Transmission of clinical risk factors through circulating DNA

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MSc Pharmaceutical Chemistry

Thesis submitted for the degree *Philosophiae Doctor* in Biochemistry at the Potchefstroom Campus of the North-West University

Promoter: Prof PJ Pretorius

Graduation October 2017

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PREFACE

Before you lies the thesis “Transmission of clinical risk factors through circulating DNA” wherein the utilisation of cell culture models in circulating DNA research is substantiated and the possibility of circulating DNA to transfer clinical risk factors during blood transfusion, in the form of pharmaceutically-induced biochemical and/or epigenetic information, is investigated. It has been written in article format to fulfil the graduation requirements for the degree Doctor of Philosophy in Biochemistry at the Potchefstroom Campus of the North-West University.

Eight articles, of which seven have been published and one has recently been submitted for publication, are provided in this thesis. These articles consist of two comprehensive review articles (paragraphs 2.2 and 3.2), two conference proceedings publications (paragraphs 3.4 and 4.1.4), one short communication article (paragraphs 4.1.2) and three regular research papers (paragraphs 4.3.6, 5.2.2 and 6.3). Information regarding the name of the journal, article submission status and author guidelines are provided in each mentioned paragraph and preceding paragraph. Documentation regarding permission from the journals to add the articles in a thesis is provided in Annexure B.

The PhD candidate is responsible for all parts of this thesis, including experimental designs, data collection and processing, result interpretation and the writing of the manuscripts. The supervisor, co-supervisor and collaborators who provided additional input (including cell culture screenings, bioenergetic analysis, three-dimensional cell cultures, natural product experiments and the critical review of article manuscripts) were included as co-authors.

Author contributions

Janine Aucamp:
Main author responsible for proposal of study, study protocol, methodology, data collection and processing, designing, planning and writing of articles, article figures and thesis and speaker or presenter of conference-related content.

Abel Bronkhorst and Dimetrie Peters:
Co-authors who assisted in the screening of cell lines, PCR-related assays, the writing and figure production of relevant content and the critical evaluation of the article content.

Dr Chris Badenhorst:
Co-author who assisted in the critical evaluation of review articles.

Prof Piet Pretorius:

Supervisor responsible for guidance, support and the critical evaluation of data and article content.

Hayley van Dyk:

Co-author and Seahorse XFe96 Extracellular Flux analyser technician that performed the bioenergetics analysis who assisted with the writing of bioenergetics analysis-related content of article manuscripts and the critical evaluation of article content.

Prof Francois van der Westhuizen:

Co-supervisor responsible for guidance, support, the critical evaluation of data and article content and supervisor of Hayley van Dyk with regards to bioenergetics analysis (paragraph 4.3.6).

Carlemi Calitz, Dr Chrisna Gouws and Prof Sias Hamman:

Co-authors involved in the performance of the three-dimensional cell cultures, assisted in the writing of related content and the critical evaluation of article content. Permission is given for the publication of article and its placement in paragraph 6.3. Permission is also given for the addition of the pilot study results in paragraph 6.4, but not for the publication of these results, as the experiments and natural product extracts involved form an integral part of Carlemi Calitz' PhD thesis.

All of the involved authors provided consent for the inclusion of the eight articles in this thesis (please refer to the next page).

AUTHOR STATEMENT

Herewith the co-authors verify their individual contributions and involvement in this study and grant permission for the inclusion of the relevant research articles in this thesis:

I hereby declare that my role, as stated above, in the article manuscripts related to the thesis, Transmission of clinical risk factors through circulating DNA, is representative of my contribution. The PhD student, Janine Aucamp, also has my consent to include the article manuscripts as part of the thesis.

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ACKNOWLEDGEMENTS

I would like to pay respect to the following people for their support and contributions during this study:

- My supervisor, Prof Piet Pretorius, for his excellent support and guidance during this study and for sharing his incredible wealth of knowledge.
- My co-supervisor, Prof Francois van der Westhuizen, for not only his support during this study, but for assisting me with the transfer from Pharmaceutical Chemistry to Biochemistry in 2013 by advising me to complete an honour's degree in Biochemistry to see whether I will have the skills and knowledge needed to continue my postgraduate studies in Biochemistry.
- The National Research Foundation (NRF) and North-West University for the financial support for this study. The financial assistance of the NRF towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.
- Abel Bronkhorst, Leslie Peters, Hayley van Dyk, Dr Chris Badenhorst and Dr Jaco Wentzel, I would like to thank you for your support and ideas. It was a pleasure to work as a research team and tackling challenges together.
- To my other colleagues and scientists at the Centre of Excellence for Pharmaceutical Sciences (Pharmacen), Carlemi Calitz, Dr Chrisna Gouws, Prof Sias Hamman, Dr Clarissa Willers and Sharlene Lowe, thank you for the utilisation of your cell culture laboratory and equipment, your cooperation, support and for allowing me to participate in your novel three-dimensional cell culture and natural product research.
- My family for their endearing support and encouragement.

ABSTRACT

Newly synthesised, actively released circulating DNA can translocate to neighbouring and remote parts of the body, enter cells and alter their biology. It has been implicated in the bystander effect, tumour development and metastasis, the blocking of tumour growth, and the spread of antibiotic- and chemoresistance, as well as bacterial and viral virulence. This raises the question of whether these intercellular messaging functions of circulating DNA can have clinical implications. There is little preventing the transfer of circulating DNA from donors to recipients during blood transfusions, making it possible that information contained in the circulating DNA of donors can elicit genetic, epigenetic and/or biochemical effects in recipients. Utilising both two- and three-dimensional cell cultures and valproic acid, paracetamol and natural plant products, this thesis demonstrates the transfer of pharmaceutically-induced biochemical and epigenetic effects from donor to recipient cells via cell-free DNA. This observed lateral transfer of information *in vitro* emphasises the clinical risk of similar events occurring during the transfusion of biological fluids, such as blood products. Furthermore, the thesis motivates the utilisation of cell-free DNA from the growth medium of *in vitro* cultures in conjunction with *in vivo* models. It is illustrated that restricting the cellular environment to the cell types, physiological system, organ or disease in question can significantly simplify the search for biomarkers and the elucidation of the biological functions of circulating DNA. In particular, the utilisation of three-dimensional cell culture technologies, in this case using spheroids developed via microgravity bioreactors, in circulating DNA research is introduced.

Keywords: cell-free DNA, circulating DNA, clinical implications, intercellular messenger, lateral information transfer, blood donation, valproic acid

OPSOMMING

Nuut gesintetiseerde, aktief vrygestelde sirkulerende DNA kan na naasliggende en veraf geleë dele van die liggaam getranslokeer word, deur selle opgeneem word en hul biologie affekteer. Hierdie bevindinge is betrokke by die omstander effek, tumor ontwikkeling en metastase, die blokkade van tumorgroei, en die verspreiding van antibiotika en chemoterapie weerstandigheid, asook bakteriële en virale kwaadaardigheid. Hierdie bevindinge kan moontlik impliseer dat die intersellulêre boodskapperfunksies van sirkulerende DNA kliniese nagevolge kan inhou. Die oordrag van sirkulerende DNA vanaf bloedskenkers na ontvangers word nie tydens oortappings volledig verhoed nie en vergroot effektief die moontlikheid dat die inligting van die skenker se sirkulerende DNA genetiese, epigenetiese en/of biochemiese gevolge in die ontvanger kan ontlok. Hierdie proefskrif beskryf die gebruik van beide twee- en drie-dimensionele sel kulture en valproësuur, parasetamol en natuurlike plant produkte om die oordrag van farmaseuties-geïnduseerde biochemiese en epigenetiese effekte vanaf skenker na ontvangende selle deur selvrye DNA te demonstreer. Hierdie *in vitro* laterale oordrag van inligting beklemtoon die kliniese risiko van soortgelyke gebeure tydens die oortapping van biologiese vloeistowwe, soos bloedprodukte en serebrospinale vloeistof. Boonop word die gebruik van selvrye DNA, afkomstig van die groeimedium van *in vitro* selkulture, saam met *in vivo* modelle gemotiveer. Die beperking van die aantal potensiële oorspronge van sirkulerende DNA deur die inperking van die sellulêre omgewing tot net die gesogde seltipes, fisiologiese sisteme, organe of siektetoestande kan die ontdekking van biologiese merkers en die verklaring van die biologiese funksies van sirkulerende DNA beduidend vereenvoudig. Die gebruik van drie-dimensionele selkultuur tegnologie, in hierdie geval die gebruik van sferoïde wat in mikrogravitasie bioreaktors ontwikkel word, in sirkulerende DNA navorsing word nadruklik voorgestel.

Sleutelwoorde: selvrye DNA, sirkulerende DNA, kliniese implikasies, intersellulêre boodskapper, laterale inligting oordrag, bloed skenking, valproësuur

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CHAPTER 1

INTRODUCTION

1.1 Problem statement and substantiation

Since the discovery of circulating DNA (cirDNA), DNA released into biological fluids from cells and tissues, in human plasma (Mandel & Métais, 1948) cirDNA research has been predominantly focusing on the utilisation of cirDNA as diagnostic, prognostic and theranostic non-invasive biomarkers, e.g. the utilisation of fetal cirDNA in maternal blood to screen for fetal genetic aberrations (Fan *et al.*, 2012; Lo *et al.*, 2010). The occurrence of varied cell-specific cirDNA levels with different sequences, sizes and genetic and epigenetic alterations, distinct from that of healthy individuals, in biological fluids has been associated with many benign and malignant diseases (Fleischhacker & Schmidt, 2007; Jahr *et al.*, 2001) and other physiological conditions, such as pregnancy (Lo *et al.*, 2010), exercise (Breitbach *et al.*, 2012) and aging (Jylhävä *et al.*, 2011), making them prime candidates as biomarkers. However, by predominantly focusing on the clinical application of cirDNA many critical questions regarding their origins, characteristics and purpose have become neglected. Despite the multiple studies regarding the application of cirDNA in clinical diagnostics, researchers have yet to find a normal reference value to correlate with any disease and the resulting findings are not yet routinely applied in a clinical setting. This is due to not only a lack of analytical consensus, but also due to an insufficiency of knowledge of the biological properties and molecular origin of cirDNA.

Furthermore, studies have demonstrated that cirDNA may act as intercellular messengers of sorts (Gahan & Chayen, 1965; Gahan & Stroun, 2010). CirDNA has been implicated to be laterally transferred between different cells in bacterial and viral virulence development (Avery *et al.*, 1944), oncogenesis, metastasis and the blocking of tumour growth (Bendich *et al.*, 1965; Garcia-Olmo *et al.*, 2015; García-Olmo *et al.*, 2010) and the development of resistance against antibiotics (Catlin, 1960), radiotherapy and chemotherapy (Chen *et al.*, 2014; Glebova *et al.*, 2015; Kostyuk *et al.*, 2012). However, the clinical implications of cirDNA as intercellular messengers have not yet been considered. CirDNA of donors have been identified in the blood of organ transplantation patients (Lo *et al.*, 1998; Lui *et al.*, 2002; Tong & Lo, 2006), indicating that the transfer of any biological sources, including blood transfusions, from donor to recipient will likely result in the presence of .donor cirDNA in the recipient's blood circulation. This raises concern over the potential of cirDNA to transfer genetic or epigenetic information of other clinical risk factors (genetically related illness, mutations or pharmaceutically-induced adverse effects) from one individual to another. If this is possible, then cirDNA could have severe clinical implications.

In this study particular focus is given to the potential of cirDNA transferring pharmaceutically-induced adverse effects. Many pharmaceutical and botanical compounds have been found to, for example, induce epigenetic alterations (Csoka & Szyf, 2009; Kacevska *et al.*, 2011; Kirk *et al.*, 2008). It is, therefore, possible that the cirDNA in patients using medication can contain these pharmaceutically-induced epigenetic alterations and that these alterations can be transferred to other individuals during blood transfusions or organ transplantations. The purpose of this thesis is, therefore, to illustrate that:

1. Pharmaceutical compounds can affect cirDNA characteristics,
2. The transfer of pharmaceutically-altered cirDNA from blood donors taking medication to recipients is a given fact, and
3. Transferred cirDNA can elicit biochemical and/or epigenetic effects in recipient cells.

However, *in vivo* means of investigation (blood plasma and/or serum) will not be used during the investigations. Thierry *et al.* (2016) illustrated that cirDNA can originate from multiple putative sources. Additionally, in this thesis a comprehensive compilation of putative sources of cirDNA and causes of cirDNA release is presented to illustrate the complexity of cirDNA samples obtained from biological fluid samples. This complexity of sample contents severely complicates the elucidation of cirDNA characteristics due to the development of unnecessary background noise from all of the putative origins of cfDNA release. Instead, *in vitro* methods are used instead of plasma samples. Similarly to the cells and tissues in organisms, cell cultures can release DNA into the growth medium and is termed cell-free DNA (cfDNA). The cells can be maintained in a controlled environment, can be easily exposed to stressors or medication and the amount of putative sources of cfDNA is limited to only the cells in the flask, making it easier to elucidate the origin, characteristics and functions of the cirDNA of that specific cell line.

This study will, therefore, investigate the feasibility of the utilisation of cell cultures to research the characteristics and function of cfDNA and illustrate the correlations between cfDNA from cell lines and cirDNA from plasma samples. Standard, two-dimensional (2D) cell cultures were treated with a pharmacological compound, the resulting cfDNA isolated and then administered to untreated cells in order to determine whether medication can affect cfDNA characteristics and, in turn, transfer pharmaceutically-induced effects to recipient cells. To bridge the gap between *in vitro* cell cultures and *in vivo* plasma samples even further, the utilisation of three-dimensional (3D) cell cultures, which have been shown to correlate more efficiently with *in vivo* samples than 2D cell cultures (Pampaloni *et al.*, 2007), will also be introduced to cirDNA research. Spheroids developed in microgravity bioreactors were treated with a pharmacological compound and a natural plant product commonly used in South Africa, respectively, to (i) illustrate that the cfDNA of this 3D cell culture technique can mirror spheroid response to treatment, (ii) provide cfDNA

characteristics comparable to that of plasma samples, and (iii) corroborate 2D cell culture findings that pharmaceutical compounds can affect cirDNA characteristics.

1.2 Research aims and objectives

The aim of this thesis is to utilise cell cultures to evaluate the ability of cfDNA to transport information from pharmaceutically-induced biochemical and epigenetic effects, in order to determine the clinical implications or risks of the presence of cirDNA in blood.

Objectives:

1 To provide empirically up to date reviews regarding the history and development of cirDNA research, the origins of cirDNA and the involvement of cellular breakdown and active release mechanisms. The reviews will substantiate the theory that cirDNA can serve as homeostatic genetic entities or messengers, which may have clinical implications.

2 To substantiate the utilisation of cell culture models in conjunction with *in vivo* samples for researching cirDNA characteristics and functions.

3 To optimise cell culture methods and cfDNA extraction and quantification by:
   a. Optimising cfDNA quantification methods through the identification of reference genes that are sufficiently and stably released in the cfDNA of the cell lines.
   b. Comparing the cfDNA release patterns of different cell lines and observe any changes in the sizes of the cfDNA fragments during cell proliferation that can identify the origin of the cfDNA, i.e. via cellular breakdown or active release mechanisms.
   c. Comparing the cfDNA fragment sizes of the different cell lines to that of plasma samples of other publications to further substantiate the utilisation of cell cultures as models for researching cirDNA characteristics and functions, as these models are not complicated by the vast number of putative sources from which DNA can be released into circulation.

4 To measure the metabolic activity of the different cell lines in order to determine whether there is any correlation between a cell line's metabolic activity and cfDNA release.

5 To determine whether pharmaceutically-induced changes in cfDNA release and characteristics can result in the transfer of biochemical or epigenetic information related to the treatment to untreated cells by:
   a. Using valproic acid in 2D cell cultures as an example of a pharmaceutical compound with known epigenetic and metabolic effects.
   b. Evaluating the ability of cfDNA to transfer induced changes in metabolic activity from treated to untreated cells.
   c. Evaluating the ability of cfDNA to transfer induced epigenetic changes from treated to untreated cells.

6 To introduce the utilisation of 3D cultures to further close the gap between cell cultures and *in vivo* samples.

1.3 Structure of thesis

This thesis is written in article format and consists of published articles, articles submitted for consideration for publication and conference proceedings publications. The articles were inserted in the text as published or submitted and are, therefore, structured according to journal submission guidelines. These submission guidelines are provided for each journal used, under the paragraphs titled “Guidelines for authors”, along with a URL link for each journal’s author guidelines page. Brief discussions regarding the purpose and outcomes precede each article and the references used in the article are not listed in the thesis bibliography as they are already listed in the article’s reference list. Other articles in which I participated as a co-author that is relevant to the research, but is not part of my study, is normally referenced in the text and separately listed in Annexure A. Permission from co-authors for the submission of the articles for degree purposes and their roles have been supplied in the preface. Permission from the journal and/or editors to include the articles in the thesis is located in Annexure B.

The thesis begins with a brief literature review regarding the history (chapter 2), origin and purpose (chapter 3) of cirDNA to introduce and summarise two accompanying comprehensive reviews (refer to articles in paragraphs 2.2 (Aucamp *et al.*, 2016a) and 3.2) and to introduce the theory of cirDNA as homeostatic genetic entities or messengers (paragraph 3.4 (Aucamp *et al.*, 2016b)). The concept of cirDNA as homeostatic genetic entities or messengers forms an integral part of the research performed and will determine whether cirDNA may have clinical implications. In chapter 4 the optimisation of cfDNA collection and quantification is described. The use of cell cultures is also substantiated here and throughout the thesis. The research is further divided into two aspects in chapter 5, namely the targeting of cell metabolism and epigenetics to determine whether cirDNA can laterally transfer information. Chapter 6 introduces the utilisation of 3D cell cultures in cfDNA research to further motivate and illustrate the utilisation of *in vitro* models. Chapter 7 serves as the final summary and conclusions.

CHAPTER 2

HISTORICAL AND EVOLUTIONARY PERSPECTIVE ON THE BIOLOGICAL SIGNIFICANCE OF CIRCULATING DNA AND EXTRACELLULAR VESICLES

To begin the literary introduction of this thesis a comprehensive review of the history and development of cirDNA research is provided (paragraph 2.2). To briefly summarise, the development of heredity theories regarding the transfer of heritable traits from parent to child via forms of freely moving particles were identified, in particular Charles Darwin's Pangenesis theory and the concept of soma-to-germline information transfer. Discussions regarding Darwin's graft hybridisation method, which was used by several researchers in support of the Pangenesis theory, followed. This method along with the discovery of DNA and both its purpose as the substance of inheritance and its existence beyond the confinement of cells inevitably led to the discovery that DNA present in the extracellular environment of an organism, later termed cirDNA, has the ability to transfer hereditary characteristics from donors to recipients.

Following these graft hybridisation studies, Gahan and Chayen (1965) and various other studies by Stroun, Pelc, Bell, Anker and Gahan have suggested that DNA in the cytoplasm of cells act as messenger DNA (Gahan, 2003; Gahan, 2006; Gahan *et al.*, 2008), possibly explaining the cellular transformation occurrences observed by several studies and the metastatic spread of cancer (Bendich *et al.*, 1965). In support of this, studies have demonstrated the active cellular release of newly synthesised cirDNA and their involvement in genometastasis and as potential treatment for cancer (Garcia-Olmo *et al.*, 2015; García-Olmo *et al.*, 2010; García-Olmo *et al.*, 2011). The ability of cirDNA to transfer effects, ranging from beneficial to damaging, between cells and the theory that there is selectivity involved in cirDNA release, due to the unequal representation of cirDNA sequences in human blood (Bronkhorst *et al.*, 2016c; Puszyk *et al.*, 2009), supports the concept of cirDNA having an intended function rather than simply being debris from cell death or damage.

However, cirDNA consists of a majority fraction of DNA derived cell death or damage, especially in patients with diseases, and a minority fraction of actively released DNA. Moreover, a study has shown that circulating apoptotic DNA can indeed induce cellular effects, in this case damage, indicating that both fractions of cirDNA have the potential or capacity to serve as some form of messenger. The question now is which of these fractions can be regarded as the main origins of cirDNA and should be targeted during research.

2.1 Guidelines for authors – Cellular and Molecular Life Sciences

(http://www.springer.com/life+sciences/cell+biology/journal/18. Date of access: 26 January 2017)

- **Article type used: Visions and Reflections**

  Visions and reflections provides a forum for expert opinions on recent developments in research fields of general interest. All manuscripts are subject to peer review by at least two external reviewers. CMLS does not have obligatory length restrictions but offers a guideline: the length should not exceed 8 typeset pages, including figures, tables and references (a printed page contains approx. 1000 words or 40 references). In the case of the following article (paragraph 2.2), the editors allowed the longer manuscript for publication.

- **Title page:**

  The title page should include the names of the authors, a concise and informative title, the affiliations and addresses of the authors, the e-mail address, and telephone number of the corresponding author and, if available, the 16-digit ORCID of the authors.

- **Abstract:**

  The abstract should consist of 150 to 250 words and should not contain any undefined abbreviations or unspecified references. Keywords (four to six) are also required.

- **Text formatting:**

  Manuscripts should be submitted in Word, using a normal, plain font (e.g., 10-point Times Roman) for text. Use italics for emphasis and the automatic page numbering function to number the pages. Do not use field functions and use tab stops or other commands for indents, not the space bar. Save your file in docx format (Word 2007 or higher). Use no more than three levels of displayed headings. Abbreviations should be defined at first mention and used consistently thereafter. Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

- **References:**

  Reference citations in the text should be identified by numbers in square brackets. The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be

mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list. The entries in the list should be numbered consecutively.

Example of a journal article: Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted. Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations.

- **Tables and figures:**

Supply all figures electronically and indicate what graphics program was used to create the artwork. For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable. Vector graphics containing fonts must have the fonts embedded in the files. Name your figure files with "Fig" and the figure number, e.g., Fig1.eps. Colour art is free of charge for online publication. Colour illustrations should be submitted as RGB (8 bits per channel). To add lettering, it is best to use Helvetica or Arial (sans serif fonts). Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt). Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label. Avoid effects such as shading, outline letters, etc. Do not include titles or captions within your illustrations.

All figures are to be numbered using Arabic numerals. Figures should always be cited in text in consecutive numerical order. Figure parts should be denoted by lowercase letters (a, b, c, etc.). Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file. Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type. No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption. Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs. Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption. Figures should be submitted separately from the text, if possible. When preparing your figures, size figures to fit in the column width. For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm.

In order to give people of all abilities and disabilities access to the content of the figures, all figures should have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware). Patterns are used instead of or in addition to colours for

conveying information (colour-blind users would then be able to distinguish the visual elements). Any figure lettering has a contrast ratio of at least 4.5:1.

