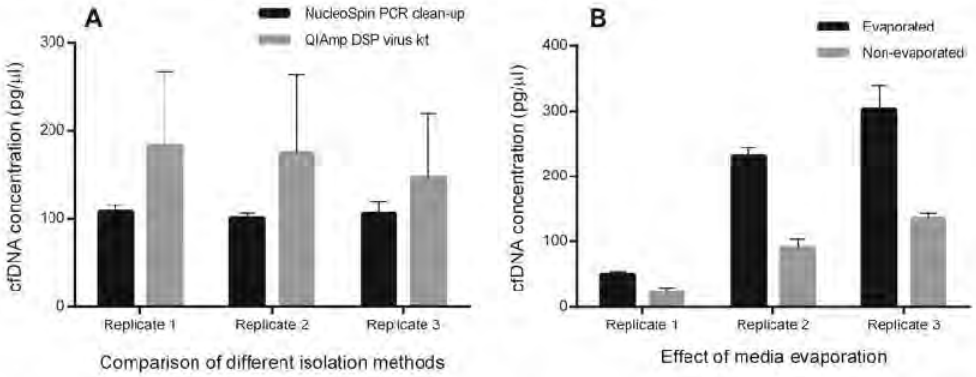

Fig. 29.4 Increasing the yield of cfDNA. (**a**). Comparing the yield of cfDNA delivered by the NucleoSpin PCR clean-up kit and the QIAmp DSP virus kit. The DSP virus kit clearly recovers more cfDNA fragments than the NucleoSpin kit. There was, however, great variation in the yield of cfDNA between the samples in each experiment. This could possibly be due to the non-equal distribution of pressure by the vacuum pump, or some other factor. On the other hand, the NucleoSpin kit was much more consistent. (**b**). The effect of media evaporation. For each replicate, 6 mL of growth medium was aliquoted into 2 mL tubes and evaporated in a SpeedVac to a total volume of 2.5 mL. The cfDNA in these samples were thus expected to be increased 2.4 fold. The average increases of the replicates were then calculated as 2.33 with a standard deviation of 0.2. This is a loss of approximately 3 %, which is rather minor

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Conflict of Interest The authors would like to declare no conflict of interest.

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Article X

A Quantitative Assessment of Cell-Free DNA Utilizing Several Housekeeping Genes: Measurements from Four Different Cell Lines

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A Quantitative Assessment of Cell-Free DNA Utilizing Several Housekeeping Genes: Measurements from Four Different Cell Lines

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Janine Aucamp, Abel Jacobus Bronkhorst, Johannes F. Wentzel, and Piet J. Pretorius

Abstract

Quantitative real-time PCR (qPCR) is regularly used to quantify cell-free nucleic acids (cfNAs) in order to identify biomarkers for various pathologies. However, studies have shown notable housekeeping gene expression variation between healthy and diseased tissues and treated versus untreated cell lines. The release of housekeeping genes by four cell lines was investigated and the housekeeping gene expression between cfNAs and mRNA of the cell lines was observed in order to elucidate their relationship.

Keywords

Housekeeping genes • Cell-free DNA • qPCR • Cell culture

Introduction

The targeting of housekeeping genes as biomarkers in cell-free nucleic acids (cfNAs) quantification is becoming quite common (Mouliere et al. 2015), but which gene serves as the most appropriate target? In quantitative real-time PCR (qPCR), housekeeping genes are primarily used as internal controls, but studies have shown notable expression variation between healthy and diseased tissues (Rubie et al. 2005) and treated versus untreated cell lines (Powell et al. 2014). These variations contradict the prerequisites for internal control housekeeping genes (Kozera and Rapacz 2013). In terms of pathology biomarker detection, however, expression variation is welcomed and the internal control prerequisites can be used to optimize cell-free DNA (cfDNA) quantification. This study focuses on the utilization of housekeeping gene expression analysis in the optimization of cfDNA quantification and the elucidation of the relationship between cfDNA and mRNA in cell lines.

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Materials and Methods

The total RNAs of melanoma (A375), osteosarcoma (143B), rhabdomyosarcoma (RD) and skin fibroblasts (ZANLP) were extracted with the NucleoSpin RNA II extraction kit (Machery Nagel), the concentrations and purity determined with the Nanodrop ID-1000 spectrophotometer (Thermo Scientific) and cDNA synthesised using the High Capacity RNA-to-cDNA kit (Applied Biosystems). The cfDNA of culture medium from the four cell lines was extracted with the NucleoSpin Gel, PCR Cleanup kit (Machery Nagel) and binding buffer NTB. The primers and probes of seven housekeeping genes from the GeNorm Reference Gene Selection Kit (Primerdesign) and β-globin (IDT, Whitehead Scientific) were used to evaluate the occurrence of housekeeping genes of the four cell lines and qPCR was performed with the RotorGene Q (Qiagen) according to the instructions of the GeNorm kit manual. Fifteen cfDNA replicates and eight cDNA replicates were prepared and the thresholds chosen to produce Ct values from the resulting raw fluorescence data was 0.028 for the cfDNA samples (as used in previous quantitative PCR studies) and 0.04 for the cDNA samples (Fig. 19.1).

Results and Discussion

ACTB was identified as the most stable gene in both cfDNA and mRNA and β-globin was stable in cfDNA, correlating with a recent study of