2.2 Article published in Cellular and Molecular Life Sciences

Aucamp, J., Bronkhorst, A.J., Badenhorst, C.P.S., Pretorius, P.J. 2016. Historical and evolutionary perspective on the biological significance of circulating DNA and extracellular vesicles. *Cellular and molecular life sciences*, 73:4355-4381. The final publication is available at Springer via http://dx.doi.org/10.1007/s00018-016-2370-3.

This article consists of a comprehensive and empirically up to date review of the history and development of cirDNA research, providing connections between four main topics or paradigms, namely heredity, DNA, messengers and the cirDNA and extracellular vesicles. A.J.. Bronkhorst and I contributed equally to the drafting of the manuscript, I drafted the figures and Chris Badenhorst and Prof Pretorius critically reviewed the manuscript. A key player in cirDNA research, Peter Gahan, also assisted in the review processes. This review has been downloaded 1 500 times within 3 – 4 months of publication due to its general applicability in both genetics and heredity research.

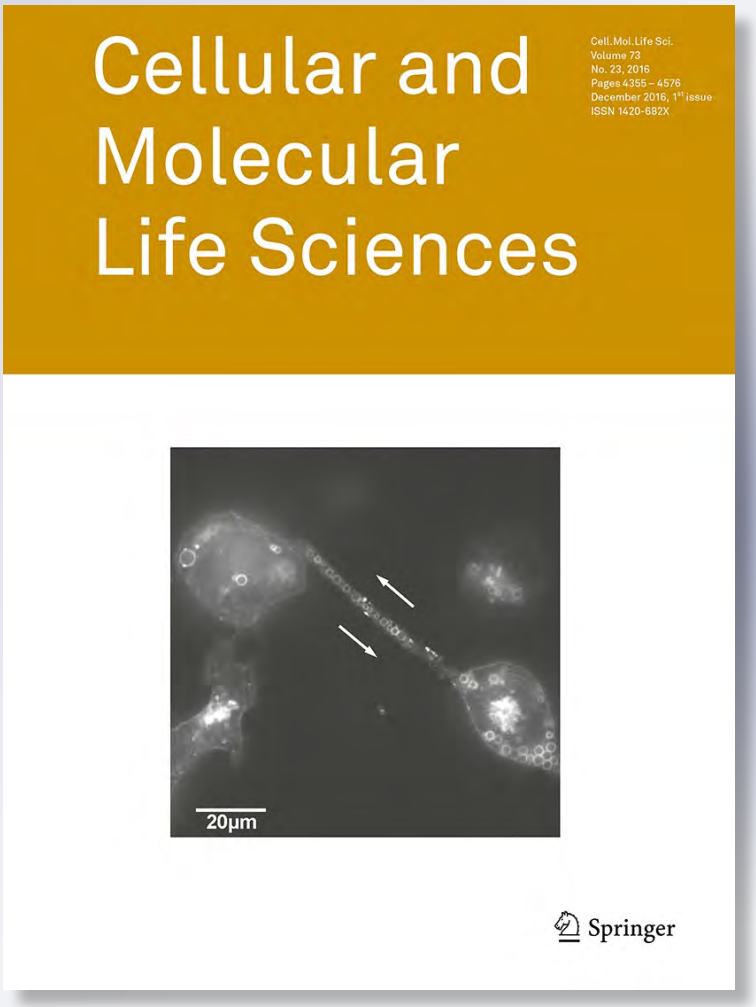
A historical and evolutionary perspective on the biological significance of circulating DNA and extracellular vesicles

Janine Aucamp, Abel J. Bronkhorst, Christoffel P. S. Badenhorst & Piet J. Pretorius

Cellular and Molecular Life Sciences

ISSN 1420-682X
Volume 73
Number 23

Cell. Mol. Life Sci. (2016) 73:4355-4381
DOI 10.1007/s00018-016-2370-3



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Cell. Mol. Life Sci. (2016) 73:4355–4381
DOI 10.1007/s00018-016-2370-3

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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Received: 14 March 2016 / Revised: 20 August 2016 / Accepted: 15 September 2016 / Published online: 20 September 2016

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.

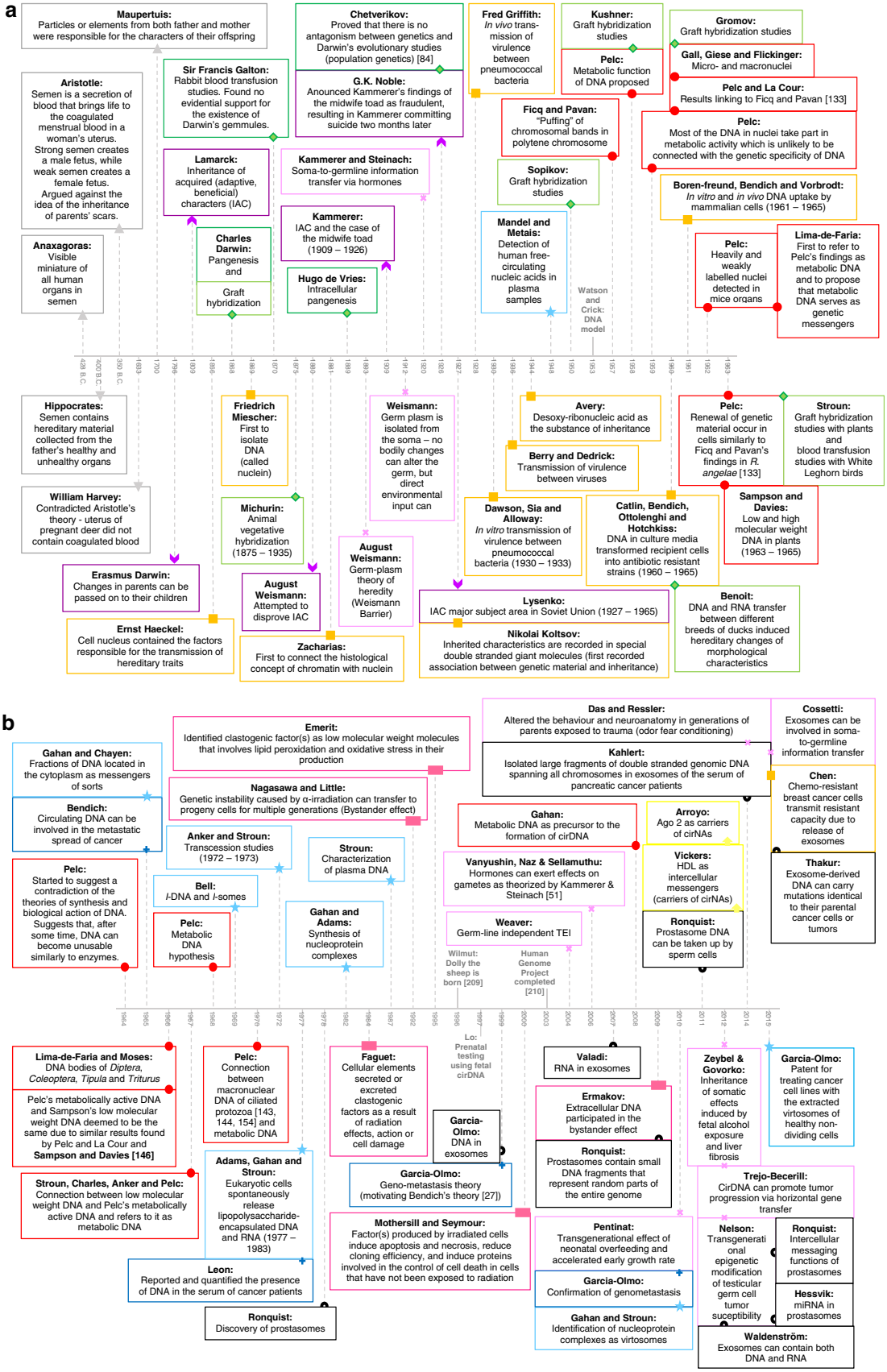
Predecessors of Charles Darwin and Jean-Baptiste Lamarck

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



◀**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

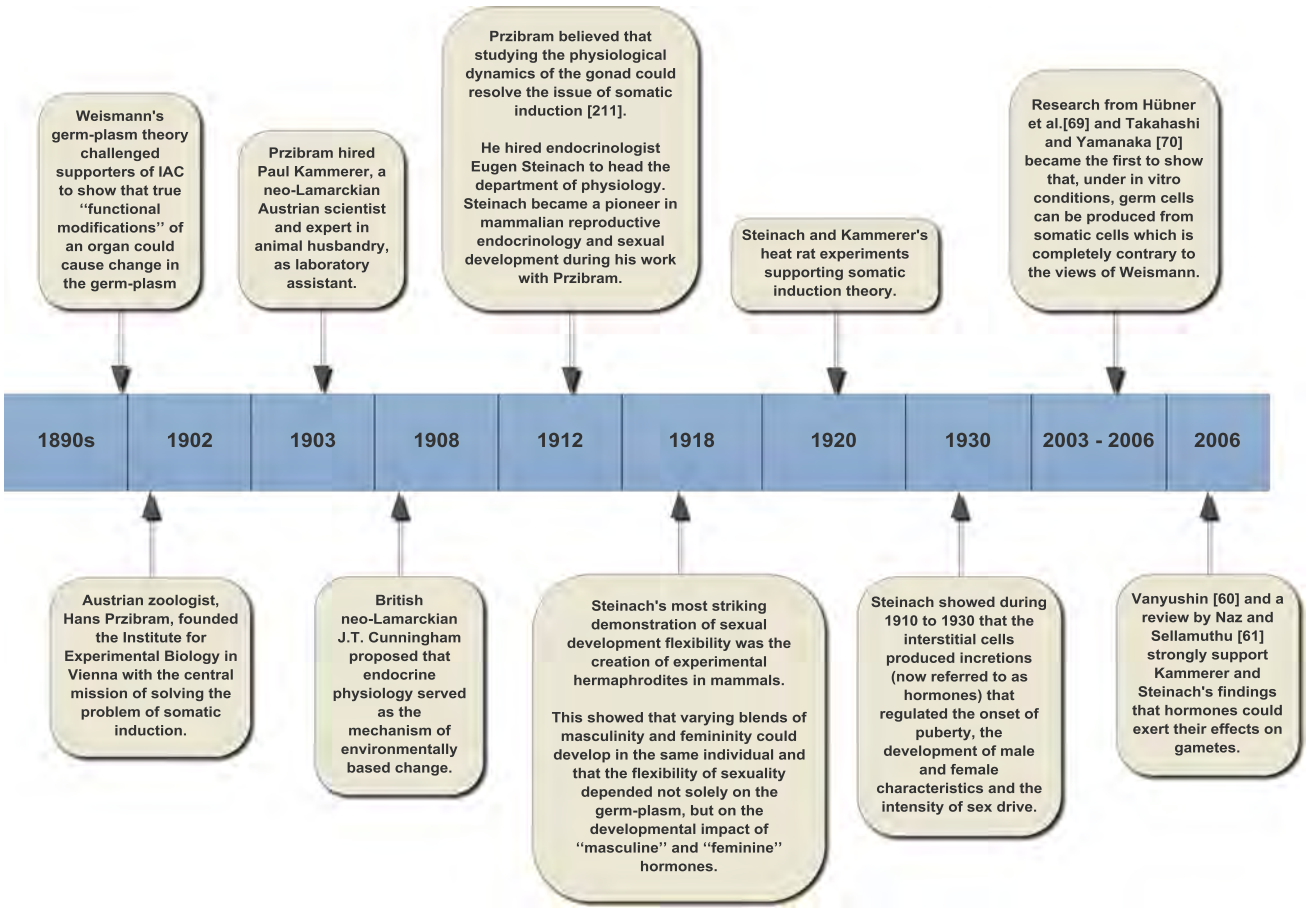


Fig. 2 Przibram and Steinach's somatic induction research [50]

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

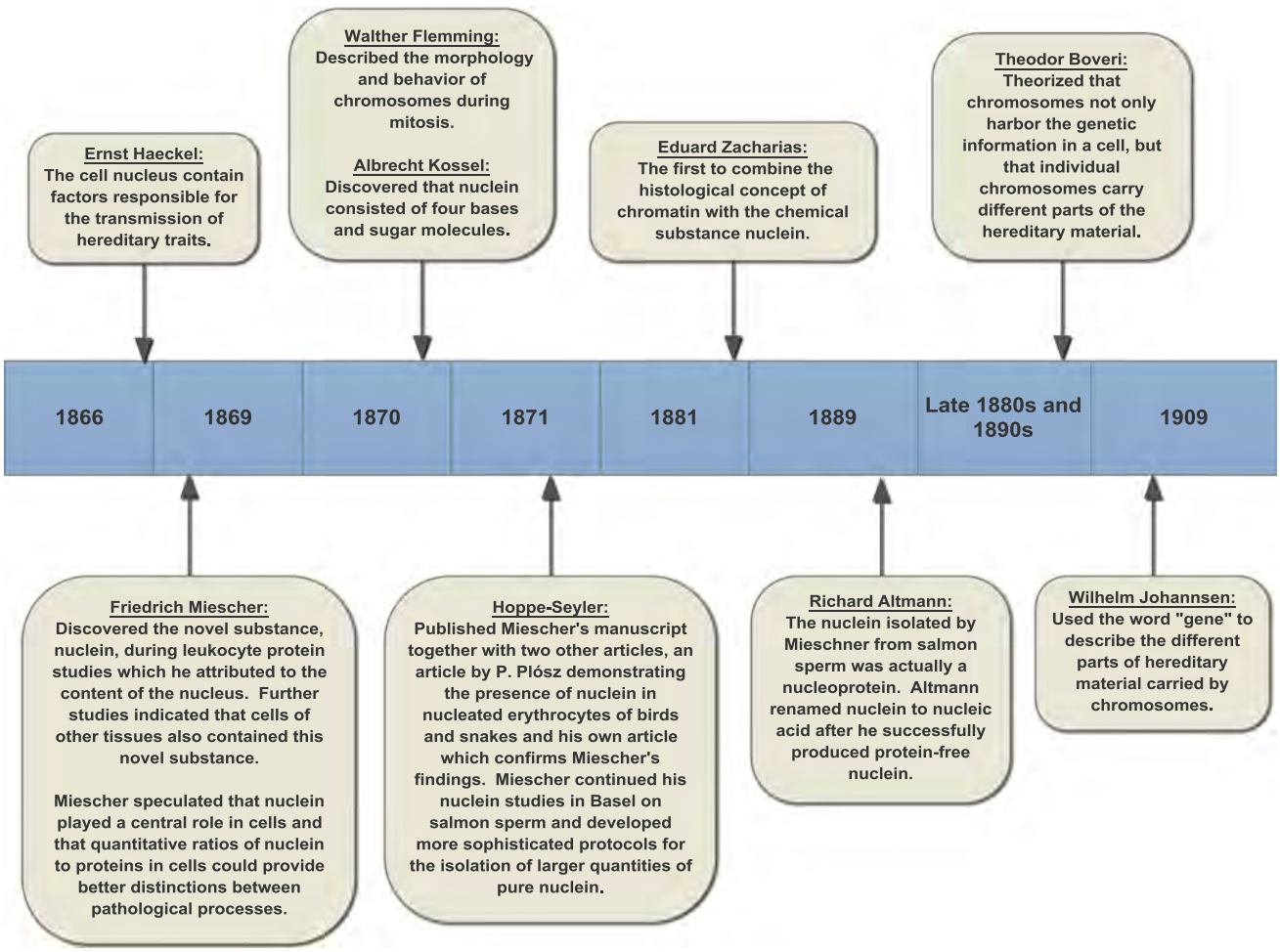


Fig. 3 Chronological summary of the events that led to the discovery and elucidation of nuclein as hereditary material in genes [83, 212, 213]

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

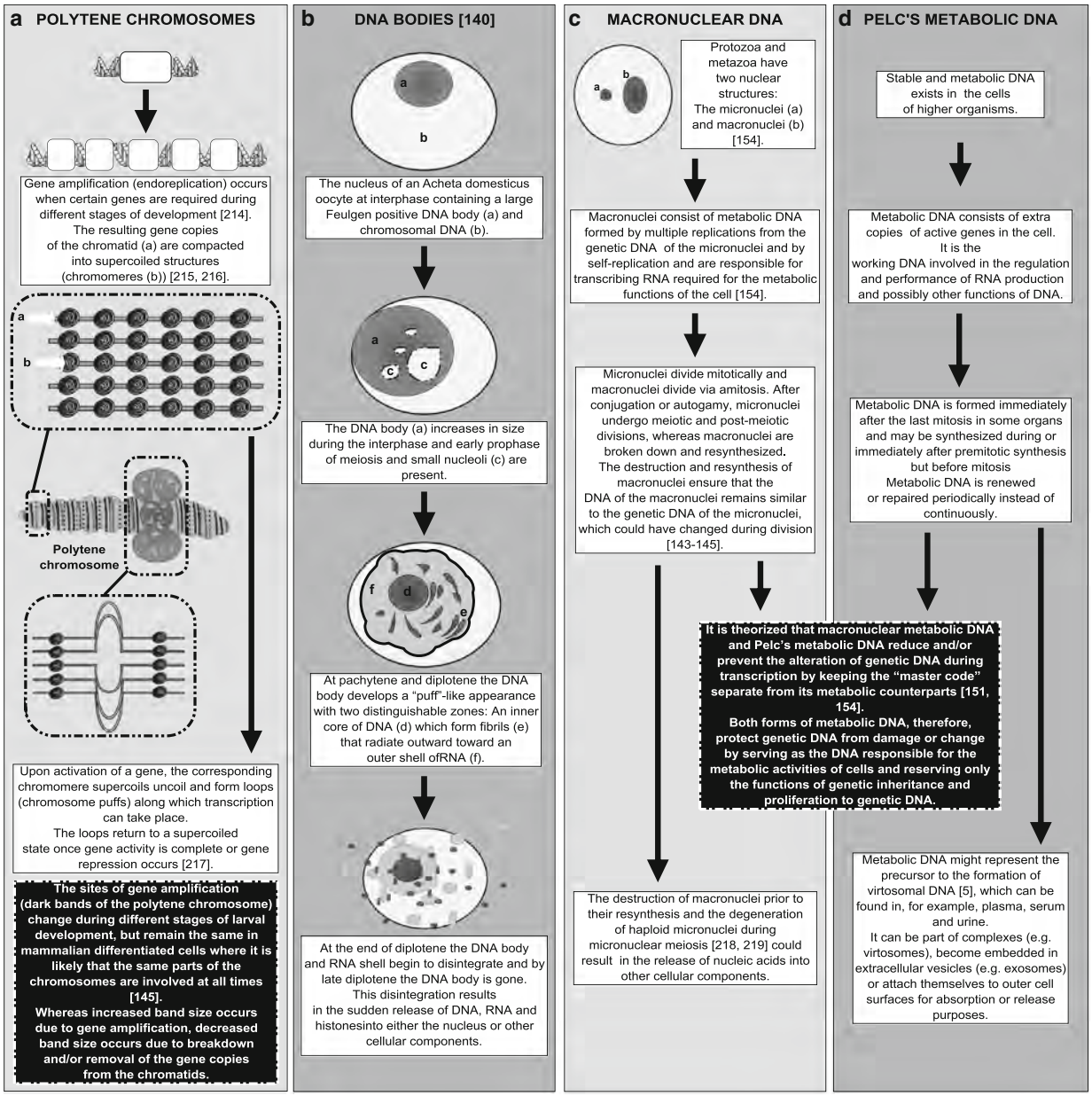


Fig. 4 Studies that contributed to the discovery and elucidation of Pelc's metabolic DNA. **a** Ficq and Pavan [133] 's discovery of DNA synthesis in the chromosomes of *R. angelae* larvae was the first indication that DNA played a very active part in the larval development. Polytene chromosomes are 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

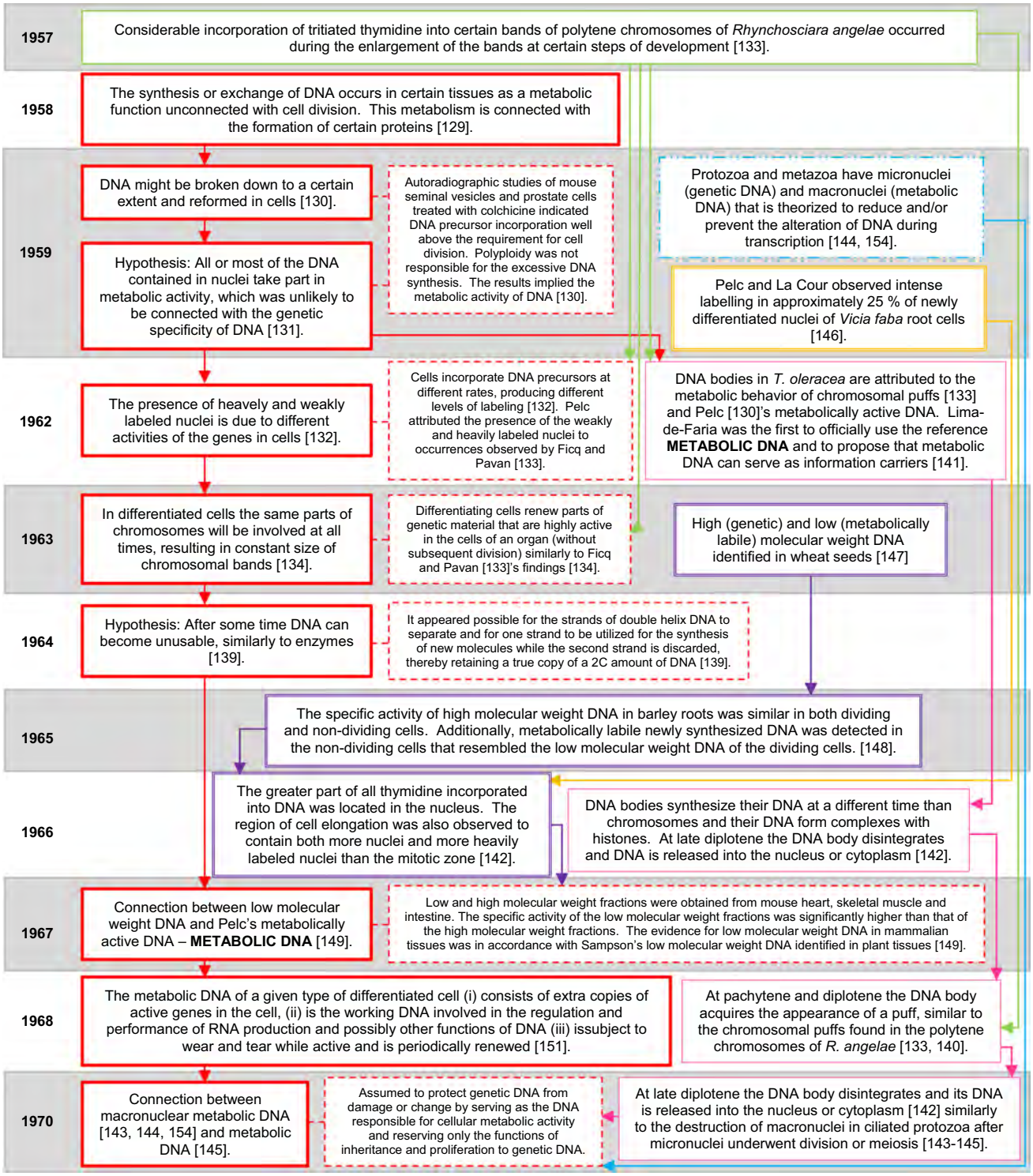


Fig. 5 Series of studies that influenced Pelc's research on metabolically active DNA and the development of Pelc's hypothesis on metabolic DNA

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.

Acknowledgments The authors would like to thank Peter B. Gahan for professional assistance with this review article. AJB (SFH13092447078) and JA (SFH14061869958) were supported by post-graduate scholarships from the National Research Foundation (NRF), South Africa. The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions, arrived at, are those of the authors and are not necessarily to be attributed to the NRF.

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CHAPTER 3

THE ORIGINS AND PHYSIOLOGICAL SIGNIFICANCE OF CIRCULATING DNA

To date knowledge regarding the origin of cirDNA is slowly progressing and the utilisation of cirDNA in the diagnosis of cancer and diseases are studied extensively. Yet there are still arguments regarding the identification of the main origin of cirDNA. As mentioned in paragraph 2.2, cirDNA consists of both a major fraction of DNA derived from cellular breakdown mechanisms and a minor fraction derived from active DNA release mechanisms, both with potential messaging capabilities. In order to determine which fraction of cirDNA should be targeted during the thesis experiments, a comprehensive compilation of the putative origins of cirDNA was prepared (paragraph 3.2) in which each mentioned biological feature and its involving mechanisms were categorised into three categories: sources of cirDNA, causes of cirDNA release and a combination thereof. In this review an argument was presented regarding whether the most abundant or most functional fraction of cirDNA should be considered in research. It was proposed that this choice will be dependent on the research objectives, but that focusing on the most functional fraction of cirDNA will serve as a better source for disease- or physiologically-specific biological markers and provide more effective insight regarding the biological function of cirDNA.

Furthermore, the categorisation of the biological features made it easier to see the relationships between these features and how they can change from sources to causes of release and *vice versa* when they interact with one another. The complexity of cirDNA contents becomes clearly visible, explaining why the elucidation and discovery of tissue origins, biological functions and biomarkers progresses so painfully slowly. With this in mind the utilisation of *in vitro* cell culture techniques, including more physiologically relevant 3D culture technologies, is proposed. It will significantly contribute in researching this minority fraction of cirDNA as *in vitro* cell cultures can produce “closed-circuit” models that restrict the putative sources of cirDNA and causes of cirDNA release to specific cell types, morphologies, tissue origins and/or physiological systems (e.g. tumours).

From both the reviews in paragraphs 2.2 (Aucamp *et al.*, 2016a) and 3.2 a pattern emerged which suggests cirDNA to form part of a homeostatic mechanism for genetic information. Paragraph 2.2 illustrated the integration of four concepts or paradigms: heredity, DNA, messaging via genetic information and cirDNA and extracellular vesicles. It provided insight regarding the development and substantiation of the concept that newly synthesised and actively released DNA has an intercellular messaging function that can laterally transmit genetic or epigenetic information between cells, tissues and organs. Paragraph 3.2 listed the different sources of cirDNA and

causes of cirDNA release, showing the characteristics and generalised involvement of active newly synthesised DNA release by multiple biological factors. Together this may implicate cirDNA as a homeostatic entity or messenger of genetic information that can promote synchrony of both adaptation and damage in tissues and organs depending on the source of the message. The article in paragraph 3.4 serves as a short review of cirDNA as a homeostatic entity or messenger of genetic information.

3.1 Guidelines for authors – Biological Reviews

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- **Article type used: Review**

  Papers should not generally exceed 20 000 words and a word count must be provided. Articles should be synthetic and critical assessments of fields of research of value to specialists in the field, and comprehensible to biologists in general

- **Title page:**

  The title should be a short (under 15 words) and clear description of your paper, avoiding the use of acronyms, abbreviations and numbers. All author first names, other names abbreviated to initials, and surnames should be provided. Identify the author for correspondence using a superscript asterisk after the surname, with contact details provided below the affiliation addresses (the institutions where the work was carried out) Affiliation addresses should follow the list of authors.

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  The abstract should consist of no more than 500 words. Five to ten keywords should also be provided.

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  Manuscripts should be submitted in an editable format (e.g Microsoft Word.docx). Text should be double-spaced throughout, with line numbering and adequate margins. The manuscript must have an Abstract, Keywords, a list of Contents, a general Introduction, and Conclusions. Sections and subsections are to be numbered I., (1), (a), (i) etc. Footnotes and boxed text are discouraged. Articles should be written in British English, avoiding informality or excessive use of technical jargon.

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  Text citations should give author or two authors and date. If several papers are listed for the same author in one year italic letters a, b, etc. should follow the date. For three-author papers all names should be given on first citation in the text, with only the first name *et al*. used subsequently. For more than three authors *et al.* is used in all citations. Only articles that have been accepted for publication or have been published can be cited. Citations of unpublished work should be cited in the text as a list of authors with forenames as initials followed by 'unpublished data' or 'in preparation'. References should be listed in alphabetical then chronological order at the end of the text. All author names should be given in the reference list; very extensive author lists may be abbreviated to the first 15 authors followed by *et al.* Full paper and journal titles, volume number, and first and last page numbers should be given. Publisher and place of publication should be given for books.

- **Tables and figures:**

  Figures may be line diagrams, drawings or photographs and the accompanying legends should be typed double-spaced on separate sheets. The position of Figures and Tables should be indicated. Figure quality is 600 – 1 000 dpi for line art and 300 dpi for colour. Figures should preferably be saved as TIFF, EPS, PDF or PNG files.

3.2 Article submitted to Biological Reviews

Aucamp, J., Bronkhorst, A.J., Badenhorst, C.P.S., Pretorius, P.J. 2017. The diverse origins of circulating DNA in the human body: Critical re-evaluation of the literature. This article was submitted to the journal editor on 2 August 2017 and is currently under review. For the final copy of the thesis, the title page of the review pdf version of the article is provided, followed by the Microsoft Office Word version of the article in order to avoid the "for review only" watermark on the pages, remove the numbering on the left margin of the paragraphs and position the figures within the text as opposed to the norm of having the articles at the end of the review copy. Other than these mentioned changes, paragraph 3.2 is an exact copy of the submitted manuscript.

The article consists of (i) a comprehensive compilation of putative cirDNA sources and causes of cirDNA release, (ii) a discussion regarding most abundant versus most functional cirDNA fractions and (iii) the motivation of using *in vitro* means to solve *in vivo*-related cirDNA research challenges. I serve as the head author and prepared the majority of the manuscript and figures. A.J.. Bronkhorst, Chris Badenhorst and Prof Pretorius critically reviewed the manuscript.

BIOLOGICAL REVIEWS
Cambridge Philosophical Society

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

Journal:	*Biological Reviews*
Manuscript ID	BRV-08-2017-0173
Manuscript Type:	Original Article
Date Submitted by the Author:	02-Aug-2017
Complete List of Authors:	Aucamp, Janine; North-West University, Human Metabonomics Bronkhorst, Abel; North-West University, Human Metabolomics Badenhorst, Christoffel; Institute of Biochemistry, Greifswald University, Department of Biotechnology and Enzyme Catalysis Pretorius, Piet; North-West University, Human Metabolomics
Keywords:	active DNA release, cellular breakdown mechanisms, circulating mitochondrial DNA, lateral information transfer, in vitro cell culture

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 the biological pathways in which cirDNA is involved, and 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; *in vitro* cell culture; lateral information transfer

Contents

1. Introduction

The presence of cell-free circulating DNA (cirDNA) in human blood was first reported by Mandel and Métais (1948). To date the utility of kinetic analysis and molecular profiling of cirDNA in the diagnosis, prognosis and therapy monitoring of cancer and other diseases are studied extensively, but the origin of cirDNA remains under debate. Various forms of biological features can not only directly contribute to the aggregate cirDNA profile (Fig. 1), but can also interact to form a cascade of effects that result in further cirDNA release. These features have been mentioned in several publications (Bryzgunova & Laktionov, 2014; Gahan, 2012; Lichtenstein *et al.*, 2001; Stroun *et al.*, 2001; Thierry *et al.*, 2016; Ulivi & Silvestrini, 2013), however many different conclusions have been reached with regard to which feature serves as the main origin of cirDNA.

Two disputable contenders for main origins of cirDNA are cellular breakdown mechanisms and active DNA release mechanisms, which also includes the vesicular transport of nucleic acids (such as exosomes, virtosomes and Argonaute 2). 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 main force 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 and categorised under (1) from living or dead cells and (2) as either sources, causes or a combination thereof.

In this regard, 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 we argue in favour of utilising both cirDNA fractions and that the lesser studied, most functional fraction of cirDNA can be of particular benefit for further cirDNA research endeavours. Secondly, we wish to emphasise the introduction of "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.

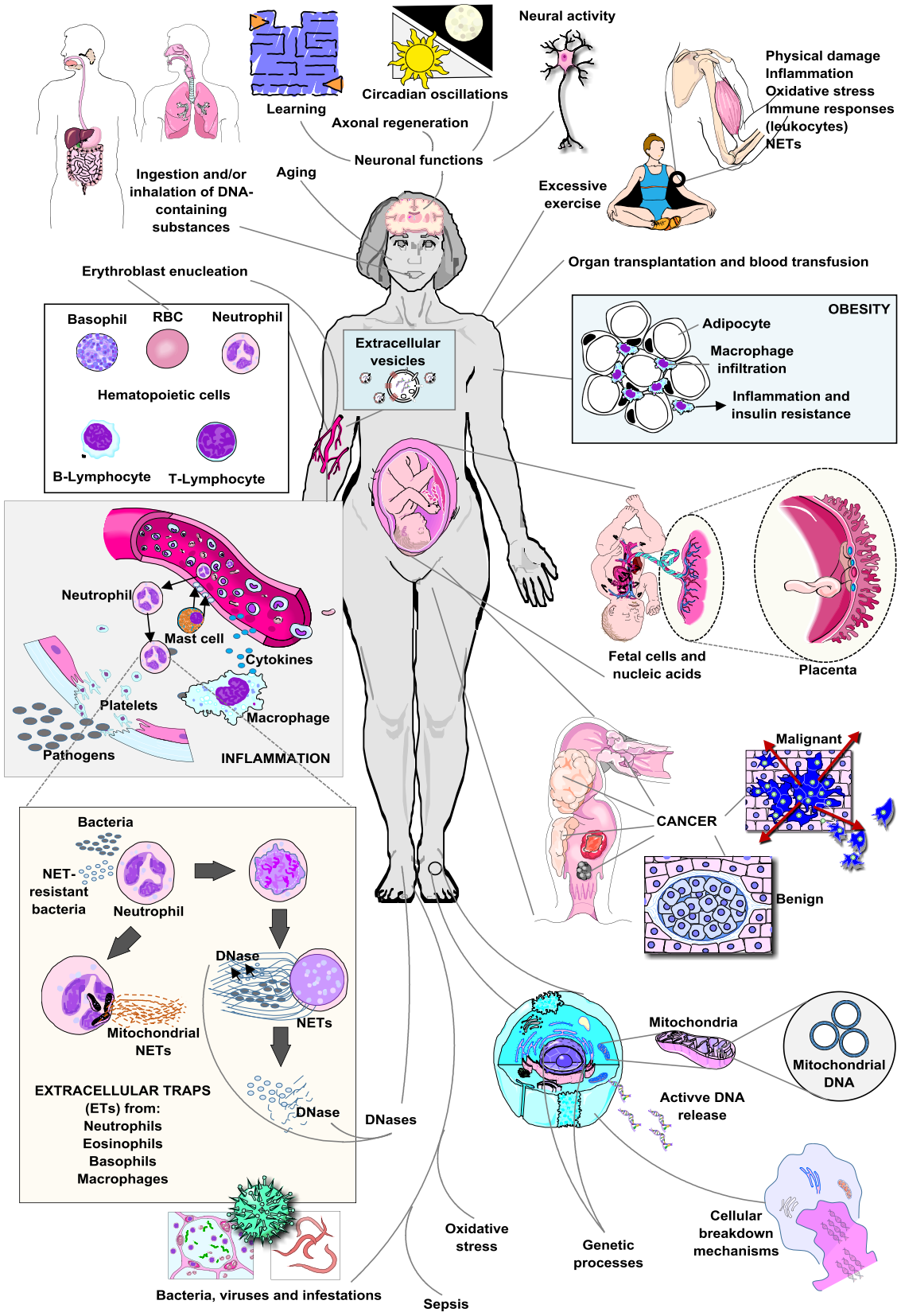


Fig. 1 The putative origins of cirDNA

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

Putative biological features contributing to cirDNA can be sorted into three categories, namely (1) sources of cirDNA, (2) causes of cirDNA release and (3) a combination of both source and cause. Table 1 provides a comprehensive summary of each putative biological features and the various mechanisms involved in the release of cirDNA. By categorising the different mechanisms involved in each biological feature, it becomes easier to determine the true origin of the cirDNA from each biological feature and how these features can change, when interacting with one another, from sources of cirDNA to causes of cirDNA release and *vice versa*. These interactions are schematically illustrated by Fig. 2, emphasising the complexity of cirDNA contents that 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 the capability to release DNA into circulation via both cellular breakdown and active DNA release mechanisms. 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, dead 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 discuss the different putative sources of cirDNA, causes of cirDNA release and the combinations thereof as listed in Table 1.

Table 1 Categorisation of a comprehensive summary of putative biological features and their cirDNA release mechanisms as sources of cirDNA or causes of cirDNA 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/tumour survival (see cancer)				
	De-polyploidisation	X			X
AUTOPHAGY	Autophagic clearance of micronuclei from mitotic catastrophe		X	X	
	Autophagic clearance of mitochondria		X		X
	Resulting cell death		X		X
	Pro-tumour activity				
	Tumour protection	X			X
	Promotion of tumour development in healthy cells	X			X
	Promotion of DNA damage in healthy cells	X	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	
	Pro-tumour effects		X	X	X
	Anti-tumour 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 oxidised 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 labour)		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
NEURONAL FUNCTIONS		X		X	X
CANCER	From tumour cells				
	Cell death due to increased tumour burden		X		X
	Active DNA release	X			X
	NETs to promote or prevent tumour development	X	X		X
	Rereplication producing short DNA fragments	X	X		X
	From cells surrounding the tumour				
	Cell death due to increased tumour burden		X		X
	NETs in response to tumour 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
	Tumour 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 tumourigenesis (see cancer)		X X		X X
	Induces apoptosis	X	X	X	
	Formation of short DNA fragments during DNA replication or transcription	X		X	
DNA ENDOREPLICATION	Polyploidisation				
	To evade apoptosis and mitotic catastrophe	X		X	
	Tumourigenesis due to chromosome instability	X	X		X
	De-polyploidisation	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
	Tumourigenesis	X	X		X
	Increases disease susceptibility	X	X		
TRANSPOSONS	Endogenous retrotransposons	X		X	
	Exogenous DNA transposons	X	X	X	

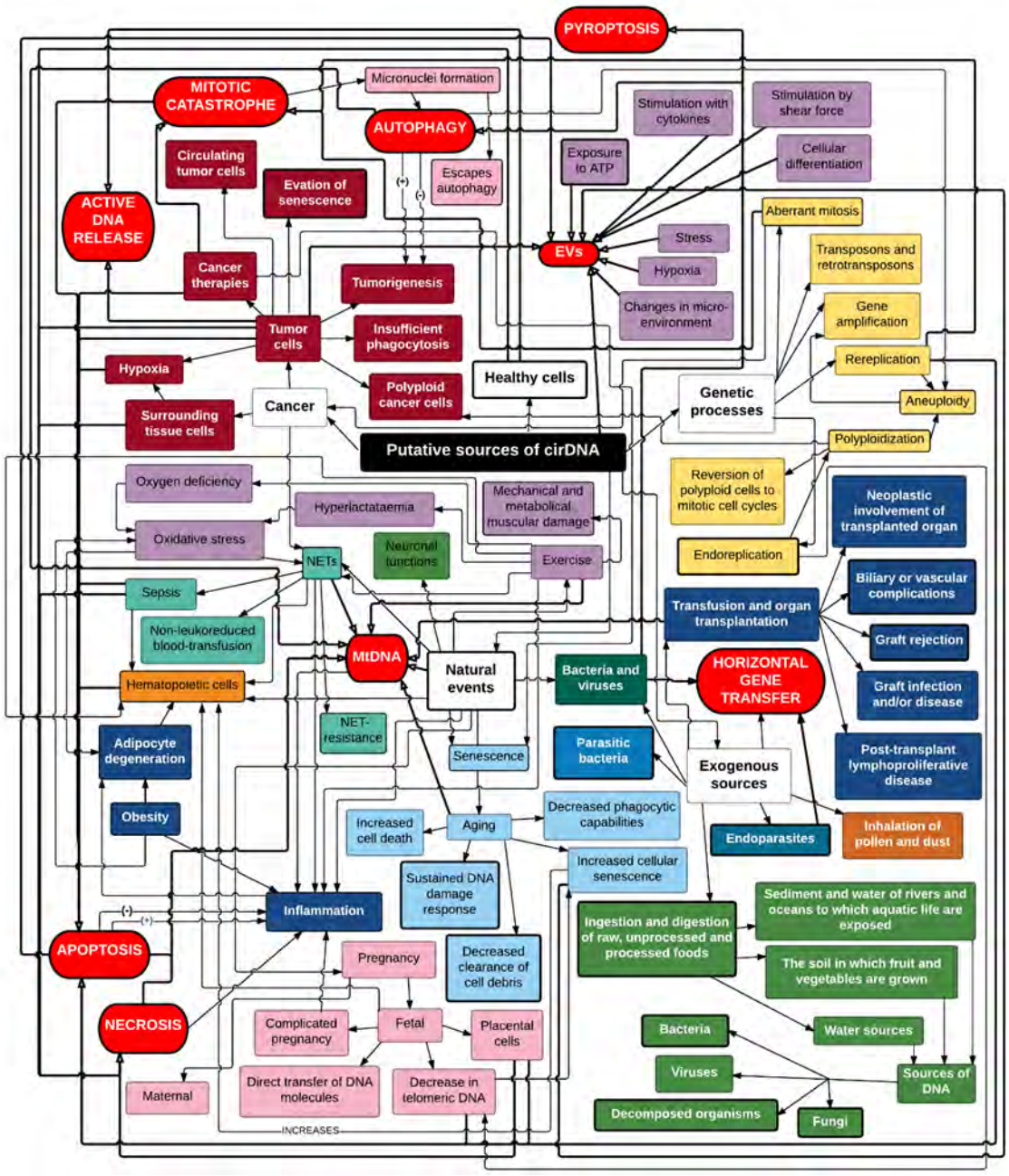


Fig. 2 Complex interactions between biological features that can further contribute to cirDNA release and complicate both the discovery of novel biological markers and the elucidation of cirDNA origins and biological functions

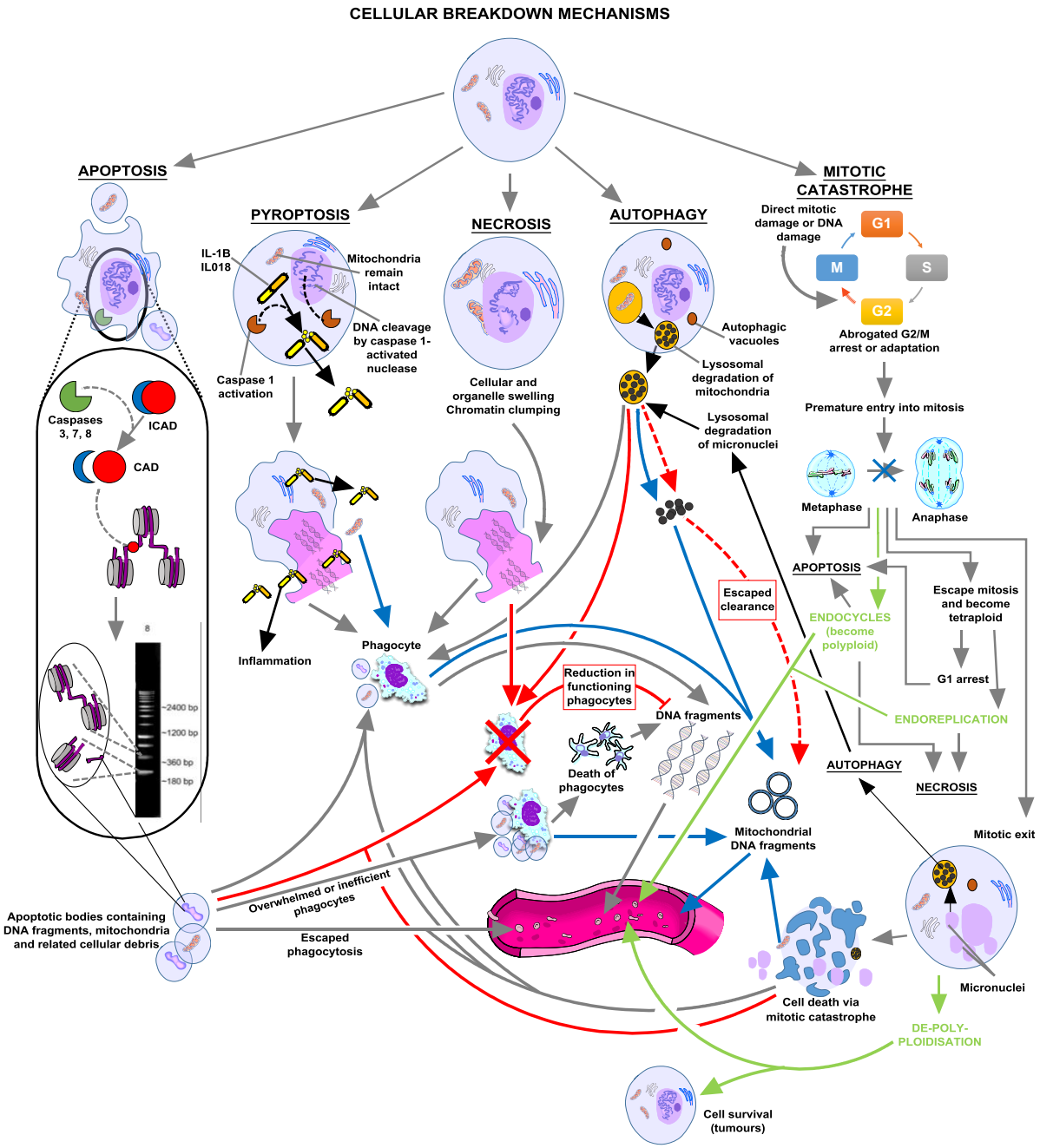


Fig. 3 CirDNA release by cellular breakdown mechanisms

3. Sources of circulating DNA

(1) Exogenous sources

Foreign DNA from putative exogenous sources (summarised in Fig. 4) may be released into the bloodstream during immune defences or metabolic digestion.