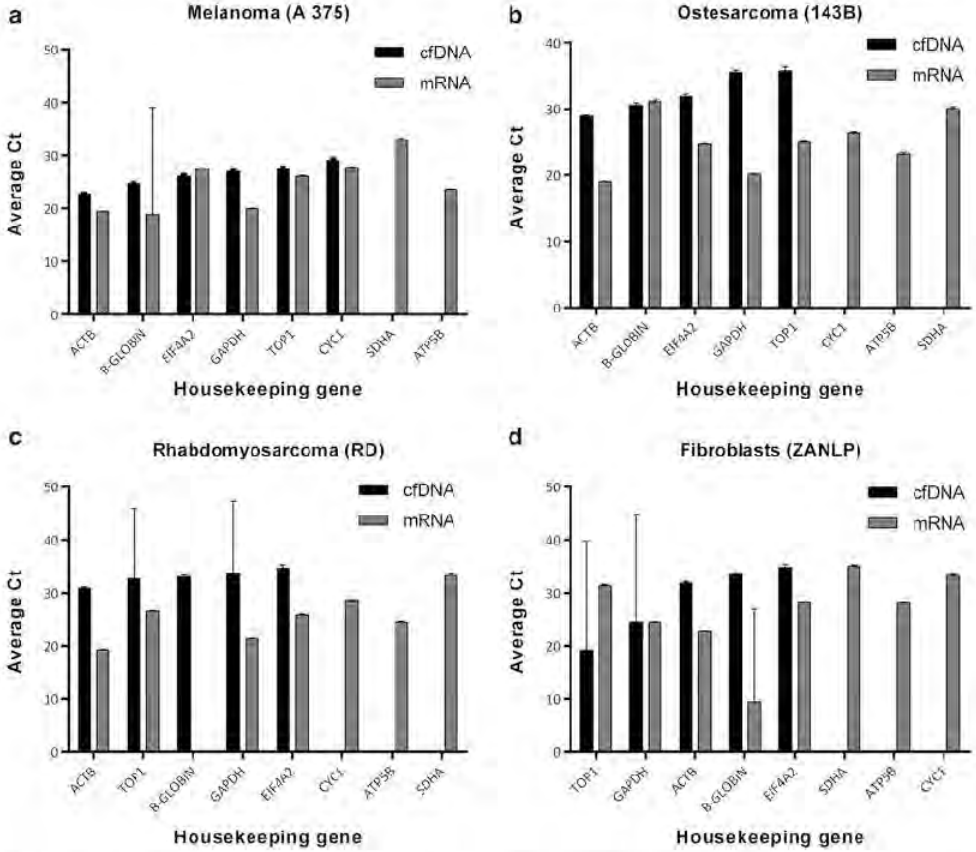

Fig. 19.1 Average Ct values of housekeeping genes that occur in the cfDNA and mRNA of the four cell lines. The cirDNA concentrations used remained constant in all four cell lines. The concentrations of cfDNA used represent the total yield of cfDNA for each cell line. Low Ct values indicate higher levels of initial PCR template and earlier cycle amplification

maternal- and fetal-derived DNA (Yang et al. 2015). The remaining housekeeping genes showed considerable variability in some cell lines and evaluating their use as controls beforehand is strongly recommended. There was little variation in the occurrence of housekeeping genes between the cfDNA of cancerous cell lines and the healthy fibroblasts. Both the cfDNA of the cancerous cell lines (with the exception of A375) and healthy fibroblasts did not release housekeeping genes related to cellular energy metabolism (SDHA, ATP5B and CYC1), but mRNA one results did show that they were expressed. The analysis of control gene stability in cfDNA samples rather than genomic DNA for qPCR will promote the optimization of cfDNA quantification.

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Conflict of Interest The authors declare no conflict of interest.

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Article XI

An Enquiry Concerning the Characteristics of Cell-Free DNA Released by Cultured Cancer Cells

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4 An Enquiry Concerning the Characteristics of Cell-Free DNA Released by Cultured Cancer Cells

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Abstract

Non-invasive screening that utilizes cell-free DNA (cfDNA) offers remarkable potential as a method for the early detection of genetic disorders and a wide variety of cancers. Unfortunately, one of the most prominent elements delaying the translation of cfDNA analyses to clinical practice is the lack of knowledge regarding its origin and composition. The elucidation of the origin of cfDNA is complicated by the apparently arbitrary variability of quantitative and qualitative characteristics of cfDNA in the blood of healthy as well as diseased individuals. These factors may contribute to false positive/negative results when applied to clinical pathology. Although many have acknowledged that this is a major problem, few have addressed it. We believe that many of the current difficulties encountered in *in vivo* cfDNA studies can be partially circumvented by *in vitro* models. The results obtained in this study indicate that the release of cfDNA from 143B cells is not a consequence of apoptosis, necrosis or a product of DNA replication, but primarily the result of actively released DNA, perhaps in association with a protein complex. Moreover, this study demonstrates the potential of *in vitro* cell culture models to obtain useful information about the phenomenon of cfDNA.

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Keywords
Cell-free DNA (cfDNA) • Apoptosis • Necrosis • Osteosarcoma • Flow cytometry

Introduction

Understanding the processes involved in the generation of cell-free DNA (cfDNA) is critical for deducing its role in biology and pathology, while advancing the translation of analyses to clinical practice. However, the origin of cfDNA still remains elusive despite the seemingly universality of it in bio-fluids. Several sources of cfDNA have been excluded including, exogenous DNA (bacterial, viral and parasitic), lysis of cells on the interface between a tumour and circulation (Sorenson 1997) and the destruction of tumour micrometastases and circulating cancer cells (Bevilacqua et al. 1998). Currently, the only remaining conceivable sources that may account for the occurrence of cfDNA are apoptosis, necrosis, or active cellular secretion (Stroun et al. 2001).

Although most evidence suggests that the release of cfDNA is mainly a consequence of apoptosis, many studies have indicated that a significant fraction of cfDNA is derived from active cellular secretions (van der Vaart and Pretorius 2008), where newly synthesized DNA in association with a lipid-protein complex is released in a homeostatic manner (Anker et al. 1975; Borenstein and Ephrati-Elizur 1969; Stroun and Anker 1972; Stroun et al. 1977, 1978). The aim of this work was to examine the release and composition of cfDNA from cultured human osteosarcoma cells (143B). The release pattern of cfDNA was characterized over time, and the sizes of the cfDNA fragments evaluated at each of these intervals. Additionally, apoptotic, necrotic and the cell cycle profiles were also investigated using flow cytometry.

Materials and Methods

Cell Culturing, Extraction and Quantification of Cellular Protein and Cell-Free DNA

The human osteosarcoma cell line 143B was obtained from the American Type Culture Collection (ATCC® CRL- 8303TM). Cells were cultured as prescribed by ATCC. Total cellular protein was isolated and quantified using the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies). cfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the manufacturer's PCR clean-up instructions and quantified using real-time quantitative PCR assay for the β-globin gene.

Fragment Size Evaluation of Cell-Free DNA

The size of cfDNA extracted at the different time intervals were analysed by capillary electrophoresis using the High Sensitivity DNA kit and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. The assay was performed according to the instructions provided by the manufacturer.

Flow Cytometric Assays

The BD Annexin V FITC assay (BD Biosciences) was utilized to determine the apoptotic/necrotic profile of 143B cells. An

APO-BrdU™ Tunel assay kit (Molecular Probes, Invitrogen) was used for the detection of DNA fragments as recommended by the manufacturer. For cell cycle analysis the Click-iT® Edu Alexa Fluor® 488 Cell Proliferation Kit (Molecular Probes, Invitrogen) was utilized according to the instructions of the manufacturer. All flow cytometric analyses were done on a FACSVerseTM bench top flow cytometer. Events were acquired on BD FACSuite™ software (Becton & Dickson, Mountain view, CA, USA).

Results and Discussion

In this study, the release of cfDNA from cultured cells was evaluated in order to gauge its potential use for elucidating the nature of cfDNA. Release of cfDNA from 143B cultured cells was characterized over time (Fig. 4.1a, b).

After growth medium renewal, the amount of cfDNA increased incrementally, notably peaked after 24 h, and plateaued at a much lower level thereafter. Since the amount of cells increase over time, this is not surprising. However, when the values were normalized in terms of total cellular protein the tendency did not change, suggesting that more cfDNA is released per cell in a time dependent manner (Fig. 4.1d, c). This suggested that the cfDNA is neither from apoptotic nor necrotic origin but originates from active release. To examine the size of cfDNA isolated at the different time points, samples were subject to capillary electrophoresis (Fig. 4.2). After 4 h of incubation a prominent peak at 166 bp, however, this peak dissipated incrementally and disappeared after 24 h when a new peak of approximately 2000 bp dominated the scene. After 40 h of incubation, the cfDNA resembles multiples of nucleosomal repeats, with small populations forming at approximately 160, 340 and 540 bp,

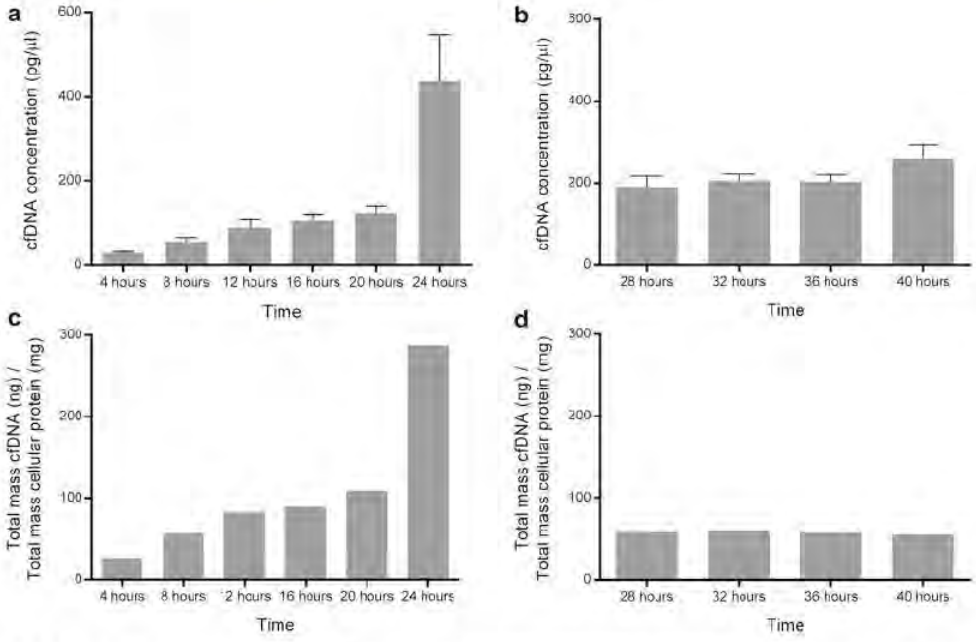

Fig. 4.1 Time-course characteristics of cfDNA released from 143 B cells. (**a**) Bar graph showing the amount of cfDNA released by 143 B cells after 4–24 h of incubation following medium renewal. (**b**) Bar graph showing the amount of cfDNA released by 143B cells after 28–40 h of incubation following medium renewal. (**c**) & (**d**) Represents the amount of cfDNA released at each time-point normalized in terms of total cellular protein

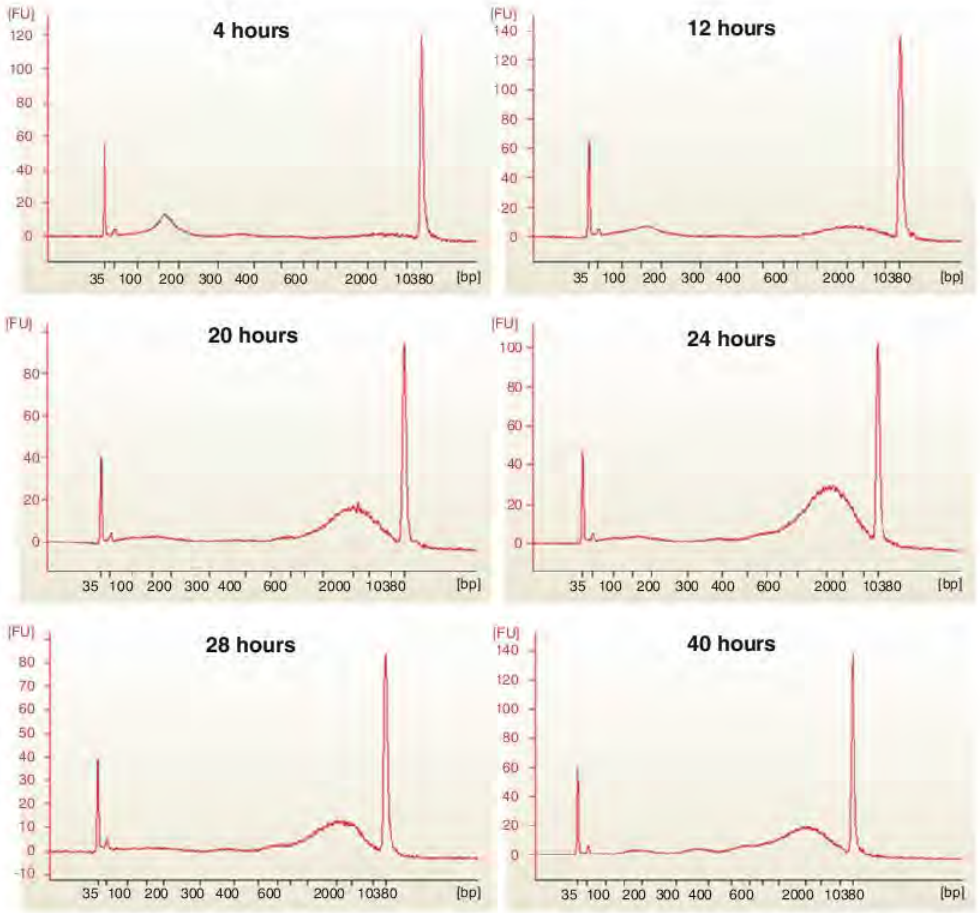

Fig. 4.2 Capillary electropherograms showing the size of cfDNA isolated after incubation at various times following medium renewal. In each electropherogram two major peaks can be seen, one at 35 bp and one at approximately 10 000 bp. These peaks correspond to the two size markers. The relative fluorescence of these markers is then used to calculate the size of the unknown samples. Thus, any deviation from the baseline, excluding the markers, indicates the size of cfDNA

suggesting an apoptotic origin. These observations demonstrate a clear correlation between an increase in the release of cfDNA and the occurrence of higher molecular weight DNA. As far as we know, this distinct size of ~2000 bp has not yet been reported. This is noteworthy, because its size suggests that it is neither from apoptotic nor necrotic origin.