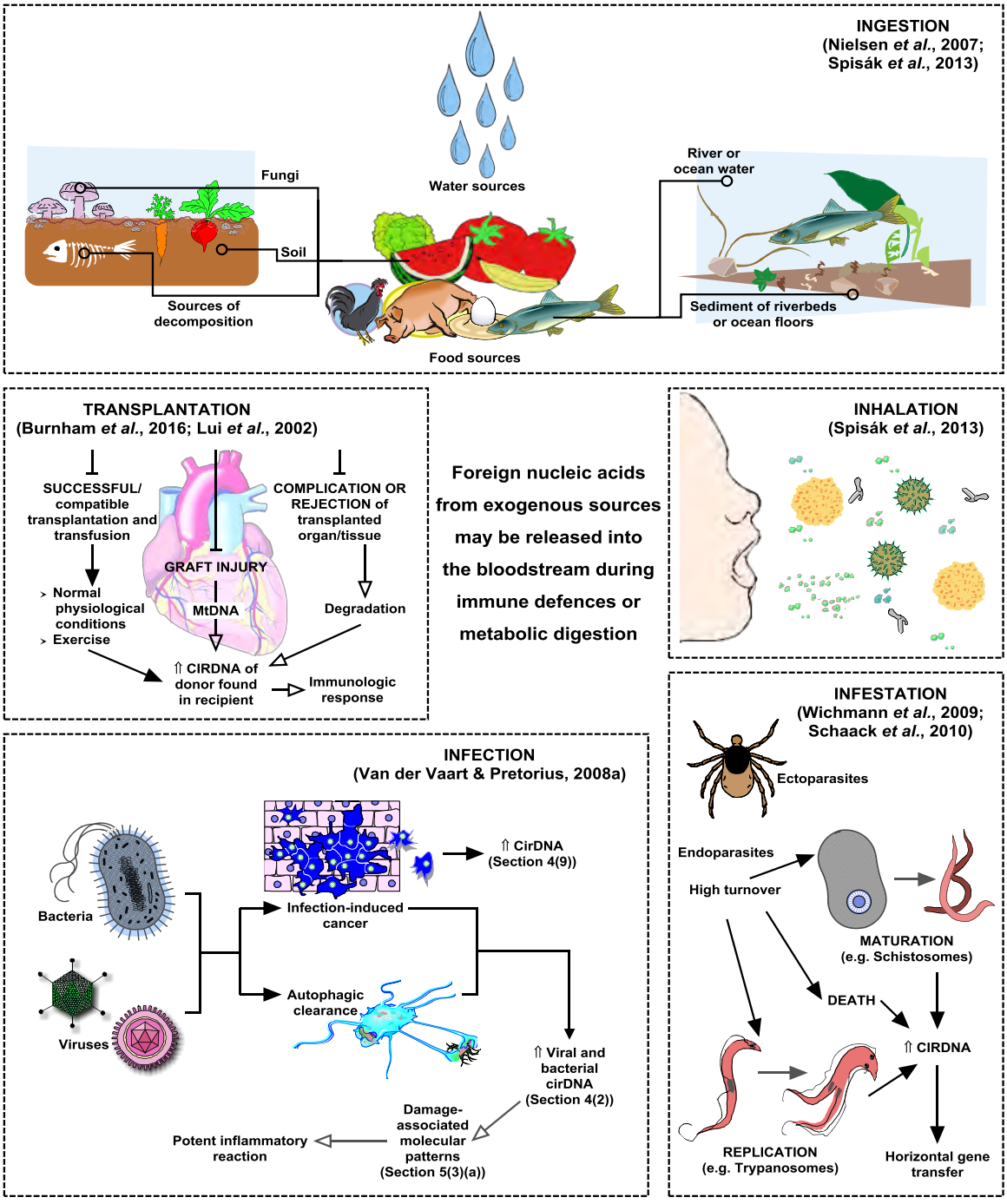


Fig. 4 Summary of putative exogenous sources of foreign cirDNA

(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 cellular and organelle swelling and disintegration, nuclear chromatin clumping and non-specific chromatin digestion (Bryzgunova & Laktionov, 2014; Holdenrieder & Stieber, 2009) (Fig. 3). There are also several forms of regulated/programmed necrosis, e.g. necroptosis (induced by death receptors, including tumour necrosis factor TNFR and Fas, with cell disintegration similar to necrosis) and pyronecrosis (induced by pathogens, e.g. *Neisseria gonorrhoeae* and *Staphyolococcus aureus*) (Yang *et al.*, 2015). Necrosis occurs more rapidly than apoptosis, but the 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) (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 phagocytic clearance is required to release necrotic DNA fragments into culture medium.

(3) Apoptosis

Studies have proposed that apoptosis serves as the main 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) (Nagata, 2000), which systematically cleaves chromosomal DNA into multiples of 160 – 180 bp nucleosomal fragments (Holdenrieder & Stieber, 2009; Jahr *et al.*, 2001; Nagata, 2000; Stroun *et al.*, 2001; Suzuki *et al.*, 2008; Van der Vaart & Pretorius, 2008*a*) (Fig. 3). A ladder pattern similar to the pattern visible after electrophoresis and sequencing reactions of cirDNA forms, indicating to many researchers that apoptosis may be the main source of cirDNA (Anker *et al.*, 1999; Jahr *et al.*, 2001; Pinzani *et al.*, 2010; Stroun *et al.*, 2001; Van der Vaart & Pretorius, 2008*b*; Van der Vaart *et al.*, 2009). However, 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). Furthermore, the discovery of the active release of newly synthesised DNA (Anker, Stroun & Maurice, 1975; Anker *et al.*, 1999; Stroun & Anker, 1972; Stroun *et al.*, 2001) (see section 3(5)) with DNA fragment sizes that is also theorized to present with a ladder pattern (Stroun *et al.*, 2001; Van der Vaart & Pretorius, 2007) further argues against apoptosis.

(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 (Hochreiter-Hufford & Ravichandran, 2013). 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 (see Section 5(4)) or in subjects performing regular exercise (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). This lack of increased cirDNA levels, 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). Certain cancer cells can also resist apoptosis and continue to proliferate with a concomitant increase in cirDNA (Anker *et al.*, 1975; Anker *et al.*, 1999; Stroun *et al.*, 2001; Van der Vaart & Pretorius, 2008*b*).

There are, however, 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 (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, pulmonary diseases, cardiovascular diseases, atherosclerosis, sepsis and septic shock, neurological conditions and cancers (Hochreiter-Hufford & Ravichandran, 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 does not cleave DNA between nucleosomes as observed in apoptosis. Instead pyroptosis-mediated cell death will likely cause autophagic clearance of mitochondria and release of mtDNA (see Section 4(2)). 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.

(5) Actively released DNA

The development of the concept of cirDNA is reviewed in Aucamp *et al.* (2016). To briefly summarise, Stephen Pelc discovered the synthesis of low molecular weight DNA molecules, dubbed metabolic DNA, in both dividing and non-dividing, differentiated cells that is independent of DNA synthesis and repair, renewed or repaired periodically instead of continuously, and closely connected with the functional activity of differentiated cells (Pelc, 1968). Pelc's studies were corroborated by Anker *et al.* (1975) and Rogers *et al.* (1972) and 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).

Gahan and Stroun (2010) have reported that the DNA released from cells can be complexed with glycolipoproteins and associating RNA, and these specific complexes were referred to as virtosomes. The DNA fraction of the virtosome is newly synthesised in the nuclei of living, dividing and differentiated cells and not dead or dying cells (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. 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.

(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 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 to: (1) 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); (3) regulate cirDNA levels in circulation via the management of the equilibrium; (4) promote the transport of cirDNA (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).

(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 in the same manner. Morozkin *et al.* (2008)indicated that the active DNA release mechanisms in primary and cancer cells can be different, the inhibition of these pathways induced different effects

in cell-surface bound DNA concentrations and cell viability between human umbilical vein endothelial cells (HUVECs) and HeLa cells,

(c) The functional aspects of actively released DNA

(1) 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, 1963*a*; Stroun *et al.*, 1963*b*) by the cirDNA transported from the mentor plant to recipient pupil plant 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 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; García-Olmo, Ruiz-Piqueras & García-Olmo, 2004; García-Olmo *et al.*, 2010; Garcia-Olmo *et al.*, 2015).

(3) Genometastasis. The cirDNA in the plasma of cancer patients have been found to transfer oncogenic information to susceptible cells *in vitro* (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 tumourigenic 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 cell cultures. NIH-3T3 cells treated with plasma from the cancer patients presented with both mutated human sequences (*K-ras*) and non-oncogenic human sequences (*p53* and β-globin-encoding sequences) soon after the start of incubation that persisted up to three weeks after the removal of human plasma from the growth medium. Further investigation showed that the treated NIH-3T3 cells did not become cancerous, but acquired the potential for oncogenic activity *in vivo*, resulting in undifferentiated carcinomas when injected into NOD-SCID mice, confirming both Bendich *et al.* (1965)'s theory and the genometastasis theory.

(4) Anticancer treatment. Where the exposure of susceptible healthy cells to tumourigenic 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 Garcia-Olmo *et al.* (2015) and Garcia-Arranz *et al.* (2016), who showed that virtosomes from non-dividing cell populations can reduce tumour size in tumour bearing BDIX rats. Due to the presence of proteins in virtosomes, histological analysis of a range of tissues were performed and confirmed that exposure to proteins from the virtosomes were not responsible for the anti-tumour effects.

(6) Vesicular transport of nucleic acids

All human cells are capable of producing extracellular vesicles (EVs) and many cells release a heterogeneous population of EVs (reviewed in Deregibus *et al.* (2007)). EVs are generally categorised as exosomes (30 – 100 nm), apoptotic bodies (ABs) (up to 4000 nm), prostasomes (40 – 490 nm) and microvesicles (MVs) (100 and 1000 nm), which are sometimes referred to as microparticles or shedding vesicles (Mause & Weber, 2010; Raposo & Stoorvogel, 2013; Ronquist *et al.*, 1978).

The functions of the different EVs are still poorly understood, but exosomes are the most thoroughly characterised (Barteneva *et al.*, 2013). 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). There are reports of MVs and exosomes of different sources containing transposable elements, single stranded DNA, genomic DNA in rats (Serrano-Heras, García-Olmo, D. & García-Olmo, D.C., 2011) and mtDNA. Waldenström *et al.* (2012) have demonstrated that cardiomyocytes can release DNA and RNA containing MVs or exosomes *in vitro*, termed cardiosomes, and proposed that MVs 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. Kahlert *et al.* (2014) identified large fragments (>10 kb) of double-stranded genomic DNA spanning all chromosomes in human exosomes isolated from 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 Thakur *et al.* (2014) reported similar findings in tumour-derived exosomes.

MV 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, San & Wickline, 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.

By carefully examining the literature cited above, it becomes clear that there are many different functional classes of EVs and not all cell-types release all EV types. There also 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) macrophages engulfing and digesting pyrenocytes in lysosomes via DNase II, (3) autophagy of nuclei (and mitochondria) of erythroblasts. As see Sections 3 and 4, apoptosis, macrophages (phagocytosis) and autophagy 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.

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 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 (Keerthivasan *et al.*, 2011)..

(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 neutrophil extracellular traps (NETs) and the snaring and accumulation of both defence-related substances and the invading microorganisms (Lögters *et al.*, 2009). The

resulting high molecular weight cirDNA fragments of NETosis are similar to that of necrosis and correlations between cirDNA levels and NETs have been found in cases of preeclampsia, sepsis, cancer and thrombosis (Pisetsky, 2012).

Macrophages play an important role in the prevention of inflammation, autoimmune responses and irreversible tissue damage induced by excessive or persistent extracellular trap (ET) formation (Boe *et al.,* 2015). The clearance process includes 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 the involvement of other mechanisms to complete degradation or incomplete degradation, resulting in the possible release of partially digested DNA fragments of NET-DNA into circulation. 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 of cirDNA and is involved in or can induce further causes of cirDNA release, such as inflammation.

(a) 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. It 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 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).

(b) Neutrophils and cancer

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. 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 (reviewed in Zawrotniak & Rapala-Kozik (2013). On the other hand, activated neutrophils can exert cytotoxic effects on tumour cells via ROS or defensin release (Granot *et al.*, 2011; Zawrotniak & Rapala-Kozik, 2013).

(c) 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 is 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) by secreting mtDNA in a catapult-like manner. Macrophages have also been shown to form different subtypes and varieties of ETs (reviewed in Boe *et al.* (2015)).

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 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 and discharge the cleaved low molecular weight DNA fragments or fail to do so, causing the phagocytes to die and release their DNA and the DNA of the engulfed cells into the bloodstream (Jiang *et al.*, 2003; Pisetsky, 2012) (Fig. 3). 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 (see Section 5(1)) and accelerated senescence.

(2) Autophagy: Clearance of cell degradation

Macroautophagy is the sequestering of portions of the cytoplasm into double-membraned vesicles that fuse with lysosomes 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 (Oka *et al.*, 2012; Rello-Varona *et al.*, 2012).

As with NETosis (Section 3(8)(b)), autophagy is said to play a tumour suppressive role in the early stages of tumourigenesis (De Bruin & Medema, 2008) and a tumour supporting role in later stages (Mathew *et al.*, 2007). The inhibition of autophagy during the later stages of tumourigenesis will result in cell death via apoptosis and tumour regression. 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 tumourigenesis (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.

(3) Aging

Total cirDNA, unmethylated cirDNA, RNase P-coding cirDNA and Alu repeat (see Section 5(6)(d)) 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 death, as well as decreased clearance and phagocytic capabilities (Jylhävä *et al.*, 2011; Jylhävä, 2013). Wu *et al.* (2002) reported that cirDNA levels were slightly higher in young individuals (<20 years) and in the elderly (~70 years), 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) revealed that total and unmethylated levels of cirDNA reflected 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 (see Section 5(3)(b)).

(4) The action of DNases

Low cirDNA levels in healthy subjects is accompanied with high levels of DNase activity and *vice versa* in patients with cancers and inflammatory diseases (Cherepanova *et al.*, 2008; Gahan, 2012; Tamkovich *et al.*, 2006; Velders *et al.*, 2014). Significantly increased cirDNA levels in healthy subjects due to training are also sufficiently reduced by adaptations of endogenously expressed DNase activity to regain homeostasis (Velders *et al.*, 2014). 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 (reviewed in Buchanan *et al.* (2006)). ExDNase results in the breakdown of the DNA framework of NETs, not only facilitating the release and systemic dispersal of the pathogens (Hawes, Wen & Elquza, 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 and endogenous sources of cirDNA in patients with infections.

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 or encapsulated in vesicles, 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

CirDNA levels are increased in sepsis patients and levels above 800 ng/ml have been associated with poor prognosis. The majority of the cirDNA originates from the host cells and the amount of bacterial DNA in the blood is negligible (Hamaguchi *et al*. 2015). NET formation has been considered as a source for the increased cirDNA levels (Lögters *et al.*, 2009; Margraf *et al.*, 2008), especially in the case of NET-resistant bacteria (e.g. *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*) (De Jong *et al.*, 2014). In this regard cirDNA is seen

as a DAMP, which is released during inflammatory stress and triggers the host immune response (Section 5(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, as 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 (see Section 3(2) and Section 3(3)).

(6) Oxidative stress

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. It can serve 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, the triggering of apoptosis and eventual cell death termed mitochondrial catastrophe or toxic oxidative stress. Oxidative stress in the mitochondria resulted in the oxidation of both released genomic DNA within the NET-like structures and mtDNA (Lood *et al.*, 2016). 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 (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.

(a) 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 a 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.*, 2011; Ermakov *et al.*, 2013).

Ermakov *et al.* (2011) observed 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 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.

(7) Sterile Inflammation

Other than through microbial infection at trauma sites (see Sections 3(3)(a) and 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 (Section 3(8)(c)). 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).

(a) Obesity and sterile inflammation

Obesity has been found to stimulate chronic sterile inflammation that, along with other obesity-related factors such as oxidative stress, induces adipose tissue cell degeneration and turnover. Nishimoto *et al.* (2016) 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 physical activity can be associated with a leukocyte inflammatory response, mechanical and metabolic muscular damage and DNA damage due to oxidative stress (see Section 4(6)), which increases cirDNA levels. However, Breitbach *et al.* (2012) proposed that cirDNA release during exercise may also be independent of inflammatory markers like leukocyte oxidative burst, leukocyte or muscle cell apoptosis. 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 (Fig. 5). 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. 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.

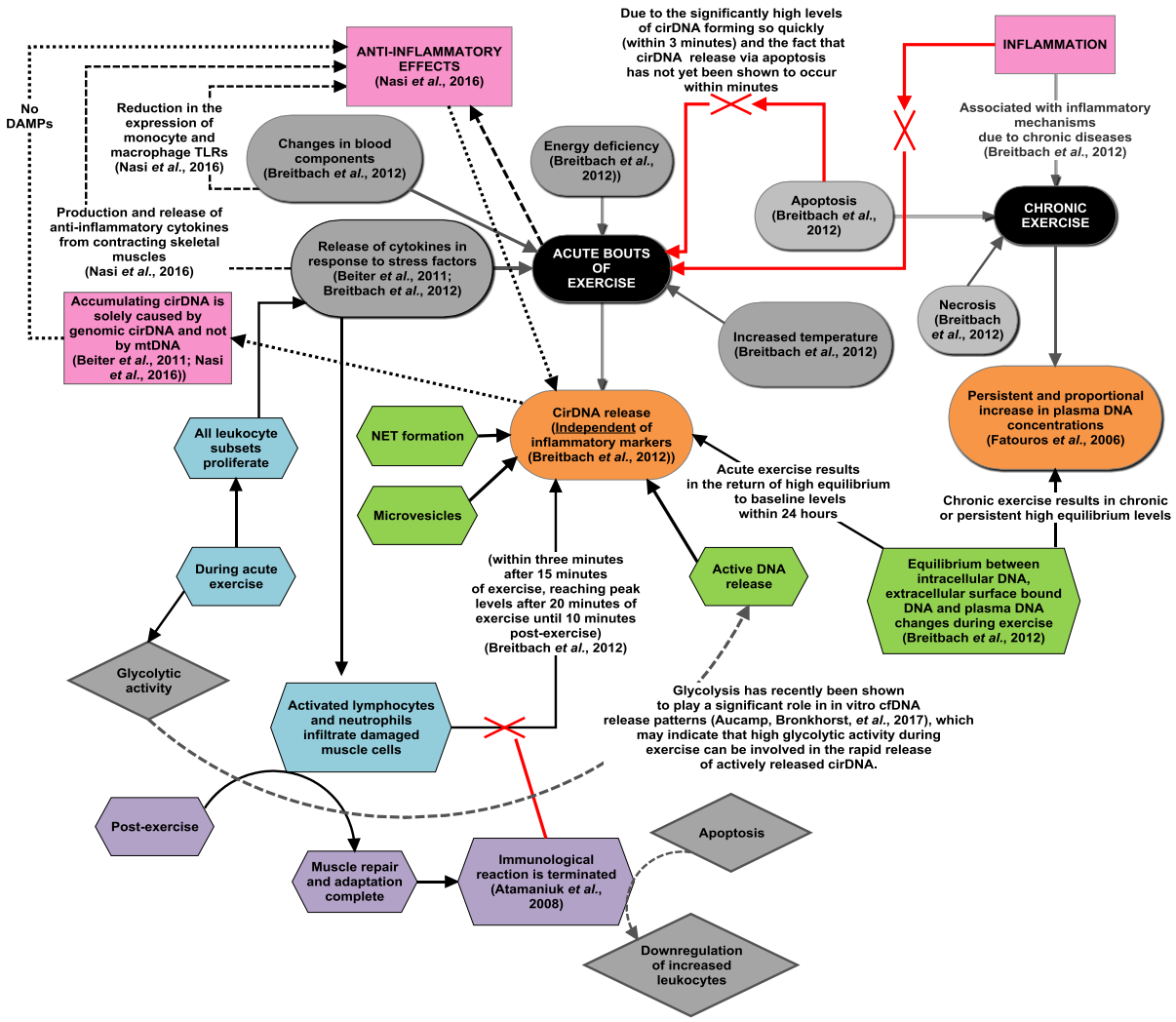


Fig. 5 Acute versus chronic exercise and resulting cirDNA release

(9) Cancer

Stroun *et al.* (1987) characterised DNA from nucleoprotein complexes in the plasma of several cancer types, while no cirDNA could be obtained from plasma samples of healthy subjects. 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. Subsequently, Stroun *et al.* (1989) determined the *in vitro* DNA synthesis pattern (induced by carcinogenic drug or chemical exposure) of the cirDNA from seven cases of patients with malignancies, revealing that five of the cases 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 (from nonmalignant host cells from the immune system in response to the malignant cells) in the plasma of these cancer patients. 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, therefore, be from two sources, the tumour cells and the surrounding tissue cells (Fleischhacker & Schmidt, 2007).

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 can allow the cells to escape senescence (Adams, 2009; 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 (Section 5(6)(b)). Paradoxically, senescence may promote tumourigenesis, possibly by secreting matrix metalloproteases, growth factors and cytokines (Chandler & Peters, 2013; Dimri, 2005).

(a) 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/degradation. These contradictions, summarised in Table 2, are 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, Bronkhorst, *et al.*, 2017) or nonmalignant host cells (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). As mentioned in Section 5(3), laboratory inconsistencies, in particular, can severely complicate the elucidation of the origins and functional roles of cirDNA.