To verify the observations made by electrophoretic analysis, and to help elucidate the origin of the cfDNA present at the different time points, the cells at each of the times correlating with the time-course study was analyzed for apoptosis, necrosis and cell cycle phase using flow cytometric assays. The FITC Annexin V assay revealed that a fraction of cells are apoptotic after 4 h, whereas 24 h showed almost no apoptosis or necrosis (Fig. 4.3).

Excluding the 4 h time point, these results were corroborated by the TUNEL assay, which is based on the detection of fragmented DNA (results not shown). The assay used to measure

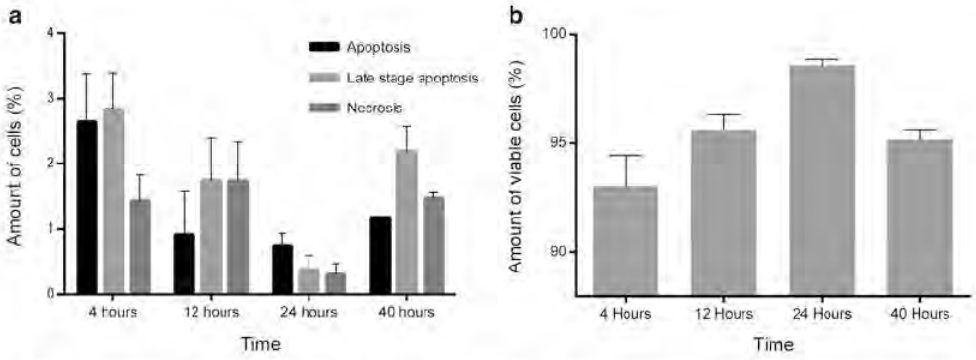

Fig. 4.3 Summary of data obtained by the FITC Annexin V flow cytometric assays. (**a**) illustrates the percentage of apoptotic, necrotic, and late-stage apoptotic 143 B cells at the different time-points. (**b**) illustrates the percentage of viable 143 B cells at each time-point. *Error bars* indicate standard deviation

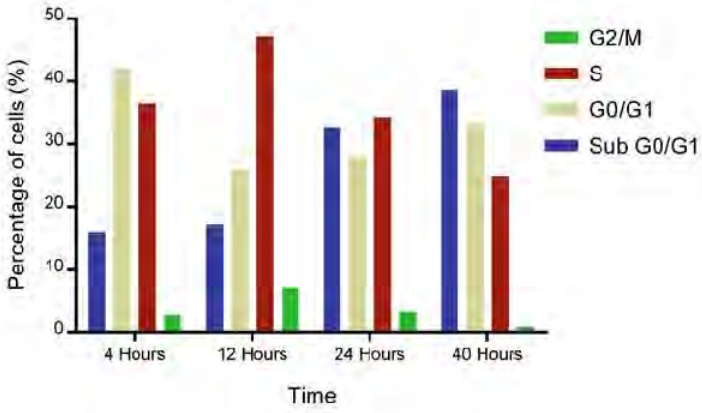

Fig. 4.4 Bar graph representing the cell cycle distribution of 143 B cells at different points in time

cell proliferation revealed that, after 24 h of growth following medium renewal, there is a decline of cells in the S phase and significant increase of cells in the sub G0/G1 phase (Fig. 4.4). This indicates that the abrupt increase of cfDNA after 24 h of incubation is not associated with the process of DNA replication.

To determine whether the cfDNA released from 143 B cells after 24 h of incubation could be similar to virtosomes, growth medium was treated with denaturing agents prior to cfDNA extraction, and were compared to untreated samples (results not shown). In all cases, the concentration of cfDNA was increased considerably by the addition of denaturing agents. These results suggest that cfDNA may be associated with proteins but whether these proteins are simply nucleosomes, lipid-protein complexes (virtosomes) or extracellular vesicles remains unclear, and requires further examination.

Conclusion

Most *in vivo* studies report that the occurrence of cfDNA is associated with apoptosis or necrosis. However, the results obtained by this study suggest that the release of cfDNA from cultured 143B cells after 24 h of incubation is not a consequence of apoptosis, necrosis or a product of DNA replication, but primarily a result of actively

released DNA, perhaps in association with a protein complex. These results also demonstrate the potential of *in vitro* cfDNA analysis to aid in the elucidation of the nature of cfDNA.

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Conflict of Interest The authors wish to declare no conflict of interest.

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Scientific Poster I

Cell-free DNA is actively released by cultured cancer cells

Abel Jacobus Bronkhorst, Johannes F. Wentzel, Lissinda H. du Plessis, Piet J. Pretorius

Presented at:

The 7th European Molecular Biology organization (EMBO) meeting, Mannheim, Germany, 10 to 13 September 2016.

Cell-free DNA is actively released by cultured cancer cells

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Introduction

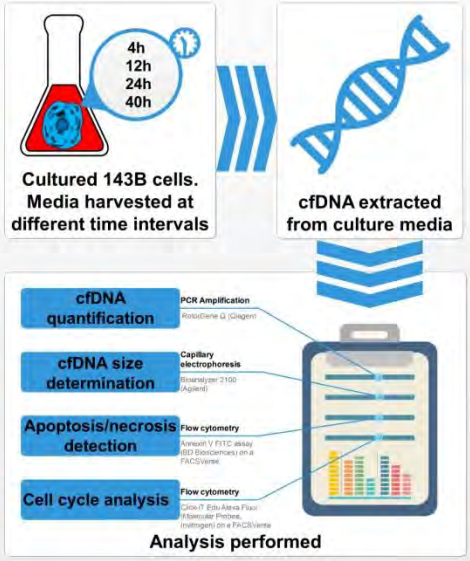
Short fragments of cell-free DNA (cfDNA) are found in human blood and can be used as potential biomarkers for non-invasive screenings such as prenatal sex-determination and detection of various pathologies[1-2]. cfDNA holds particular promise in cancer screening as cancer patients generally present with elevated levels of cfDNA in their blood[3].

Understanding the processes involved in the generation of cfDNA is critical for deducing its role in biology and pathology, and ultimately applying it in a clinical setting. However, this basic knowledge is lacking, and the origin of cfDNA is still elusive.

The aims of this study were to assess the release and composition of cfDNA from cultured human osteosarcoma cells (143B) and to gauge the potential use of *in vitro* models in assisting in the elucidation of the nature of cfDNA.

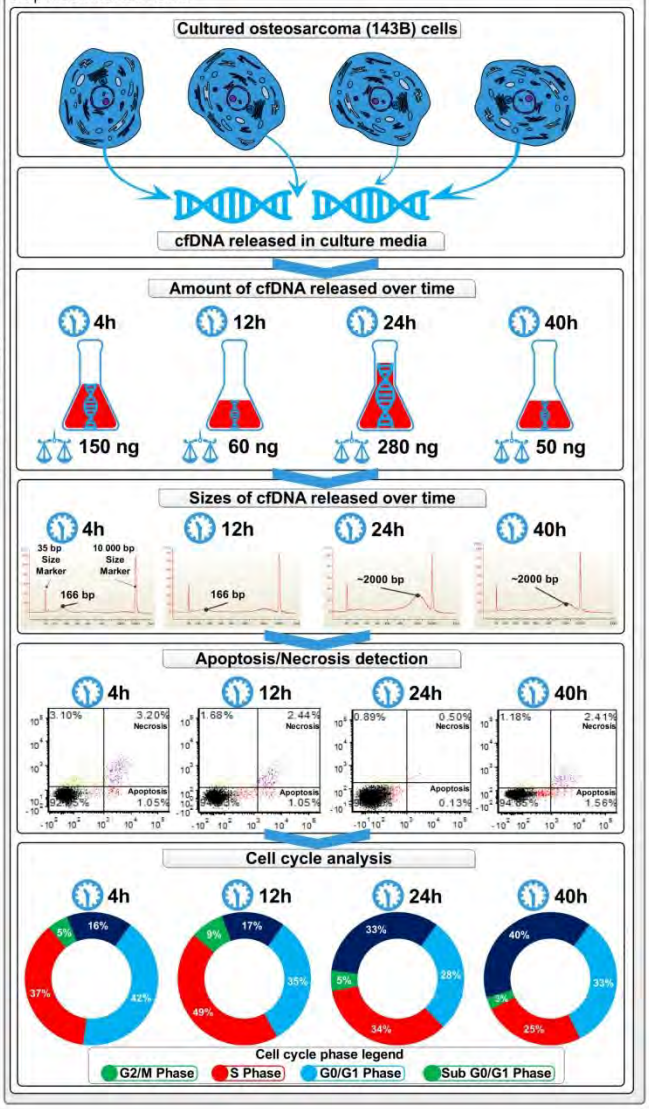
Methods

In this study, the release pattern of cfDNA from cultured 143B cells was examined over time, and the sizes of the cfDNA fragments at each of these intervals were evaluated. Apoptosis, necrosis and cell cycle profiles were also investigated at the corresponding time intervals using flow cytometry.



Results

After 4 hours of incubation a prominent cfDNA population of 166 bp was observed. This peak diminished and disappeared after 24 hours and was replaced by a cfDNA population of ~2000 bp. Flow cytometry revealed that a small fraction of cells are apoptotic after 4 hours, whereas 24 hours showed almost no apoptotic or necrotic populations. Cell cycle analysis showed a shift from the S to the sub G0/G1 phase after 24 hours.



Conclusion

After 4 hours, a prominent cfDNA population of 166 bp was observed, suggesting an apoptotic origin. However, this population diminished and disappeared after 24 hours and was replaced by a cfDNA population of ~2000 bp. Flow cytometry revealed that a small fraction of cells are apoptotic after 4 hours, whereas 24 hours showed almost no apoptotic or necrotic populations[4]. Cell cycle analysis showed a shift from the S to the sub G0/G1 phase after 24 hours, suggesting that the increase of cfDNA is not associated with DNA replication. These results suggest that the release of cfDNA from cultured 143B cells after 24 hours is not a consequence of apoptosis, necrosis or a product of DNA replication, but a result of actively released DNA. This suggests that the occurrence of cfDNA is a result of actively released DNA, signifying a specific biological function that still eludes us.

Presented at: the 'in' EMBO meeting 2016 Mannheim 10-13 September

References: [1] Sci Transl Med. 2010 Dec 8;2(61):61ra91; [2] JAMA. 2011 Aug 10;306(6):627-36; [3] Mol Diagn Ther. 2015 Dec;19(6):339-50; [4] BBA. 2016 Jan;1863(1):157-65

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Scientific Poster II

Molecular Characterization and Profiling of the DNA released by Cultured Cancer Cells using Massively Parallel Semiconductor Sequencing

Abel Jacobus Bronkhorst, Johannes F. Wentzel, Piet J. Pretorius

Presented at:

The 7th European Molecular Biology organization (EMBO) meeting, Mannheim, Germany, 10 to 13 September 2016.

Molecular Characterization and Profiling of the DNA released by Cultured Cancer Cells using Massively Parallel Semiconductor Sequencing

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Background

Since the discovery of cell-free DNA (cfDNA) in blood, these molecules have been extensively studied as potential biomarkers for non-invasive disease screening [1]. Furthermore, numerous studies have shown that cfDNA can enter target cells and genetically or epigenetically alter their biology [2], indicating a capacity for various intercellular messaging functions and a possible role in evolution [3] (**Fig. 1**.).

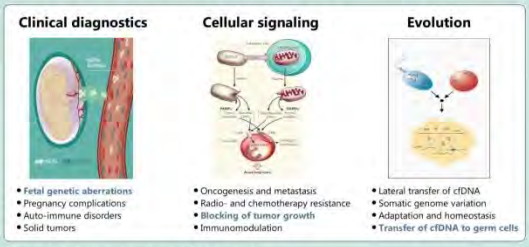


Figure 1: The biological significance of cell-free DNA

However, the development of comprehensive clinical tests utilizing cfDNA, as well as gauging its role in biology, requires a firm understanding of its biological properties, but this is largely lacking. Detailed sequence analysis may provide crucial knowledge; however, the establishment of a consensus on sequence information is deterred by the arbitrary fluctuation of its characteristics *in vivo*, wherein the cfDNA population in the blood of an individual at any given time is a mixture derived from different cells, tissues and organs, and its release dependent on various environmental cues.