Table 2 Contradictions regarding the origins of plasma tumour DNA

<table>
<tr><th colspan="3">CELL DEATH MECHANISMS</th><th>LIVING CELLS</th></tr>
<tr><td colspan="3">Cell death as a probable source for plasma tumour DNA (García-Olmo et al., 2004; Jahr et al., 2001).
Once increases in cancer cell development leads to an increase in cancer burden, cell death rates and cancer cell proliferation, cell death becomes a likely contribution to cirDNA release (Van der Vaart & Pretorius, 2007).</td><td>During 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).
Active DNA release can also be responsible for the presence of cirDNA (Gahan et al., 2008; Stroun et al., 2001) (Section 3(5)).</td></tr>
<tr><th>NECROSIS</th><th>APOPTOSIS</th><th>PHAGOCYTOSIS
and</th><th>ACTIVE DNA RELEASE</th></tr>
<tr><td>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 (reviewed in Thierry et al. (2016)).
Invasive tumours, therefore, generally have larger necrotic regions than benign tumours.</td><td>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).</td><td>Apoptotic cells can ESCAPE phagocytosis and lead to increased cirDNA levels (Section 3(3)(a)).
On the other hand, phagocytosis can also be a REQUIREMENT as necrotic DNA is not detectable in blood unless macrophages are present (Choi et al., 2005; Diehl et al., 2005) (Section 4(1)).
Apoptotic cells can also interact with proteins, causing the cells to behave or appear necrotic and thereby promoting the modification in the interactions of these cells with macrophages followed by increased DNA release (Choi et al., 2005).</td><td>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).
Tumours can also 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.</td></tr>
</table>

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

(1) Mitotic catastrophe

As mentioned in Section 3(3), certain cancers can resist apoptosis yet still undergo treatment-induced cell death. Experiments where apoptotic activity has been inhibited by genetic manipulation showed increased morphological markers of mitotic catastrophe (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. 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. Fig. 3 summarises the different mechanisms of cell degradation (Roninson *et al.*, 2001), survival (in the case of tumours (Vakifahmetoglu *et al.*, 2008)) and cirDNA release resulting from the abrogation of G1 and/or G2 checkpoints.

Mitotic catastrophe has been characterised as the main form of cell death in radiation therapy (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). Micronuclei can also be degraded by autophagy as shown by the studies of Rello-Varona *et al.* (2012). 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, therefore, serve as sources for cirDNA due to the micronuclei either being broken down or escaping the breakdown process and should the micronuclei be subjected to autophagy, then autophagy will become the source of cirDNA and no longer be categorised as a cause.

(2) 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). 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 *vice versa* were determined and it was concluded that cirDNA is predominantly of donor (hematopoietic) origin. Quantitative PCR studies of the plasma DNA of 34 non-pregnant and 31 pregnant women by Chan *et al.* (2004) revealed that circulating fetal DNA from non-hematopoietic placental cells in maternal plasma was shorter than hematopoietic circulating maternal DNA. 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, also observed that non-hematopoietic cirDNA is shorter than hematopoietic DNA. Tug *et al.* (2015) further implicated hematopoietic cells as an origin for exercise-induced cirDNA.

Recently Snyder *et al.* (2016) determined that fragmentation patterns of cirDNA can be used to identify the origin of this DNA. 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. 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 corroborating 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.

(3) Cell-free circulating mitochondrial DNA

MtDNA can be released from mitochondria into the cytoplasm and extracellular environment during cellular clearance or repair processes, particularly autophagy, apoptosis, or necrosis, see Sections 4(2), 3(3) and 3(2), respectively (Zhang *et al.*, 2016). The presence of circulating mtDNA was first reported by Zhong *et al.*, (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- (Section 4(8)) and age-related inflammatory responses (Section 4(3)), and NETs (Section 3(8)). Chiu *et al.* (2003) have shown that circulating mtDNA is present in plasma samples in both intact cell-free mtDNA and particle-associated forms. It was proposed that the particles with which the mtDNA can be associated, 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. 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).

It is possible that the abovementioned different sizes and 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. Changes in sample storage and processing can, for example, affect the amount of cell-free DNA extracted from cell culture growth medium (Bronkhorst *et al.*, 2015). 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.*, 2016). It is obviously a major issue that different analytical protocols produce 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 research field.

(a) Mitochondrial DNA as DAMPs and NETs during trauma

MtDNA has been shown to contain inflammatogenic unmethylated CpG motifs (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 (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). The inflammatory reactions produce cell damage that releases more mtDNA, resulting in a vicious cycle (Nasi *et al.*, 2016).

McIlroy *et al.* (2015) showed that absolute plasma levels of mtDNA decline in the immediate postoperative period (up to seven hours postoperatively). 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 also 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 were responsible for the release of mtDNA into the circulation after major trauma and subsequent surgical interventions (McIlroy *et al.*, 2015).

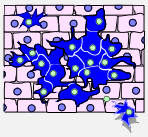
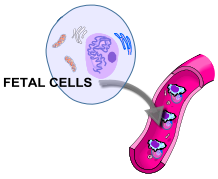
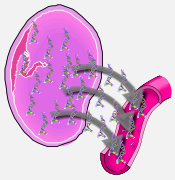
(b) Mitochondrial DNA and aging

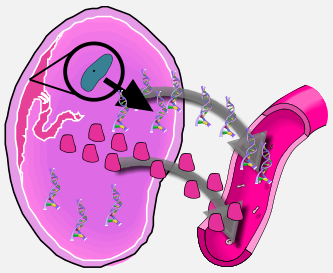
MtDNA plasma levels gradually increase after 50 years of age and a role for familiar/genetic background in controlling circulating mtDNA levels has also been proposed, as the mtDNA values of two members of the same sibling relationship older than 90 years were found to be directly correlated (Pinti *et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

(4) Pregnancy

CffDNA has been detected in amniotic fluid and maternal plasma, serum, urine, cerebrospinal fluid and peritoneal fluid (reviewed in Bianchi (2004)). Putative origins of this fetal DNA in maternal body fluids and contradicting theories are summarised in Table 3. It 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.*, 1998). 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 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 entire fetal and maternal genomes are represented in the maternal plasma at a constant relative proportion.

Table 3 Putative origins of cffDNA in maternal biological fluids and contradicting theories

PUTATIVE SOURCES OF CFFDNA	CHARACTERISTICS
Fetal cells proliferation similar to that of cancer cells 	Theory: 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 (Lo *et al.*, 1997).
Freely-circulating, intact fetal cells in maternal circulation 	Destruction of freely-circulating, intact fetal cells in maternal circulation due to immune reactions (Bianchi, 2004). **HOWEVER** Intact fetal cells in maternal blood are rare. 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 erythroblasts and cell-free fetal DNA (cffDNA) levels. (Bianchi, 2004; Zhong, Holzgreve & Hahn, 2002)
Concentration gradient allows exit of cffDNA from amniotic fluid 	The amount of amniotic fluid fetal DNA is two hundred times that of the amount in the maternal plasma. DNA could, therefore, be transferred directly across the placenta or membranes via a concentration gradient (Bianchi, 2004).
Placental- and pregnancy-related complications 	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). Fetal DNA is hypomethylated and can serve as DAMPS as they are detectable by TLR9, resulting in NF-κB activation and subsequent inflammatory reactions (Section 5(3)(a)) and the induction of preterm birth (Yuen *et al.*, 2010).

The placenta	
	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 (Bianchi, 2004). 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)).
	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).
	In normal pregnancies, grams of placental material are shed daily into the maternal bloodstream without eliciting inflammation (Taglauer, Wilkins-Haug & Bianchi, 2014).
	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)

(5) 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 (see 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, consisting of: (1) Increases in the number of glial nuclei of the hypoglossal nucleus that contained newly synthesised DNA during axonal regeneration; (2) Increased glial DNA synthesis in response to induced changes in neural activity; (3) Significantly increased and persistent newly synthesised brain DNA levels in response to learning (passive avoidance task training); (4) Circadian oscillations of sleep and waking affects brain DNA levels in different areas of the body (e.g. adult 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).

(6) Genetic processes

Fig. 6 summarises mechanisms of the genetic processes, rereplication, endoreplication, gene amplification and transposons, which may be involved in cirDNA production and/or release.

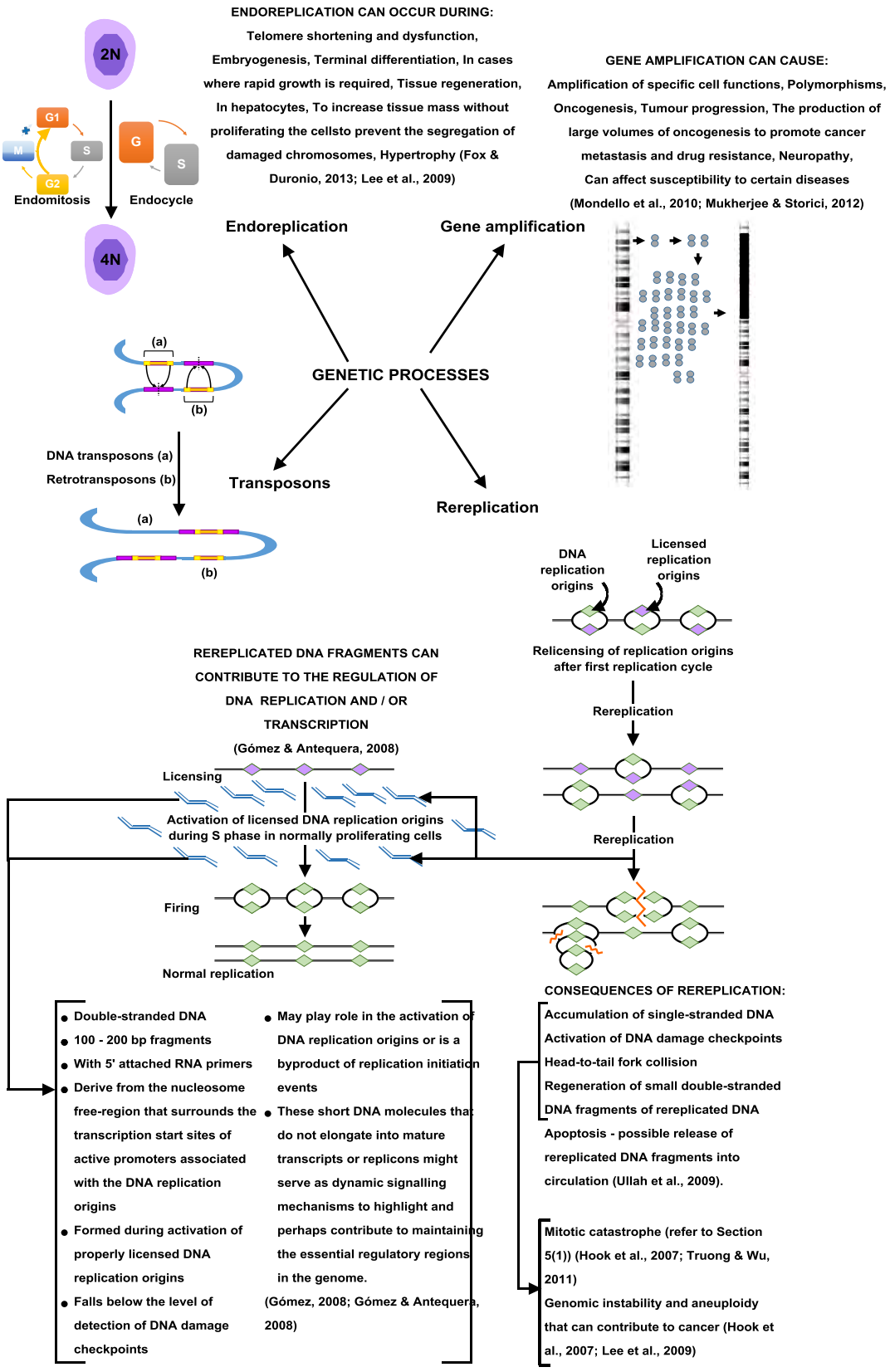


Fig. 6 Summary of genetic processes that can contribute to cirDNA production and/or release

(a) 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). Typically, replication initiation occurs once and only once per cell cycle (Gómez, 2008; Hook, Lin & Dutta, 2007; 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. 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 (Tatsumi *et al.*, 2006).

Consequences of rereplication include cell death and tumourigenesis, but it has also been speculated that rereplicated DNA can contribute to the regulation of DNA replication and/or transcription (Fig. 6). Gómez and Antequera (2008) have identified short rereplicated DNA fragments that are not formed 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 these rereplicated DNA fragments are not limited to cell cultures, they can also form in organisms. It is theorized that these DNA fragments are involved in the activation of licensed DNA replication origins during the S phase in normally proliferating cells. Their characteristics are strikingly similar to that of Pelc's metabolic DNA and virtosomal DNA (see 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, i.e. metabolic and virtosomal DNA are independent of active cell proliferation (and can, therefore, be found in both dividing and non-dividing cells), whereas the rereplication of short DNA fragments require active cell proliferation (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 (see Section 4(9)) and the shorter forms of cffDNA of developing fetuses (see Section 5(4)).

(b) 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; Lee *et al.*, 2009). Endoreplication can occur through developmentally controlled endocycles 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, or through endomitosis, where cells enter but do not complete mitosis (Fox & Duronio, 2013; Lee *et al.*, 2009) (Fig. 6). 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). 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 (see 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). 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 (see Section 5(1)) can also form cirDNA.

(c) Gene amplification

DNA amplification results in an increase in copy number of a discrete chromosomal DNA region (Mukherjee & Storici, 2012) (Fig. 6). 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). 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).

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) (see 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.

(d) Transposons

Gahan and Stroun (2010) proposed that transposons and retrotransposons may be possible sources of circulating nucleic acids (cirNAs). 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. 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) (Bronkhorst, A.J., Wentzel, J.F, Aucamp, J., Peters, D.L., De Villiers, E.P. & Pretorius, P.J., in preparation). 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.

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.*, 2016; Delgado *et al.*, 2013; Gahan, 2006; Holdenrieder *et al.*, 2005; Lichtenstein *et al.*, 2001; Stroun *et al.*, 2001; Ulivi & Silvestrini, 2013; Van der Vaart & Pretorius, 2007; Van der Vaart & Pretorius, 2008*a*). 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 grouping biological features into putative sources of cirDNA and causes of cirDNA release it became clear that a seemingly unintended lack of consensus regarding the very definition of the term "main origin" emerged 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 the most abundant fraction must be the main origin of cirDNA; and (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, which theoretically implies that actively released DNA is the original origin of cirDNA masked by the overabundance of debris-related DNA fragments. Therefore, abundance versus function is where the core of the "controversy" resides. 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 may also be functional. The transfer of tumour DNA to cells via the phagocytosis of tumour apoptotic bodies can promote transformation, malignant development, and angiogenesis (Bergsmedh *et al.*, 2006; Ehnfors *et al.*, 2009). However, this only occurred in Chk2-, p53- and p21-deficient cells and when DNase II is inhibited, indicating that tumour cells are susceptible to information transfer via apoptotic bodies whereas normal cells are effectively protected from this messaging effect unless malfunction of checkpoint controls in DNA replication occurs (Holmgren, 2010). Mittra *et al.* (2015) 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 (see Section 4(6)(a)). The mechanisms of the effects of DNA from cellular breakdown processes on recipient cells still requires investigation. Nevertheless, it is clear that this fraction of cirDNA does have the ability to act as an intercellular messenger under specific favourable conditions. This apparent functionality 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 cell degradation being the main origins of cirDNA is easily corroborated.

However, this says nothing about the biological purpose of cirDNA, 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. The tumour promoting effects of apoptotic bodies in immunodeficient and tumour cells (Holmgren, 2010) are also most likely mere coincidence rather than intentional, due to the cells' compromised/altered defences and clearance mechanisms. 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 was pointed out in this review (Table 1 and Fig. 2) and in our cell culture research, cells

have the potential to release both actively released DNA and DNA from cellular breakdown processes (Aucamp, Bronkhorst, *et al.*, 2017; Bronkhorst *et al.*, 2016) and cellular breakdown levels are increased in cancer patients (see Section 4(9)). It is, therefore, possible that the cirDNA from 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 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 see 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) protected from nucleases via complexation, encapsulation or adherence to cell membranes and (4) their levels are controlled or regulated, reaching maximum levels that cannot be exceeded (see 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 cirDNA, 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 pro-inflammatory DNA (Rock & Kono, 2008) debris (i.e. DAMPs), 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. In addition, by focusing on the functional fraction of cirDNA, keeping in mind its capacity to act as an intercellular messenger, could

provide both multi-purpose biomarkers and 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 a particular cirDNA fraction 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 via whole genome sequencing and cirDNA fragmentation patterns and sizes (Jiang and Lo, 2016; Snyder *et al.*, 2016; Ulz *et al.*, 2016)). 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 "closed-circuit" model of the physiological, biological or pathological area in question to remove the "background noise" produced by the cirDNA from the rest of the organism.

Cell cultures have revealed useful cfDNA characteristics, e.g. different cfDNA release patterns between cell types or origins, the release of cfDNA via multiple mechanisms at different time intervals and strong correlations between active cfDNA release and both glycolytic activity and the G0/G1 phase of the cell cycle (Aucamp, Bronkhorst, *et al*., 2017; Bronkhorst *et al*., 2016; Wang *et al.*, 2017). The theory of the intercellular messaging functions of cirDNA has been strongly supported, as cfDNA has recently been shown to laterally transfer pharmaceutically-induced effects (Aucamp, Van Dyk, *et al.*, 2017) and promote breast cancer cell proliferation via a non-inflammatory TLR9-NF-κB-cyclin D1 pathway (Wang *et al.*, 2017). Furthermore, 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 cfDNA

from several two-dimensionally cultured cell lines (Aucamp, Bronkhorst, *et al.*, 2017; Bronkhorst *et al.*, 2016) and cirDNA from human plasma samples (Applied-Biosystems, 2015). 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 (e.g. fibercell systems and three-dimensional (3D) cell cultures (Antoni *et al.*, 2015; Hughes, 2015; Wrzesinski & Fey, 2013)). The excuse of not using *in vitro* models because they are not properly reflective of *in vivo* conditions are, therefore, becoming increasingly irrelevant. Our recent 3D cell culture study, for example, revealed that cfDNA release mirrors brief and/or minor changes in growth and glucose consumption during both spheroid development and toxicological studies.

8. Conclusions

(1) By replacing the term "main origins of cirDNA" with that of most abundant (i.e. DNA from cellular breakdown mechanisms) and most functional fractions (i.e. actively released DNA) one can provide a clearer picture regarding cirDNA origins.

(2) It is evident that there are various different sources of cirDNA and causes of cirDNA release in the mammalian body.

(3) Most, if not all, of these features have the capability to release DNA into circulation via both cellular breakdown and active DNA release mechanisms.

(4) 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.

(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, environment and the pathophysiology of a disease or disorder.

(6) A more holistic view of cirDNA research is encouraged, taking into consideration both cirDNA fractions and all the biological pathways in which cirDNA is involved.

(7) The use of "closed-circuit" *in vitro* models are strongly encouraged in conjunction with *in vivo* confirmation to effectively isolate and fractionate tissue, target or physiological system-specific cirDNA.

(8) This approach can strongly contribute to the elucidation of the cellular origin and physiological purpose of cirDNA.

(9) Expanding the knowledge regarding the origin and purpose of cirDNA can significantly contribute to the discovery of novel and/or specific biomarkers for diagnostics and prognostics, as well as the discovery of other novel therapeutic uses.

9. Acknowledgements

This work was supported by the National Research Foundation (NRF), South Africa, under grants SFH14061869958 (JA) and SFH13092447078 (AJB) and the George Forster Research Fellowship by the Alexander von Humboldt Foundation (CPSB). The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF.

10. Conflict of interest

The authors report no declarations of interest.

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3.3 Guidelines for authors – Advances in Experimental Medicine and Biology

The following guidelines were provided by the editor of the book series "Circulating nucleic acids in serum and plasma – CNAPS IX", Peter B. Gahan, for publication as part of Advances in Experimental Medicine and Biology:

Each article should be of not more than five pages long (approximately 450 words per page). Each extended poster should not be longer than two pages (approximately 450 words per page). Tables and Figures can be included. Where possible, figures should be in black and white. Manuscripts should have a title, authors' names and addresses, corresponding author's e-mail address, not more than six keywords, an abstract of 200 words, introduction, materials and methods, results, discussion, acknowledgements, conflict of interest and references.

Cite references in the text with author name/s and year of publication in parentheses (Harvard system). E.g. Gahan PB, Anker P, Stroun M. (1973) An autoradiographic study of bacterial DNA in *Lycopersicum esculentum*. Ann Bot 37: 681-685.

3.4 Circulating DNA as homeostatic entities or messengers of genetic information: CNAPS IX conference proceedings publication

Aucamp, J., Bronkhorst, A. & Pretorius, P. 2016. Historical and evolutionary perspective on circulating nucleic acids and extracellular vesicles: Circulating nucleic acids as homeostatic genetic entities. (*In* Gahan, P.B., Fleischhacker, M. & Schmidt, B., *eds.* Circulating nucleic acids in serum and plasma – CNAPS IX. Advances in experimental medicine and biology, 924. Switzerland: Springer International Publishing. p. 91-95).

This review serves as a shortened version of the empirically up-to-date schematic summary of paragraph 2.2, consisting of only the major events that developed and integrated the concepts of heredity, genetic information and cirDNA. The schematic summary paints a picture of how cirDNA can promote synchrony in tissues and organs depending on the source of the message. I serve as the main author and speaker for the article, and as this article stems from the original review in paragraph 2.2, A.J. Bronkhorst and Prof Pretorius were also involved in critically reviewing the manuscript. The article was presented at the CNAPS IX conference in Berlin, Germany, in 2015.

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

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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P.B. Gahan et al. (eds.), *Circulating Nucleic Acids in Serum and Plasma – CNAPS IX*, Advances in Experimental Medicine and Biology 924, DOI 10.1007/978-3-319-42044-8_17

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.

Acknowledgements The postgraduate studies of JA and AB are supported by scholarships from the North-West University and National Research Foundation (NRF), South Africa. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.

Conflict of Interest The authors declare no conflict of interest.

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CHAPTER 4

THE UTILISATION OF CELL CULTURE MODELS IN CIRCULATING DNA RESEARCH

To date there are a vast number of research publications regarding the potential use of cirDNA in the samples of body fluids, but a severe lack of the clinical application thereof. Comparisons between data obtained from different patients with the same cancer or illness from different laboratories yield discrepancies (Fleischhacker & Schmidt, 2007; Malentacchi *et al.*, 2015) and it is not just due to lack of standard operating procedures. Paragraph 3.2 illustrates the vast number of putative sources of cirDNA and causes of cirDNA release, emphasising the complexity of cirDNA contents and characteristics. Studying specific fractions of cirDNA (e.g. the minority functional fractions of newly synthesised and actively released DNA) and/or identifying unique markers of a cancer, disease or disorder within a physiological environment capable of providing multiple sources of cirDNA is no easier than searching for a needle in a haystack. By reducing sources of cirDNA to the areas where disease or cancer is present or to the areas affected by it will greatly improve the chances of discovering novel and specific disease, disorder or cancer biomarkers and elucidating the biological functions and characteristics of cirDNA.

The advantages of using *in vitro* means to overcome *in vivo*-related challenges have been provided in paragraph 3.2. The utilisation of immortal commercial or primary cell lines provides the opportunity of collecting DNA released by only interested cell origins, types or morphologies (2D cell cultures) or tissues or physiological systems such as tumours (3D cell cultures). Using these techniques in conjunction with biological fluid samples (e.g. plasma, serum and urine) can provide much needed insight regarding the characteristics and functions of cirDNA. Restricting cirDNA sources by *in vitro* disease and cancer models can also simplify the discovery of specific biomarkers. For this thesis DNA released into the growth medium of cell cultures (cfDNA) were utilised to introduce the merits of the utilisation of *in vitro* cfDNA in cirDNA research. This chapter focuses on (i) the optimisation of cfDNA collection and quantification, (ii) the screening of cfDNA release patterns and fragment sizes and (iii) the screening of multiple cell lines for the identification of relationships between cfDNA and cell types, origins or activity.

4.1 Optimisation of cell-free DNA collection and quantification methods

In the matter of optimised cfDNA collection methods, the parameters for growth medium collection, storage and cfDNA extraction provided by Bronkhorst *et al.* (2015), Bronkhorst *et al.* (2016a) and Bronkhorst *et al.* (2016b) (in which I participated as co-author), were used as

guidelines. An appropriate quantification method, however, was still required for the quantification of cfDNA.

For the screening of cfDNA release patterns, the quantification of total cfDNA is an appropriate method and for this purpose the Qubit high sensitivity DNA quantification kit was ideal. However, when focusing on the quantification of particular genes in cfDNA, a screening of appropriate reference genes is required for cell line cfDNA. In order to optimise the quantification of cfDNA via quantitative PCR (qPCR), housekeeping genes with sufficient expression stability, levels and reproducibility in cfDNA must be identified. Four cell lines were chosen for the optimization of cfDNA quantification methods, namely two skin cells (melanoma (A375) and normal fibroblasts (ZANLP)), osteosarcoma (143B), which was used in our previous publications (Bronkhorst *et al.*, 2015; Bronkhorst *et al.*, 2016a; Bronkhorst *et al.*, 2016d) in which I participated as co-author, and Rhabdomyosarcoma (RD), larger cells with a slower growth rate than the A375 and 143B, but faster growth rate than ZANLP. The potential of using differences in housekeeping gene levels between the cfDNA of cancer cell lines and that of normal fibroblasts when the cell lines are treated with one another's cfDNA was also evaluated. The results were published in Clinical Biochemistry (paragraph 4.1.2), and summarised in a poster (paragraph 4.1.3) and conference proceedings publication (paragraph 4.1.4) for the CNAPS IX conference in Berlin, 2015.

The presence and release of housekeeping genes involved in metabolism, DNA transcription, oncogenesis and cell motility and structure, in mRNA and cfDNA were evaluated. It was determined that B-actin 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. Interestingly, the cell lines did not release housekeeping genes related to cellular energy metabolism, but were expressed in the cells, suggesting that there is some selectivity involved in the release of cfDNA. This may give some insight on the possible origin of cfDNA. With little variation in the occurrence of housekeeping genes between the cfDNA of cancer cell lines and that of normal fibroblasts, it is clear that housekeeping genes cannot be used to study induced changes in cfDNA content under various experimental conditions.

4.1.1 Guidelines for authors – Clinical Biochemistry

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4.1.2 Article published in Clinical Biochemistry

Bronkhorst, A.J., Aucamp, J., Wentzel, J.F., Pretorius, P.J. 2016. Reference gene selection for *in vitro* cell-free DNA analysis and gene expression profiling. *Clinical biochemistry*, 49(2016):606-608.

A.J. Bronkhorst, J.F. Wentzel and I contributed equally to the article. Prof Pretorius critically reviewed the manuscript

The presence and release of housekeeping genes involved in metabolism, DNA transcription, oncogenesis and cell motility and structure, in mRNA and cfDNA were evaluated in cancer cell lines, A375, 143B and RD, and normal fibroblasts. It was determined that B-actin 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. B-globin came in close second as a candidate reference gene as control for cfDNA analysis and its utilisation in cfDNA quantification is appropriate. However, B-globin showed considerable variation in mRNA expression profiling. The other housekeeping genes also exhibited significant variation in both cfDNA and mRNA analysis, and evaluating their use as controls before their use as reference genes is strongly recommended.

Interestingly, the cell lines did not release housekeeping genes related to cellular energy metabolism, but were expressed in the cells, suggesting that there is some selectivity involved in the release of cfDNA. This may give some insight on the possible origin of cfDNA. With little variation in the occurrence of housekeeping genes between the cfDNA of cancer cell lines and that of normal fibroblasts, it is clear that housekeeping genes cannot be used to study induced changes in cfDNA content under various experimental conditions.

Clinical Biochemistry 49 (2016) 606–608

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Short Communication

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

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ARTICLE INFO

Article history:
Received 10 November 2015
Received in revised form 13 January 2016
Accepted 28 January 2016
Available online 2 February 2016

Keywords:
Cell-free DNA
Cancer
Biomarkers
Tumor markers
Housekeeping genes
Real-time PCR

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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http://dx.doi.org/10.1016/j.clinbiochem.2016.01.022
0009-9120/

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.

Acknowledgments

AJB (SFH13092447078) and JA (SFH14061869958) were supported by post-graduate scholarships from the National Research Foundation (NRF), South Africa. The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

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)

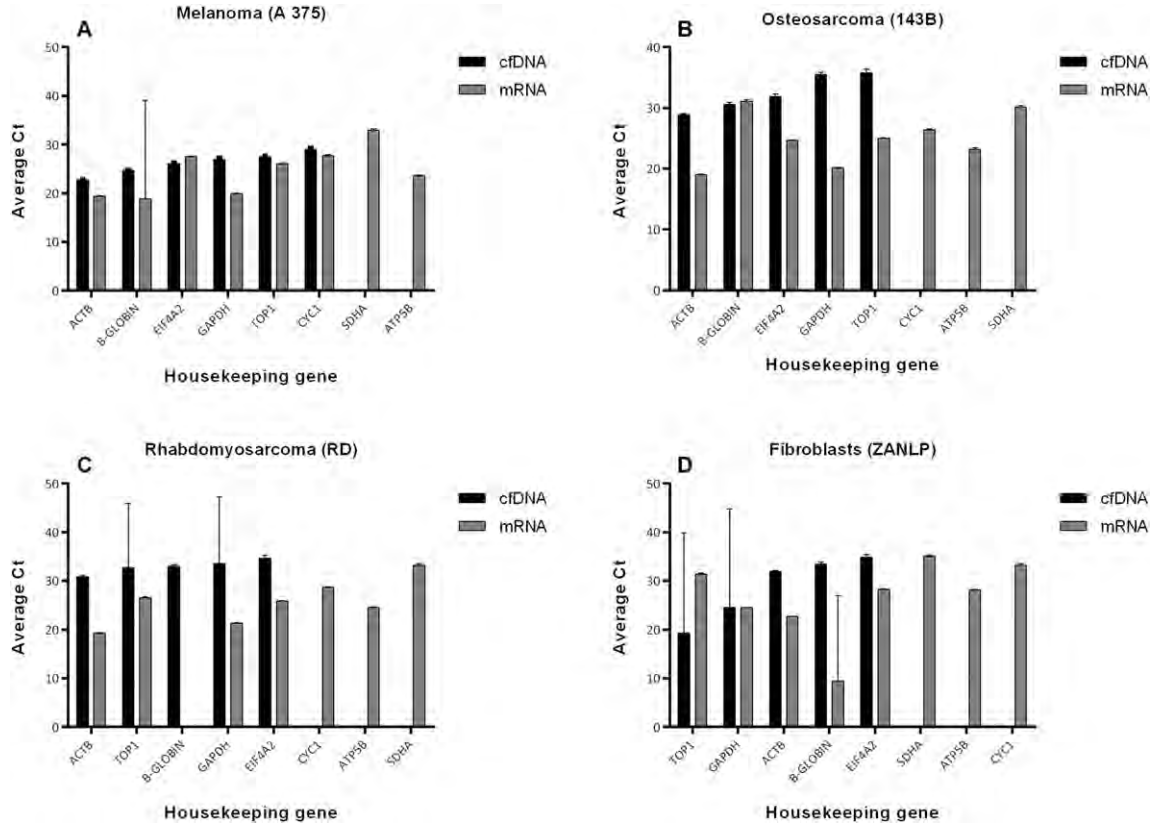

Fig. 1. Average Ct values of HKGs that occur in the cfDNA and mRNA of (*A*) A375, (*B*) 143B, (*C*) RD, and (*D*) ZANLP fibroblast cells. The concentration of cDNA used was kept constant for all four cell lines. The amount of cfDNA used represents the total yield of cfDNA for each cell line. Low Ct values 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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4.1.3 CNAPS Conference poster presentation

The housekeeping gene study of paragraph 4.1.2 (Bronkhorst *et al.*, 2016c) was presented at the CNAPS IX conference in Berlin, Germany, in 2015 as a poster. I serve as the main author and presenter of the poster, and as this article stems from the original article in paragraph 4.1.2, A.J. Bronkhorst, J.F. Wentzel and Prof Pretorius were also involved in critically reviewing the manuscript.

A quantitative assessment of circulating nucleic acids utilizing several housekeeping genes:
Measurements from four different cell lines

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1. INTRODUCTION

Quantitative real-time PCR (qPCR) is regularly used to quantify circulating nucleic acids (cirNAs), referred to as cell-free nucleic acids (cfNAs) in the case of cell cultures, in order to identify biomarkers for various pathologies. The targeting of housekeeping genes as biomarkers, especially β globin, in cirNA and cfNA quantification is becoming quite common [1], but is it the most appropriate gene to target? In qPCR, housekeeping genes are primarily used as internal controls as they are expected to be expressed in all cells regardless of the tissue type, developmental stage, cell cycle state or external signal. However, this is not always the case as studies have shown notable expression variation between healthy and diseased tissues [2], treated and untreated cell lines [3], as well as inter-individual expression [4]. The prerequisites for internal control housekeeping genes are: (i) adequate expression in the target tissues, and (ii) minimal expression variability between both the samples and the experimental conditions used [5]. This promotes the normalization of sample differences generated during sample preparation. In terms of biomarker detection quite the opposite is required, as the goal is to search for variations in gene expression to identify pathologies. However, the internal control prerequisites can be used to optimize cirNA and cell-free DNA (cfDNA) quantification and to study gene expression changes after administering external signals. This study focuses on the utilization of housekeeping gene expression analysis in the optimization of cfDNA quantification.

2. MATERIALS AND METHODS

Cell cultures:

143B (osteosarcoma) and RD (rhabdomyosarcoma) cells were each seeded at 50 % confluence into one T175 flask in 36 ml DMEM (HyClone) supplemented with 10% fetal bovine serum (Biochrom) and 1 % penicillin/streptomycin (Lonza) and incubated at 37 °C in humidified air and 5 % CO_2 for 12 hours. The media was then replaced and the cells incubated for a further 24 hours. Skin fibroblasts (ZANLP) were seeded at 50 % confluence into two T175 flasks in 36 ml DMEM and incubated for 72 hours. The media was then replaced and the cells incubated for a further 72 hours. A375 (melanoma) cells were seeded at 30 % confluence into two T175 flasks in 36 ml of DMEM medium with additional supplementation of 1 % each Amphothericin B (Biochrom), L-Glutamine (Lonza) and non-essential amino acids (Lonza) for 4 hours. The media was then replaced and the cells incubated for a further 16 hours. The culture media of these cell lines were collected, centrifuged at 5 000 x g for 10 min and stored at -80 °C. The cells were trypsinated, 5 x 10^6 cells collected, pelleted at 300 x g for 5 min and the total RNA immediately extracted.

Total RNA extraction and cDNA synthesis:

The total RNA of the four cell lines were extracted with 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 extracted 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 synthesised from the extracted RNA samples with the High Capacity RNA-to-cDNA kit (Applied Biosystems).

CfDNA extraction:

The culture medium of the four cell lines were thawed at 37 °C and concentrated using the Savant ISS110 SpeedVac Concentrator (Thermo Electron Corporation) to a third of the original volume. CfDNA was extracted with the NucleoSpin Gel and PCR Cleanup kit (Machery Nagel) and binding buffer NTB according to the instructions provided by the PCR clean-up user manual.

Real-time PCR:

The primers and probes of seven housekeeping genes from the GeNorm Reference Gene Selection Kit (Primerdesign) and β-globin (IDT, Whitehead Scientific) was used to evaluate the occurrence of housekeeping genes of the four cell lines and real-time PCR was performed according to the instructions of the GeNorm kit manual. Real-time PCR was performed with the RotorGene Q (Qiagen). Fifteen cfDNA replicates and eight cDNA replicates were prepared. 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.

TABLE: Housekeeping genes used during real-time PCR

GENE SYMBOL	GENE	FUNCTION
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Important enzyme in glycolysis with diverse non-glycolytic functions, incl. the binding of DNA and RNA, regulation of transcription and facilitating vesicular transport [6].
ACTB	Beta-actin	Cell motility, structure and integrity and serves as a major constituent in the contractile apparatus [7].
EIF4A2	Eukaryotic translation initiation factor 4A2	Involved in the binding of messenger RNA to the ribosome. Plays a vital role in cancer genesis [8].
SDHA	Succinate dehydrogenase	Part of the Kreb's cycle and respiratory chain (complex II) [9].
CYC1	Cytochrome C-1	Mobile electron carrier between complex III and complex IV of respiratory chain. Also involved in apoptosis [10].
ATP5B	ATP synthase	Complex V of the respiratory chain which produces ATP using the energy provided by the proton electrochemical gradient [11].
TOP1	Topoisomerase 1	Enzyme that controls and alters the topologic states of DNA during transcription [12]
β-GLOBIN	Beta globin	Forms part of the heteromeric hemoglobin protein-complex involved in protein transport [13].

3. RESULTS

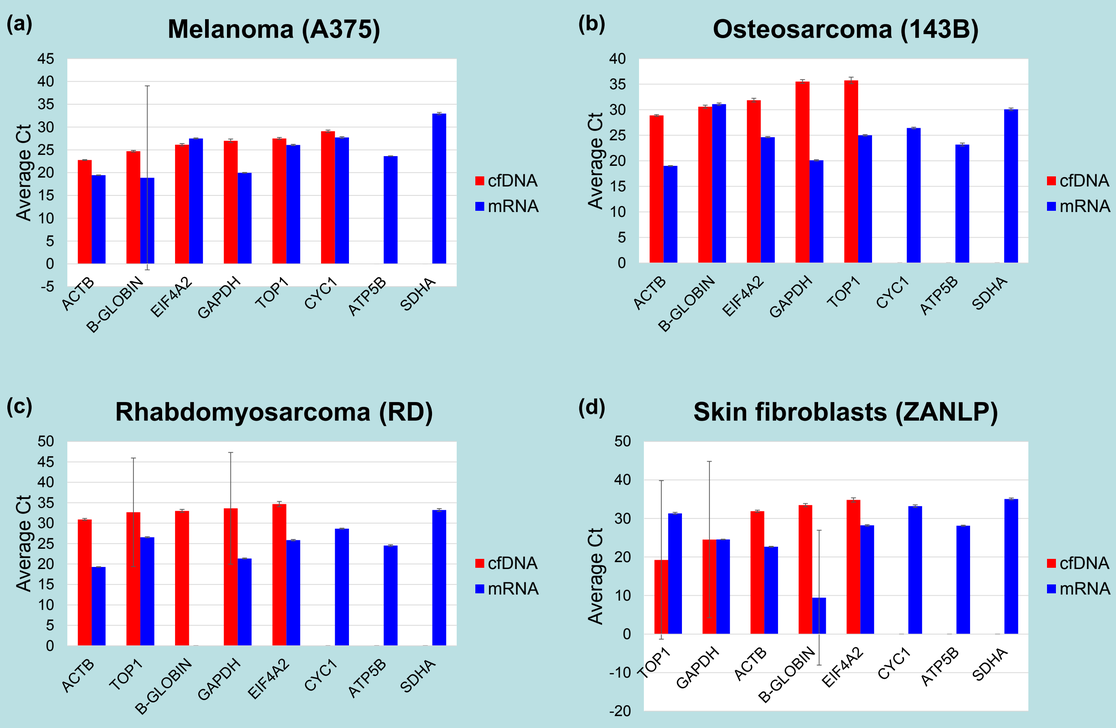

Figure: Average Ct values of housekeeping genes that occur in the cfDNA and mRNA of (a) A375, (b) 143B, (c) RD and (d) ZANLP fibroblast cells. The cDNA concentrations used remained constant in all four cell lines. The concentrations of cfDNA used represent the total yield of cfDNA for each cell line. Low Ct values indicate higher levels of initial PCR template and earlier cycle amplification.

4. CONCLUSIONS

- ACTB was identified as the most appropriate housekeeping gene to use as control for qPCR.
- The remaining housekeeping genes, including β-globin, 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, showing that studying the occurrence of housekeeping genes may not necessarily be the correct method to use when intending to monitor induced changes in cfDNA content during analysis under various experimental conditions.
- 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 results did show that they were expressed.
- Analyzing the stability of target gene amplification in cfDNA samples rather than genomic DNA before using the gene as control for the quantification via qPCR will promote the optimization of cfDNA quantification.

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6. ACKNOWLEDGEMENTS

A special thanks to the North-West University and National Research Foundation (NRF) for their financial support.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

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4.1.4 CNAPS Conference proceedings publication

Aucamp, J., Bronkhorst, A.J., Wentzel, J.F., Pretorius, P.J. Quantitative assessment of cell-free DNA utilizing several housekeeping genes: Measurements from four different cell lines. (*In* Gahan, P.B., Fleischhacker, M. & Schmidt, B., *eds.* Circulating nucleic acids in serum and plasma – CNAPS IX. Advances in experimental medicine and biology, 924. Switzerland: Springer International Publishing. p. 101-103).

The guidelines for authors regarding this publication are provided in paragraph 3.3. The conference proceedings article is based on the CNAPS IX conference poster and serves as a less detailed version of the article of paragraph 4.1.2 (Bronkhorst *et al.*, 2016c). I serve as the main author of the article, and as this article stems from the original article in paragraph 4.1.2, A.J. Bronkhorst, J.F. Wentzel and Prof Pretorius were also involved in critically reviewing the manuscript.

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

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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P.B. Gahan et al. (eds.), *Circulating Nucleic Acids in Serum and Plasma – CNAPS IX*, Advances in Experimental Medicine and Biology 924, DOI 10.1007/978-3-319-42044-8_19

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

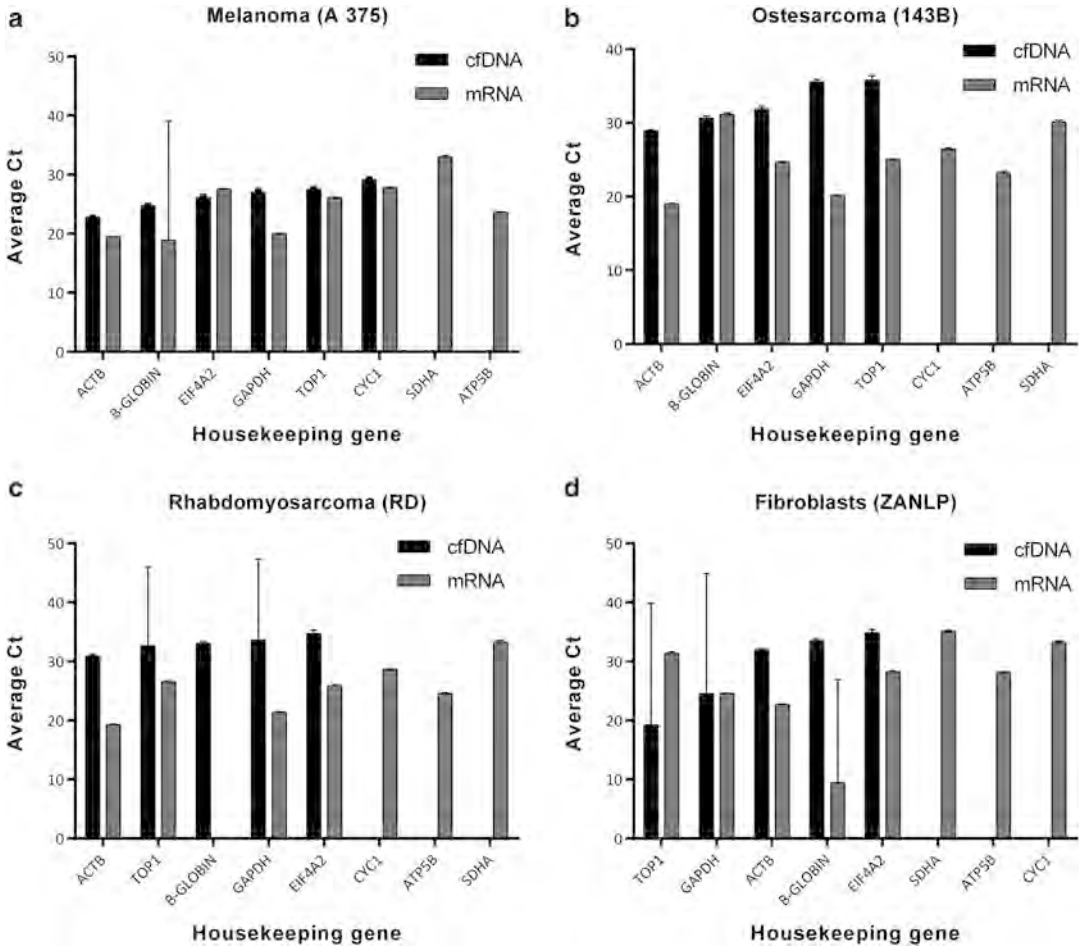

Fig. 19.1 Average Ct values of housekeeping genes that occur in the cfDNA and mRNA of the four cell lines. The cirDNA concentrations used remained constant in all four cell lines. The concentrations of cfDNA used represent the total yield of cfDNA for each cell line. Low Ct values indicate higher levels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

Acknowledgements This work was supported by the North-West University and National Research Foundation (NRF).

Conflict of Interest The authors declare no conflict of interest.

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4.2 Osteosarcoma cells as *in vitro* cell culture model for cell-free DNA research

Osteosarcoma (143B) cells were originally used to evaluate cfDNA release levels, fragmentation patterns, the involvement of cellular breakdown mechanisms in cfDNA release and in which cell cycle stage cfDNA is released (Bronkhorst *et al.*, 2016d; Bronkhorst *et al.*, 2016e). I participated as co-author in these publications.