The aim of this work was to explore the prospect that the difficulties encountered in *in vivo* studies can be overcome by *in vitro* cell culture models

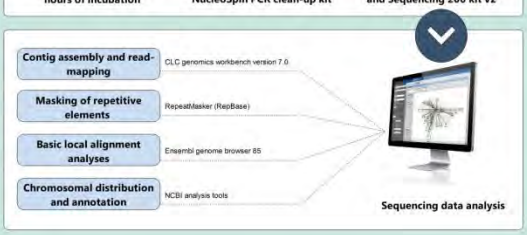
Materials and methods

In a previous study [4], we have demonstrated that the cfDNA present in the growth medium of cultured 143B osteosarcoma cells after 24 hours of incubation is primarily a product of active release mechanisms, and not a consequence of cellular demise. In this study we have sequenced and characterized this DNA using massively parallel semiconductor sequencing. Experimental details are outlined below.

Cultured 143B cells. Growth medium harvested after 24 hours of incubation

Extraction of cfDNA from growth medium using a NucleoSpin PCR clean-up kit

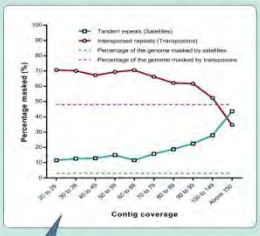
DNA sequencing on the Ion torrent PGM using a 318v2 chip and Sequencing 200 kit v2



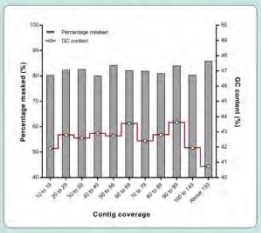
Results

- Satellite DNA and transposable elements are significantly overrepresented.
- Regarding SINEs and LINES, the level of occurrence corresponds with transposition activity. Active transposons are overrepresented and inactive transposons are underrepresented.
- Although thought to be inactive, MaLR and DNA/TcMar-Tigger elements are overrepresented, which may relate to tissue specific copy number/activity variations.
- BLAST analysis showed that nearly half of the unique sequences still originate from the centromeres, while the rest originates from one gene of which only a very small percentage are comprised of protein coding sequences. Furthermore, these sequences originate from specific chromosomes.

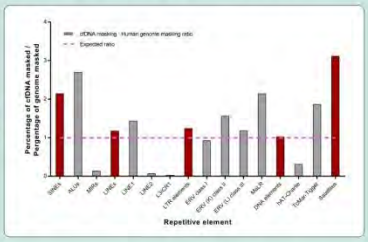
Overrepresentation of Satellite DNA and Transposable elements in cfDNA



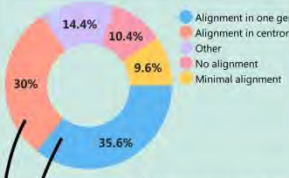
Overrepresentation of repeat elements is not a result of sequencing bias



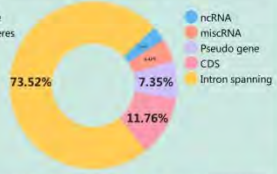
Overrepresentation of satellite DNA, active SINE and LINE elements, and underrepresentation of elements that have no transposition activity



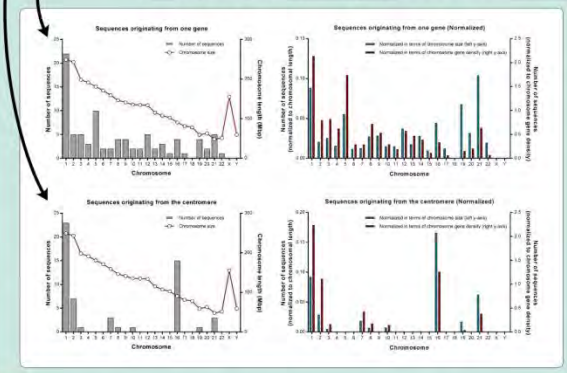
BLAST analyses of 250 masked cfDNA sequences



Annotation of cfDNA sequences originating from one gene



Overrepresentation of specific chromosomes for sequences originating from both one gene and the centromeres



Conclusion

Results indicate that cultured 143B cells actively and purposely release satellite DNA and transposable elements into the extracellular environment, a phenomenon heretofore unknown to occur in eukaryotic cells. Furthermore, this study exemplifies the capacity of *in vitro* models to fill gaps in our understanding of the phenomenon of cfDNA, and to partially circumvent the difficulties encountered in *in vivo* experiments

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[2] Cell Biochem Funct 2010;28:529-38.
[3] Epigenomics 2015;7:1165-71.
[4] BBA mol cell res 2016;1863:157-65.

Appendix II: Data article

Article XII

Adjustments to the preanalytical phase of quantitative cell-free DNA analysis

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Data Article

Adjustments to the preanalytical phase of quantitative cell-free DNA analysis

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ABSTRACT

Evaluating the kinetics of cell-free DNA (cfDNA) in the blood of cancer patients could be a strong auxiliary component to the molecular characterization of cfDNA, but its potential clinical significance is obscured by the absence of an analytical consensus. To utilize quantitative cfDNA assessment with confidence, it is crucial that the preanalytical phase is standardized. In a previous publication, several preanalytical variables that may affect quantitative measurements of cfDNA were identified, and the most confounding variables were assessed further using the growth medium of cultured cancer cells as a source of cfDNA ("Cell-free DNA: Preanalytical variables" [1]). The data accompanying this report relates to these experiments, which includes numerous changes to the sample handling and isolation protocols, and can be used for the interpretation of these results and other similar experiments by different researchers.

Specifications table

Subject area	Biochemistry, molecular biology
More specific subject area	Clinical biochemistry, translational oncology, prenatal diagnostics

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http://dx.doi.org/10.1016/j.dib.2015.12.009

Type of data	Excel spreadsheet, table
How data was acquired	PCR amplification of cell-free DNA was measured using a real-time quantitative assay for the β-globin gene. All assays were performed on a Rotor-Gene Q detection system (Qiagen) using a 72 well ring-setup.
Data format	Analyzed
Experimental factors	Centrifugation, medium storage temperature, medium thawing temperature, medium storage tube type, treatment with denaturing agents, combining snap freezing with proteinase K, binding buffer type, elution volume, elution regime and elution tube type.
Experimental features	Cell-free DNA was extracted directly from growth medium collected from 143B osteosarcoma cells in culture, and then quantified by real-time PCR. Several variations to the standard procedure were evaluated.
Data source location	South Africa
Data accessibility	The data is with this article

Value of the data

- This data will be useful considerations when optimizing protocols and setting up a standard operating procedure, which should expedite the translation of cfDNA analyses to clinical practice.
- This data could be compared to other studies that investigated the effect of methodological variables on quantitative measurements of cfDNA.
- This data could be used to interpret studies that investigated the effect of methodological variables on qualitative measurements of cfDNA.