To summarise Bronkhorst *et al.* (2016d), it was determined that 143B cells released increasing levels of cfDNA as incubation time progresses, reaching the highest amount after 24 hours. Further incubation results in a significant decrease in cfDNA levels. Capillary electrophoresis of cfDNA samples revealed that cfDNA is both actively released and released via apoptosis due to the presence cfDNA fragment sizes associated with the typical apoptotic ladder pattern and a 2 000 bp peak not yet reported in cirDNA research. Confirmation of identification of these peaks as, respectively, apoptotic and actively released were provided via flow cytometric apoptosis and necrosis measurements. It was further determined that cfDNA is associated with proteins, as treatment of collected cell growth medium with denaturing agents results in increased cfDNA levels. Whether these proteins are nucleosomes, virtosomes or extracellular vesicles have yet to be determined.

The amount of cells at 24 hours after growth medium renewal were shown to decrease in the S phase and significantly increase in the sub G0/G1 phase, suggesting that the increased 2 000 bp sized cfDNA levels after 24 hours of incubation is not associated with the process of DNA replication. This supports Gahan and Stroun's (2010) theory that the G0/G1 phase is the realm in which actively released cfDNA is synthesized and that it is not likely related to mitotic DNA synthesis (Bronkhorst *et al.*, 2016d).

4.3 Kinetic analysis, size profiling and bioenergetic association of cell-free DNA released by cell lines

To determine whether the cfDNA release patterns described in paragraph 4.2 are general or 143B cell-specific occurrences, several cell lines were screened for their cfDNA release levels, patterns and fragment sizes. The following information was published in the article of paragraph 4.3.6.

4.3.1 The three cell-free DNA release patterns of *in vitro* cell cultures and their fragment size distributions

Three non-cancerous and four cancerous human cell lines with varying tissue origins, growth rate and cancer status were chosen for screening and their results were compared with one another and the results obtained for 143B cells (Bronkhorst *et al.*, 2016d): (i) rhabdomyosarcoma (RD),

(ii) skin fibroblast (FIBRO) (also referred to as ZANLP in our previous study (Bronkhorst et al., 2016c)), (iii) melanoma (A375), (iv) keratinocytes (HaCat), (v) cervical adenocarcinoma (HeLa), (vi) human embryonic kidney (HEK-293) and (vii) hepatocellular carcinoma (HepG2). The screening results (refer to article in paragraph 4.3.6) revealed that there are three distinct cfDNA release patterns: (i) HepG2 and 143B cells showed a tendency of increasing cfDNA levels, (ii) HeLa, RD and HEK-293 showed a tendency of decreasing cfDNA levels and (iii) the skin cells HaCaT, FIBRO and A375 showed varied levels of increasing and decreasing cfDNA release. The varying levels and unstable growth of HaCaT, FIBRO and A375 hinted towards the release patterns being skin origin-specific. The other two release patterns, on the other hand, did not seem to show peculiarities suggesting a correlation with tissue origins. The results also confirmed the presence of nucleosomal fragments (which we believe serves as a sign of apoptosis (Bronkhorst *et al.*, 2016d)) and a 2 000 bp peak (which we believe represents actively released cfDNA (Bronkhorst *et al.*, 2016d)) in HepG2, 143B, HEK-293 and FIBRO. The different cfDNA release patterns were determined to likely be due to fluxes in actively released cfDNA levels, but the electropherogram data did not provide an explanation as to why there are different cfDNA release patterns.

4.3.2 Strong similarities in electropherogram data between cell cultures and plasma samples

CfDNA fragment size electropherograms obtained for each cell line were compared to that of cirDNA extracted from human plasma samples in an application note from Applied Biosystems (2015). The electropherogram data were strikingly similar, with the presence of both the nucleosomal fragments and a 2 000 bp in the cell culture samples being confirmed to occur in plasma samples (refer to article in paragraph 4.3.6). The similarities between the cfDNA of cell cultures and cirDNA of plasma samples, therefore, 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) plasma or serum samples for cirDNA research.

4.3.3 Bioenergetic parameters reveal correlations with cell line growth rate, origin and cancer status

Cellular metabolism was also investigated to determine whether there is any correlation between cfDNA release levels, cfDNA release patterns, glycolysis and OXPHOS. It was observed that:

1. HepgG2 and RD metabolic activity correlated well with the theory of cancer cell lines utilizing aerobic glycolysis as a predominant energy source (De Preter *et al.*, 2015).
2. Fast growing A375 cells require higher levels of glycolysis, but can also use OXPHOS.

3. Aerobic glycolysis dependence of HeLa was also less prominent than that of the other cancer cell lines.
4. 143B cells were shown to have slightly lower OXPHOS and more significantly lower glycolysis levels compared to A375.
5. HaCaT and HEK-293 showed high levels of both OXPHOS and glycolysis activity, which is expected for normal cell lines, with HEK-293 showing potential signs of increased anabolism.
6. FIBRO shared metabolic similarities to that of the cancer cells

4.3.4 Cell-free DNA levels correlate with glycolysis activity

There were no statistically significant correlations between cfDNA levels and OXPHOS activity, but there does appear to be a tendency toward an inverse correlation. A statistically significant correlation between the cfDNA release and glycolysis activity has, however, been identified (refer to article in paragraph 4.3.6). This correlation was detected when the eight cell lines were divided into group 1 (HEPG2, 143B, RD and FIBRO) and group 2 (HEK-293, A375, HaCaT and HeLa). This grouping of the cell lines correlated with: (i) the obtained bioenergetics analysis data, (ii) the cfDNA release patterns of each cell line and (iii) the cell line's glycolysis dependence as energy source. 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 glycolysis activity.

4.3.5 Lack of correlation between cfDNA levels and oxidative phosphorylation correlates with reference gene study results

Interestingly, 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 (with the exception of A375 releasing CYC1), but were expressed by the cultured cells (refer to article in paragraph 4.1.2 (Bronkhorst *et al.*, 2016c)), which may explain or contribute to the lack of correlations found between cfDNA release and OXPHOS activity (refer to article in paragraph 4.3.6).

4.3.6 Article published in Cellular and Molecular Life Sciences

Aucamp, J., Bronkhorst, A.J., Peters, D., Van Dyk, H.C., Van der Westhuizen, F.H., Pretorius, P.J. 2017. Kinetic analysis, size profiling and bioenergetic association of DNA released by selected cell lines *in vitro*. *Cellular and molecular life sciences*, 74:2689-2707. The final publication is available at Springer via https://link.springer.com/article/10.1007%2Fs00018-017-2495-z.

The guidelines for authors regarding this publication are provided in paragraph 2.1. I performed the majority of the cfDNA extractions, quantifications and fragment size analysis and drafting of the manuscript. H.C. van Dyk performed the bioenergetics analysis as initiated by me. H.C. van Dyk, D. Peters and A.J. Bronkhorst assisted with the experiments and in the final editing of the manuscript. A.J. Bronkhorst drafted the figures and Prof Pretorius and Prof Van der Westhuizen critically reviewed the manuscript.

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

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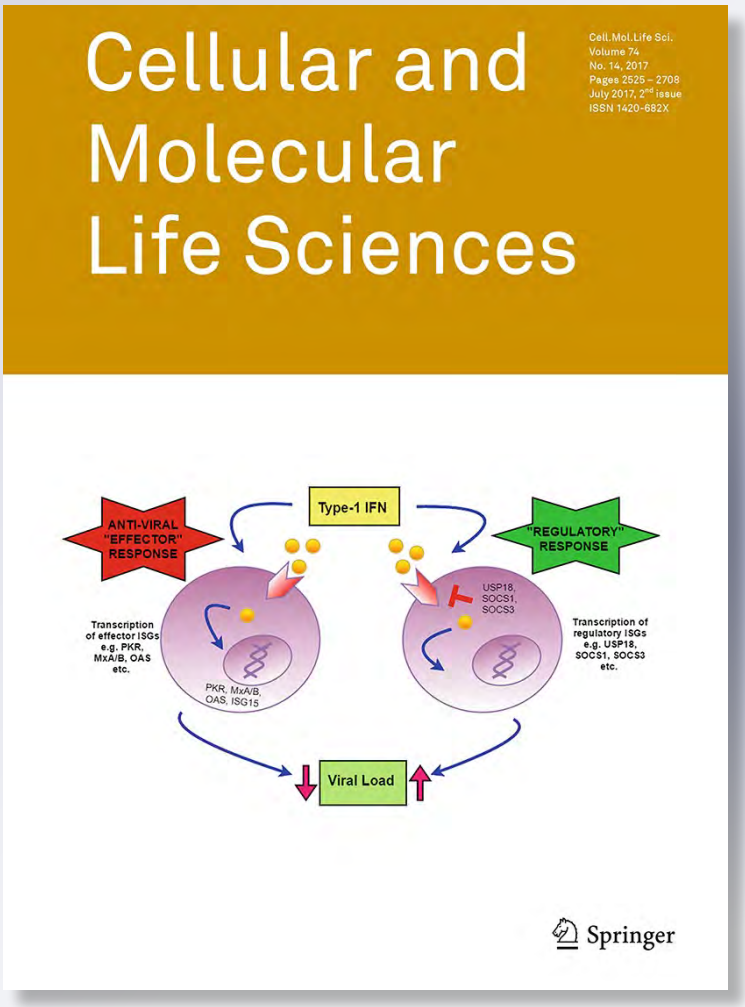
Cellular and Molecular Life Sciences

ISSN 1420-682X
Volume 74
Number 14

Cell. Mol. Life Sci. (2017) 74:2689-2707
DOI 10.1007/s00018-017-2495-z



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Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

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

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Received: 15 November 2016 / Revised: 19 February 2017 / Accepted: 22 February 2017 / Published online: 18 March 2017

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

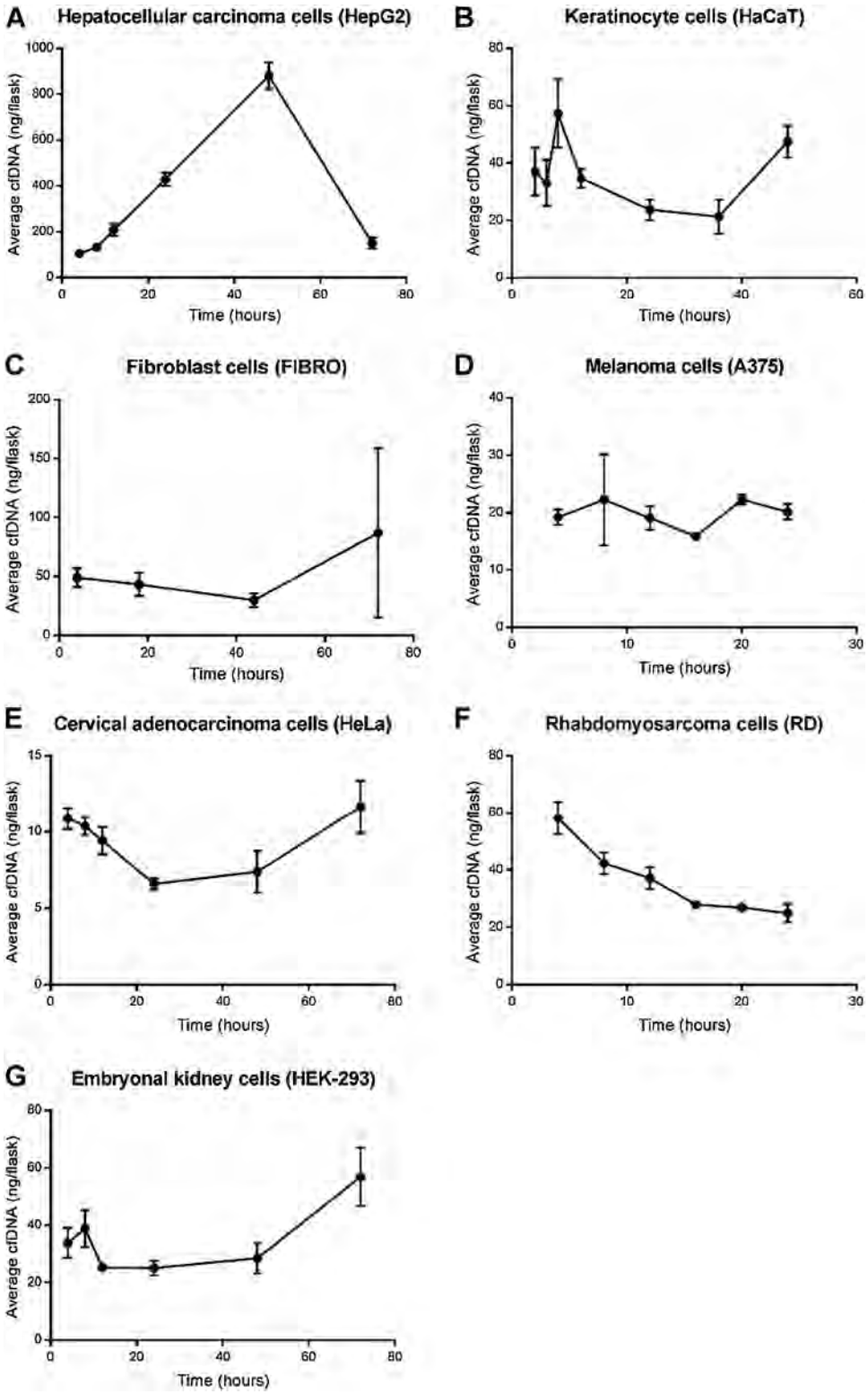

Fig. 1 CfDNA levels (ng/flask) of **a** HepG2, **b** HaCaT, **c**FIBRO, **d** A375, **e** HeLa, **f** RD, and **g** HEK-293 cell lines after several time intervals of incubation following medium renewal. The value of each bar represents the average (±SD, $n=6$) amount of cfDNA released in 12 mL of growth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

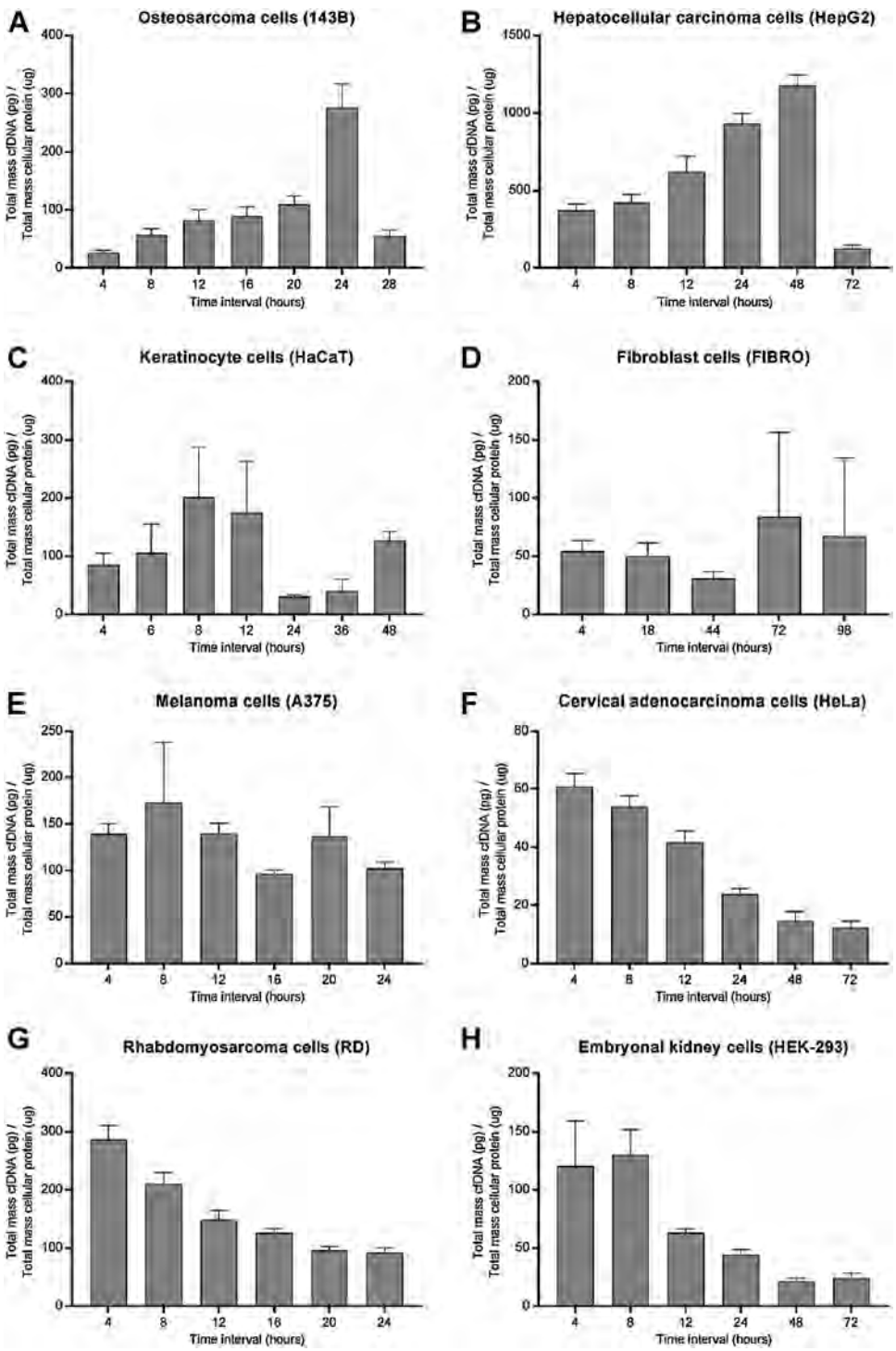

Fig. 2 CfDNA release patterns of **a** 143B quantified by qPCR using β-globin (from Bronkhorst et al. [14]), **b** HepG2, **c** HaCaT, **d** FIBRO, **e** A375, **f** HeLa, **g** RD, and **h** HEK-293 cell lines (quantified using the Qubit high sensitivity assay) after the indicated hours of incubation 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.

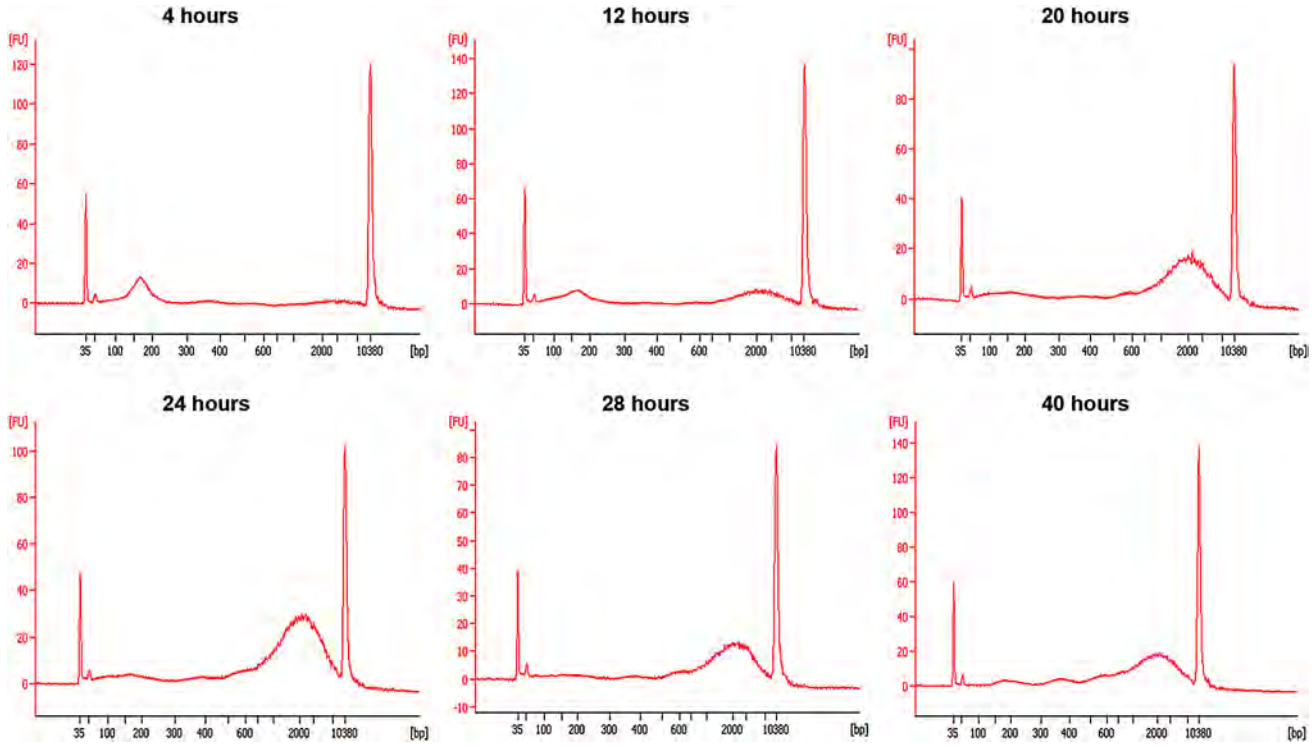

Fig. 3 Capillary electropherograms showing the fragment sizes of cfDNA isolated from 143B cells after 4–40 h of incubation following medium renewal [14]. The peak at 150–200 bp was shown to be representative of apoptosis, while larger peaks arising after 12 h of incubation and reaching a maximum at 24 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