1. Data

In order to investigate the effects of several adjustments to the preanalytical phase of quantitative cfDNA measurements, the growth medium of cultured cancer cells was used as a source of cfDNA. The data in this report was obtained by amplifying cfDNA with real-time PCR, after it had been extracted under different preanalytical conditions. The data is presented in a supplementary file as a single table, which includes several quantitative measurements of cfDNA following modifications to the standard protocol followed. These changes are described in Table 1.

2. Experimental design, materials and methods

2.1. Cell culturing

Culture medium of the human bone cancer (osteosarcoma) cell line 143B (ATCC® CRL-8303™) was used as a source of cfDNA. Given that DNA levels in growth medium fluctuate much like cfDNA in the blood of humans, we could use it as a model to evaluate the effect of different variables on both high and low concentrations of cfDNA. Cells were cultured in T75 flasks in Dulbecco's Modified Eagle's medium (DMEM) (HyClone; SH30243.01) supplemented with 10% fetal bovine serum (Biochrom; S0615) and 1% penicillin/streptomycin (Lonza; DE17-602E) at 37 °C in humidified air and 5% CO_2. After the cells have reached the necessary confluency, the culture medium was removed, processed and stored at −80 °C in 15 ml tubes.

Table 1
Modifications to standard procedure.

Modifications to sample handling	
Modification to standard procedure	**Description**
Centrifugation regime	Growth medium was centrifuged for 10 min at different forces (1000, 5000, 10 000 and 20,000 × g). Other samples were subject to two rounds of centrifugation, first at 1000 × g and then transferred to new tubes before the next centrifugation at 5000, 10,000 and 20,000 × g, respectively. After centrifugation all samples were transferred to new tubes.
Growth medium storage temperature	After centrifugation, growth medium was transferred to fresh tubes and stored until cfDNA was extracted. Three storage schemes were tested: −20 °C, −80 °C, and snap-freezing in liquid nitrogen followed by storage at −80 °C.
Growth medium thawing temperature	Prior to cfDNA extraction, the growth medium is thawed. Two approaches were tested: thawing of growth medium at room temperature, and at 37 °C in a temperature controlled water bath for 5 min.
Growth medium storage tube type	After collection and processing, growth medium was stored in three different tubes: 15 mL nuclease free tubes (Ambion), regular 1.5 mL tubes (Eppendorf), and DNA LoBind tubes (Eppendorf).
Modifications to cfDNA extraction protocol	
Treatment with denaturing agents	Prior to cfDNA extraction, growth medium was treated with SDS (0.05%), proteinase K (1.5 mg/mL), and a combination of the two for 30 min at 50 °C, respectively. In the cases where SDS was used, buffer NTB was used instead of buffer NTI. As the kit makes no suggestions regarding the use of proteinase K, buffer NTI was used in this case.
Effect of combining snap freezing with proteinase K	Four different scenarios were compared: (1) cfDNA was extracted from growth medium directly after collection, (2) Growth medium was treated with proteinase K immediately after collection, followed by cfDNA extraction, (3) Growth medium was snap frozen before cfDNA extraction, and (4) Growth medium was snap frozen and then thawed and treated with proteinase K prior to extraction.
Binding buffer type	After thawing, growth medium is mixed with binding buffer before it is added to the spin column. Here, we compared two binding buffers, NTI and NTB. In the case of buffer NTB, the ratio of sample to buffer is 1:5. In the case of extractions where buffer NTI is used, the sample to buffer ratio is only 1:2.
Elution volume	CfDNA was extracted and eluted into 20 μL, 40 μL, 60 μL, and 100 μL of elution buffer, respectively.
Elution regime	CfDNA was extracted and eluted into 20 μL of elution buffer and repeated twice more to have a final volume of 60 μL. This was followed by the elution of DNA into 30 μL elution buffer and repeated once more to achieve a final volume of 60 μL. The former was compared to DNA eluted into 60 μL of elution buffer once.
Elution tube type	To examine the loss of eluted DNA due to adsorption to tube walls, regular 1.5 mL tubes (Eppendorf) were compared with 1.5 mL DNA LoBind tubes (Eppendorf)
Comparing different protocols	
Non-optimized	Media was collected and centrifuged at 1000 × g and transferred to fresh 1.5 mL Eppendorf DNA LoBind tubes and stored at −20 °C. Before extraction, the medium was thawed at room temperature, and no denaturing agent was added thereafter. CfDNA was then extracted and eluted into 20 μL of elution buffer in one step. Samples were stored in 1.5 mL DNA LoBind tubes (Eppendorf)
Optimized	Media was collected and centrifuged at 10,000 × g and transferred to fresh 15 mL tubes (Ambion). The media was then snap-frozen in liquid nitrogen and stored at −80 °C. The samples were then thawed at 37 °C, and incubated with proteinase K (1.5 mg/mL) for 30 min at 37 °C. CfDNA was extracted and eluted into 60 μL of elution buffer in three steps (3 × 20 μL) into regular 1.5 mL tubes (Eppendorf)
QIAmp DSP virus kit	CfDNA was extracted according to the instructions provided by the manufacturer.
Increasing the yield of cfDNA	
Effect of media evaporation	For each replicate, 6 mL of growth medium was aliquot into 2 mL tubes and evaporated in a SpeedVac to a total volume of 2.5 mL

2.2. Extraction of cfDNA

CfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; 1502/001), according to the instructions described by the PCR clean-up user manual. Briefly, samples were removed from the −80 °C freezer and thawed at 37 °C and then vortexed and centrifuged briefly. For each biological replicate, cfDNA was extracted in triplicate. For every sample, 600 μL of growth medium was mixed with 1200 μL of binding buffer. Samples were then vortexed, the entire volume of growth media was added to the spin column in small regiments, and centrifuged at 11 000 × g for 1 min at room temperature. The columns were then washed twice, followed by the elution of cfDNA into 20 μL of elution buffer.

2.3. Quantification of cell-free DNA

PCR amplification of cfDNA was measured using a real-time quantitative assay for the β-globin gene. All assays were performed on a Rotor-Gene Q detection system (Qiagen) using a 72 well ring-setup. The reaction mixture consisted of 2 μL DNA and 23 μL master mix, which was composed of 8.1 μL H2O, 12.5 μL TaqMan Universal MasterMix (Life technologies; 1502032), 0.4 μL of 10 μM dual fluorescent probe 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3′, and 1 μL of 10 μM forward and reverse primers, respectively. The primers used were: F1, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, and R1, 5′-CCT TGA TAC CAA CCT GCC CAG-3′. These probe and primers were synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific). PCR conditions were set to: 95 °C for 10 min, followed by 45 cycles of 15 s denaturation at 95 °C, 1 min annealing at 60 °C, followed by 30 s extension at 72 °C. Sequence data of β-globin is attainable from GenBank (accession number: U01317). The absolute concentration of the target gene was calculated by using a standard curve. In this study, a standard curve was generated using five-fold serial dilutions of genomic DNA (50,000, 5000, 500, 50 and 5 pg/μL). Each biological replicate was quantified in duplicate, and triplicates of the standard curve were included in each run (only assays with R^2 values > 0.99 for the standard curve were used).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dib.2015.12.009.

References

[1] A.J. Bronkhorst, J. Aucamp, P.J. Pretorius, Cell-free DNA: preanalytical variables, Clin. Chim. Acta 450 (2015) 243–253.

Appendix III: List of publications and scientific posters

Peer reviewed publications

1. J Aucamp, AJ Bronkhorst, CPS Badenhorst, PJ Pretorius. A historical and evolutionary perspective on the biological significance of circulating DNA and extracellular vesicles. *Cellular and Molecular Life Sciences* (2016), Volume 73, pp 4355-4381. (**Article I**)

2. AJ Bronkhorst, J Aucamp, PJ Pretorius. Cell-free DNA: Preanalytical variables, *Clinica Chimica Acta* (2015), Volume 450, pp 243-253. (**Article III**)

3. AJ Bronkhorst, J Aucamp, JF Wentzel, PJ Pretorius. Reference gene selection for *in vitro* cell-free DNA analysis and gene expression profiling, *Clinical biochemistry* (2016), Volume 49, pp 606-608. (**Article IV**)

4. AJ Bronkhorst, JF Wentzel, J Aucamp, E van Dyk, LH du Plessis, PJ Pretorius. Characterization of the cell-free DNA released by cultured cancer cells, *Biochimica et Biophysica Acta- Molecular Cell Research* (2015), Volume 1863, pp 157-165. (**Article V**)

5. J Aucamp, AJ Bronkhorst, DL Peters, HC Van Dyk, FH Van der Westhuizen, PJ Pretorius. Kinetic analysis, size profiling and bioenergetic association of DNA released by selected cell lines *in vitro*, *Cellular and Molecular Life Sciences (Issue not yet assigned).* (**Article VI**)

6. AJ Bronkhorst, J Aucamp, PJ Pretorius. Adjustments to the preanalytical phase of quantitative cell-free DNA analysis, *Data in Brief* (2016), Volume 6, pp 326-329. (**Article XII**)

7. JF Wentzel, A Lewies, AJ Bronkhorst, E van Dyk, LH du Plessis, PJ Pretorius. Exposure to high levels of fumarate and succinate leads to apoptotic cytotoxicity and altered global DNA methylation profiles *in vitro*, *Biochimie* (2017), Volume 135, pp 29-34. *

Submitted manuscripts

1. J Aucamp, AJ Bronkhorst, CPS Badenhorst, PJ Pretorius. The diverse origins of circulating DNA in the human body: A Critical re-evaluation of the literature (*Submitted to Biological Reviews*). (**Article II**)

2. AJ Bronkhorst, JF Wentzel, DL Peters, J Aucamp, E van Dyk, EP de Villiers, PJ Pretorius. Alpha-satellite DNA and active transposable elements are spontaneously released by bone osteosarcoma (143B) cells *in vitro* (*Submitted to Clinica Chimica Acta*). (**Article VII**)

3. J Aucamp, HC Van Dyk, AJ Bronkhorst, PJ Pretorius. Lateral transfer of pharmaceutically-induced effects *in vitro*: Clinical implications of the putative messaging functions of cell-free DNA (*Submitted to Biochimie*). *

4. J Aucamp, AJ Bronkhorst, PJ Pretorius. Pharmaceutically-induced epigenetic changes of cell-free DNA *in vitro* and the risk of clinical implications *in vivo* (*Submitted to Biochimie*).*

5. J Aucamp, C Calitz, AJ Bronkhorst, K Wrzesinski, C Gouws, PJ Pretorius. Cell-free DNA in a three-dimensional spheroid cell culture model: A preliminary study (*Submitted to International Journal of Biochemistry & Cell Biology*).*

Published conference proceedings

1. J Aucamp, AJ Bronkhorst, PJ Pretorius. A Historical and Evolutionary Perspective on Circulating Nucleic Acids and Extracellular Vesicles: Circulating Nucleic Acids as Homeostatic Genetic Entities, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 91-95. (**Article VIII**)

2. AJ Bronkhorst, J Aucamp, PJ Pretorius. Methodological Variables in the Analysis of Cell-Free DNA, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 157-163. (**Article IX**)

3. J Aucamp, AJ Bronkhorst, JF Wentzel, PJ Pretorius. A Quantitative Assessment of Cell-Free DNA Utilizing Several Housekeeping Genes: Measurements from Four Different Cell Line, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 101-103. (**Article X**)

4. AJ Bronkhorst, JF Wentzel, J Aucamp, E van Dyk, LH du Plessis, PJ Pretorius. An Enquiry Concerning the Characteristics of Cell-Free DNA Released by Cultured Cancer Cells, *Advances in Experimental Medicine and Biology* (2016), Volume 924, pp 19-24. (**Article XI**)

Scientific posters presented at a conference

Presented at the 7th European Molecular Biology Organization (EMBO) meeting, which was held in Mannheim, Germany on 10-13 September 2016:

1. AJ Bronkhorst, JF Wentzel, LH du Plessis, PJ Pretorius. Cell-free DNA is actively released by cultured cancer cells. (**Poster I**)

2. AJ Bronkhorst, JF Wentzel, PJ Pretorius. Molecular characterization and profiling of the DNA released by cultured cancer cells using massively parallel semiconductor sequencing. (**Poster II**)

* *Published or submitted papers that share points of contact with this study, but were not included in the thesis.*

Appendix IV: List of figures

Chapter 2, Article I

Chapter 2, Article II

Chapter 3, Article III

Chapter 3, Article IV

Chapter 4, Article V

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Chapter 5, Article VII

Appendix I, Article VIII

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Chapter 4, Article VI

Appendix II, Article XII

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