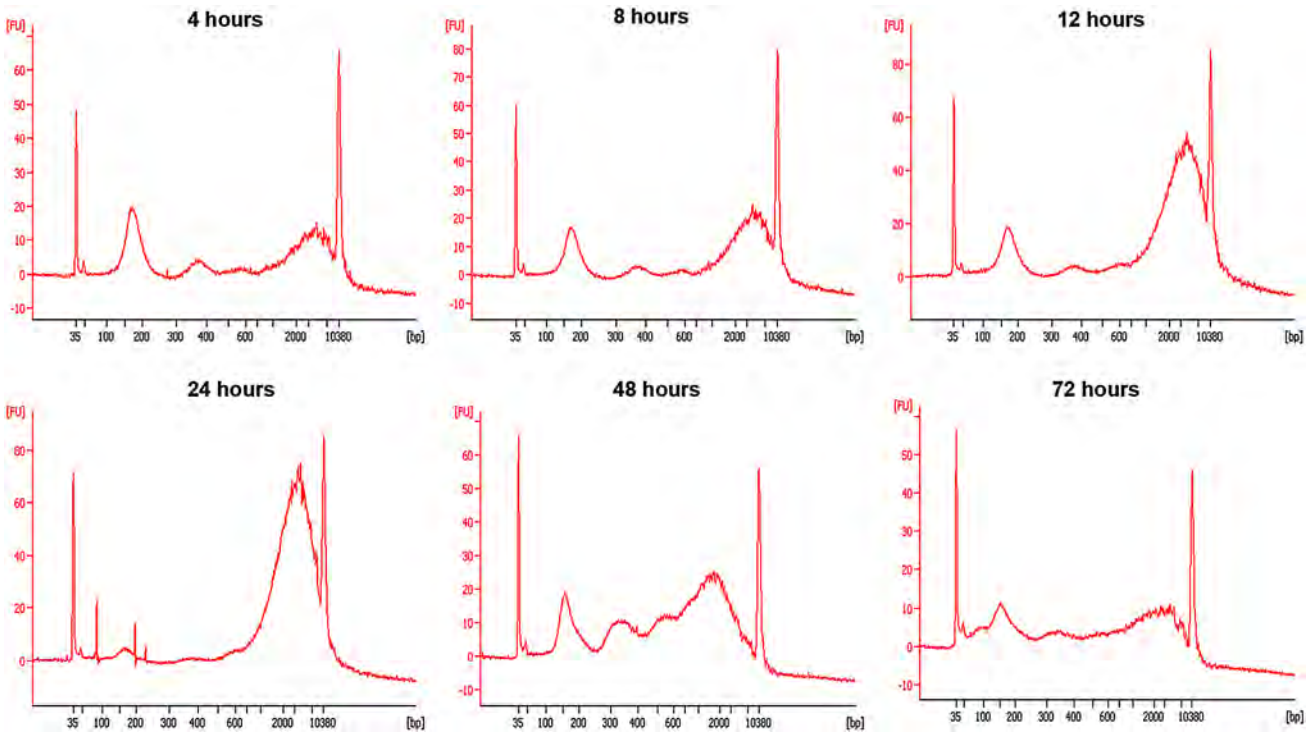

Fig. 4 Capillary electropherograms showing the fragment sizes of cfDNA isolated from HepG2 cells after 4–72 h of incubation following medium renewal. Each shows a typical ladder pattern associable with nucleosomal subunits of predictable sizes that are most prominent at around 150–200, 300–400, and 500–600 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

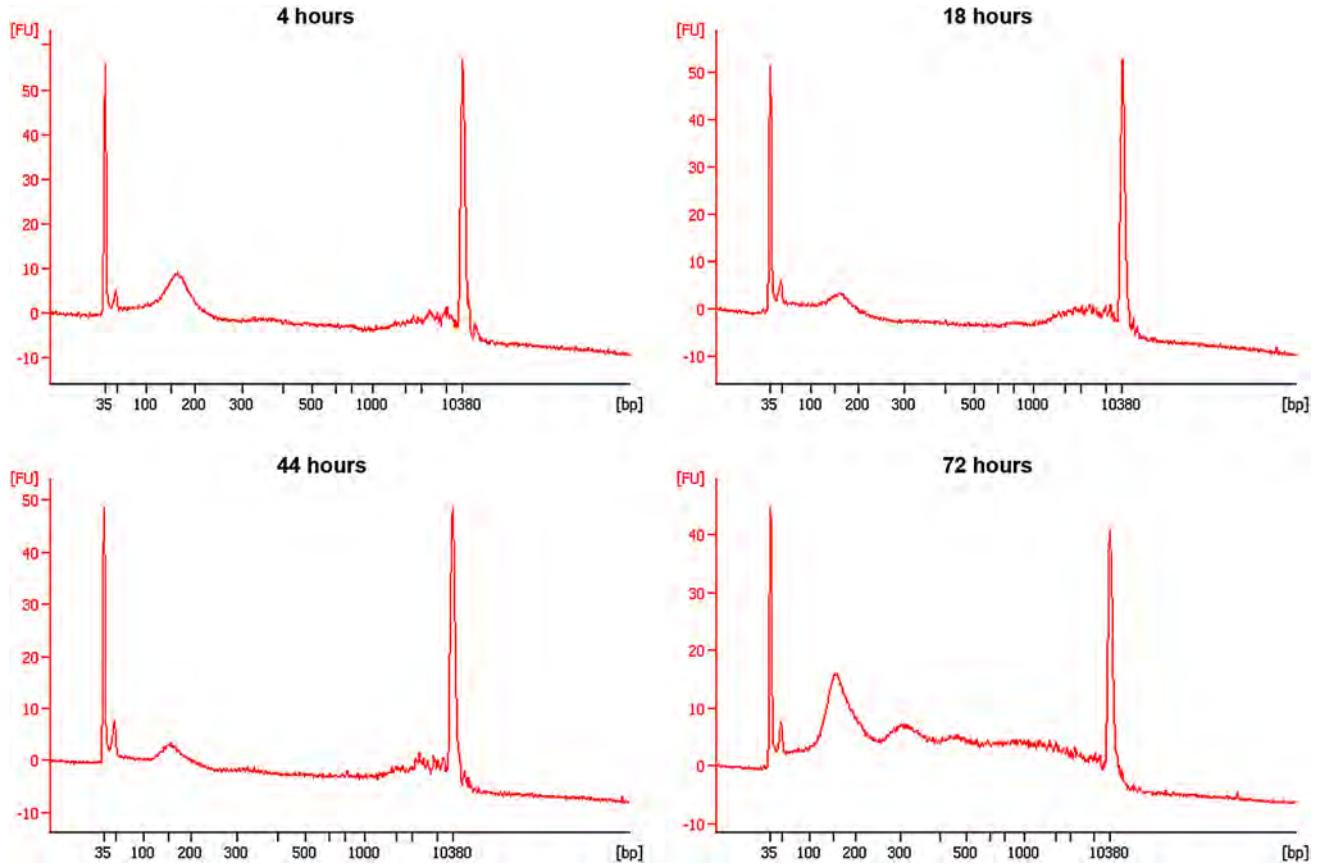

Fig. 5 Capillary electropherograms showing the sizes of cfDNA isolated from FIBRO cells after 4–72 h of incubation following medium renewal. Very prominent in each electropherogram is the peak at 100–200 bp. This peak is associable with apoptosis and becomes exceptionally prominent at 72 h of incubation. 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

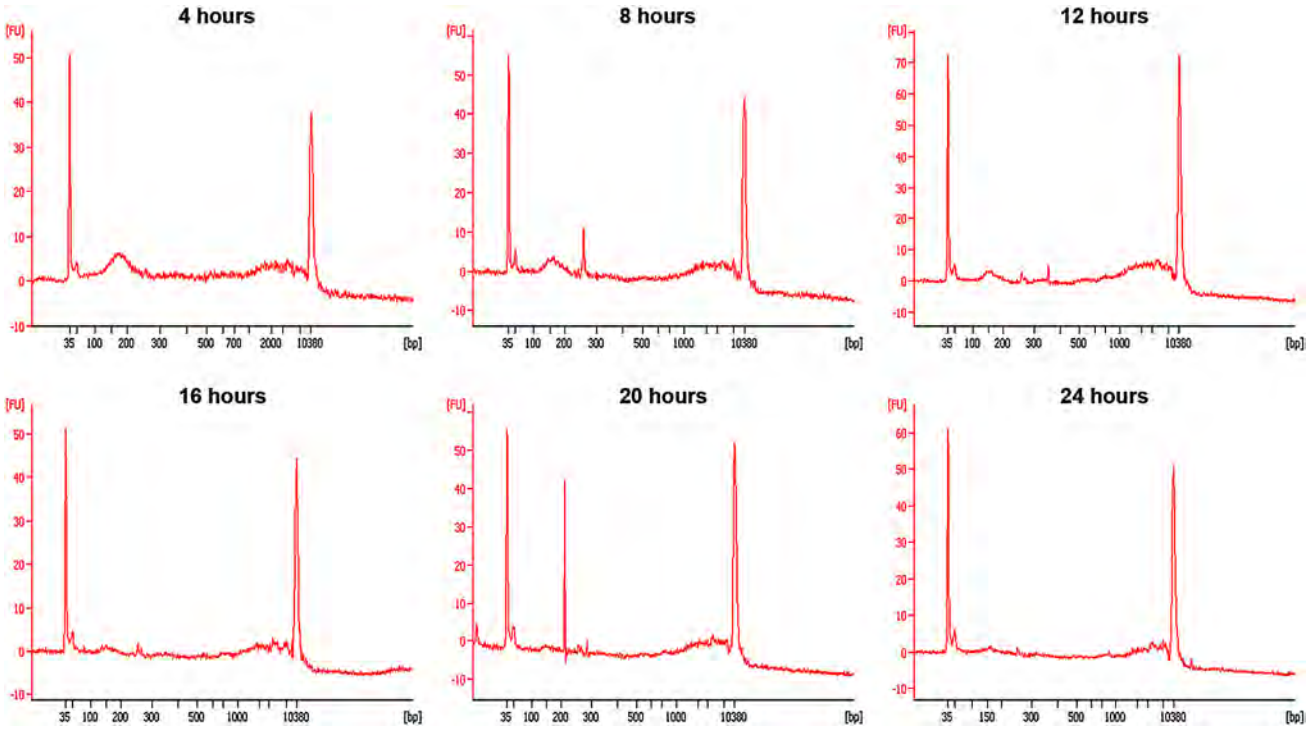

Fig. 6 Capillary electropherograms showing the fragment sizes of cfDNA isolated from RD cells after 4–24 h of incubation following medium renewal. A peak is prominent at 100–200 bp from 4 h of incubation that has been shown to be associable with apoptosis [14]. This peak can be seen declining over time, 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

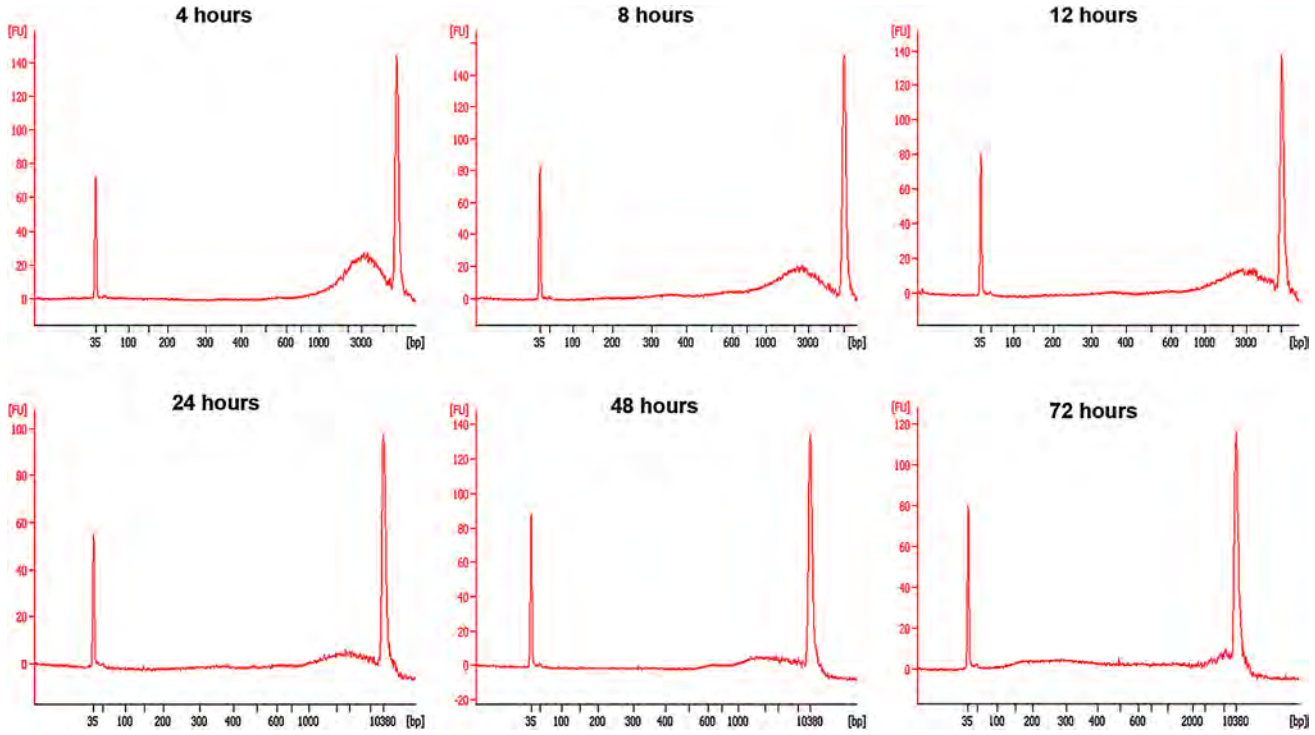

Fig. 7 Capillary electropherograms showing the sizes of cfDNA isolated from HEK-293 cells after 4–72 h of incubation following medium renewal. The electropherograms show no indications of smaller peaks. Instead, a peak with maximal fluorescence is visible at 2000–3000 bp. Once again, this peak diminishes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

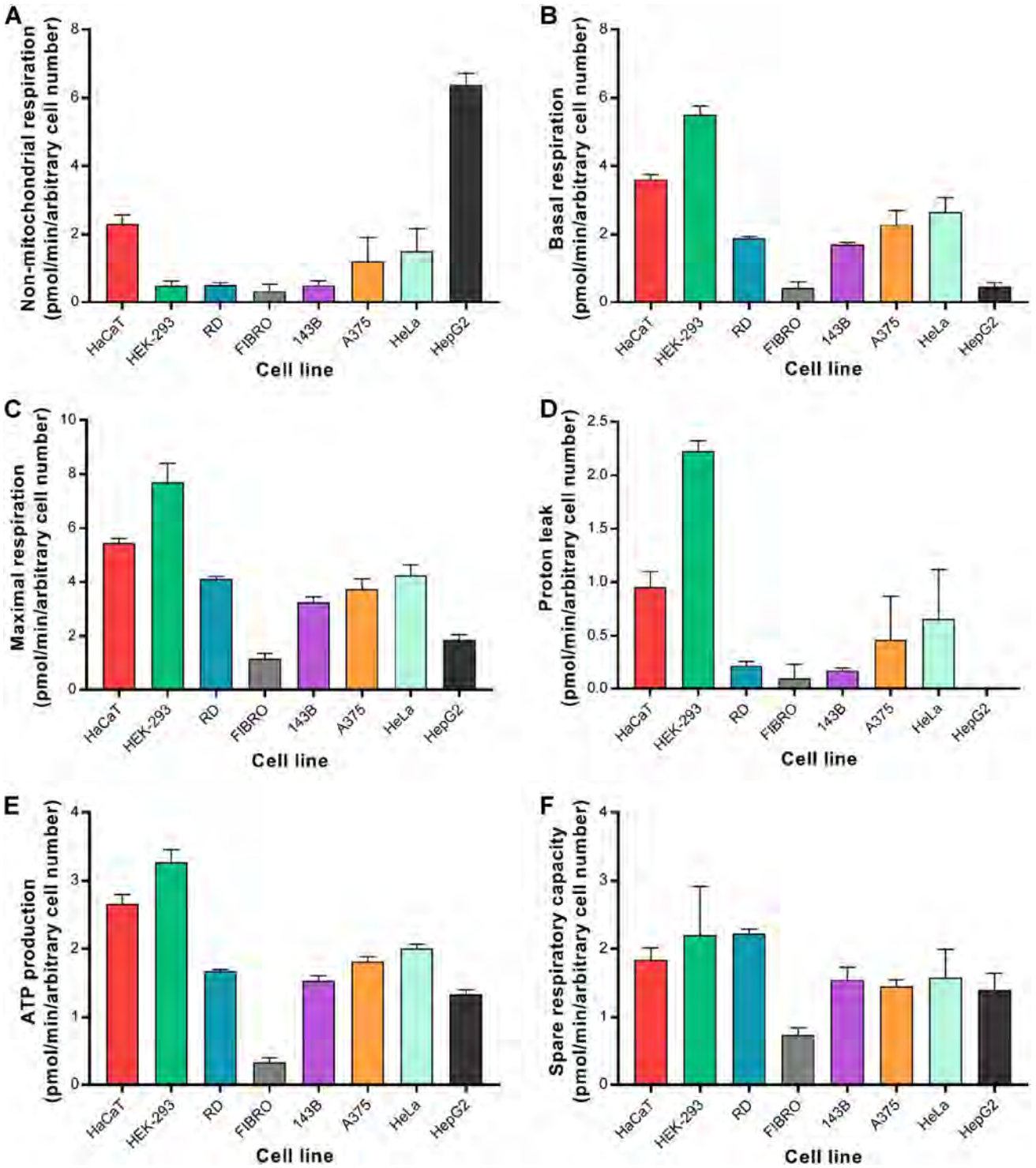

Fig. 8 Histograms depicting eight cell lines for each of the OXPHOS parameters determined using the Mito stress test ($n = 5$–6). **a** Non-mitochondrial respiration, **b** basal respiration, **c** maximal respiration, **d** proton leak, **e** ATP production, and **f** spare respiratory capacity. *Error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

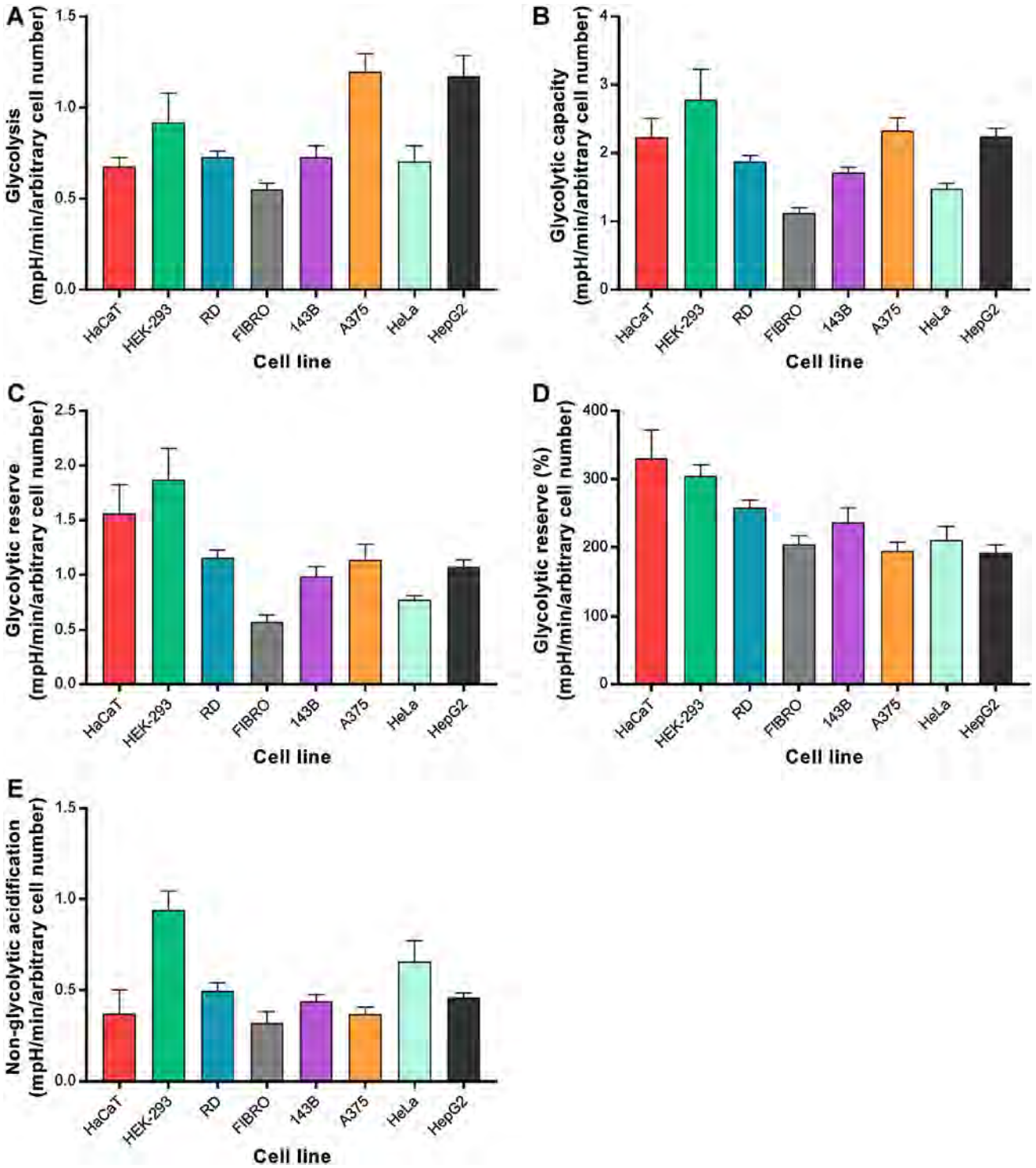

Fig. 9 Histograms depicting eight cell lines for each of the glycolytic parameters determined using the glycolysis stress test ($n = 5$–6). **a** Glycolysis, **b** glycolytic capacity, **c** glycolytic reserve, **d** glycolytic reserve (%), and **e** non-glycolytic acidification. *Error bars* indicate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

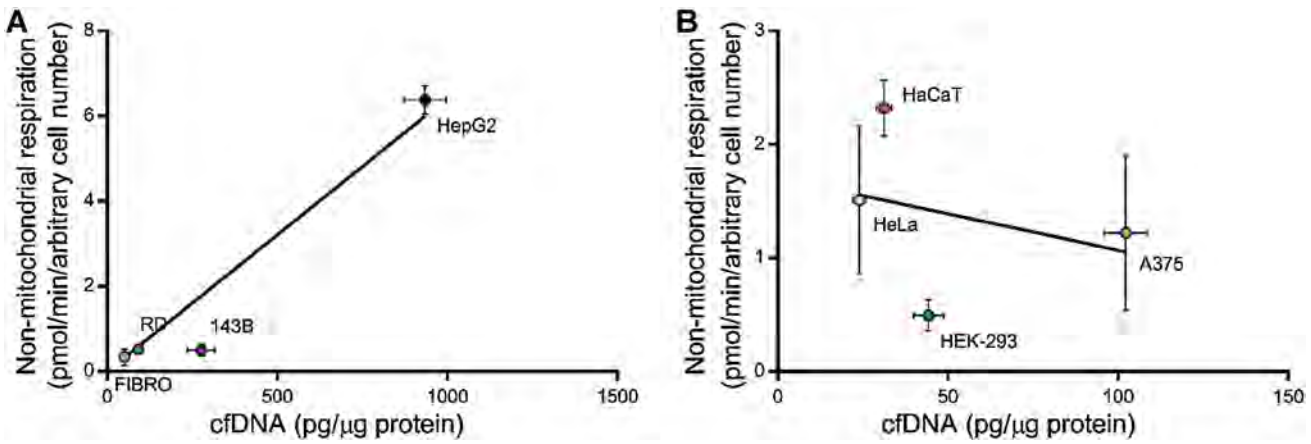

Fig. 10 Scatter plot depicting the correlation between non-mitochondrial respiration and cfDNA release for **a** group 1 (HEPG2, FIBRO, RD, and 143B) and **b** group 2 (HeLa, HaCaT, A375, and HEK-293). Values indicate the mean ± standard deviation

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

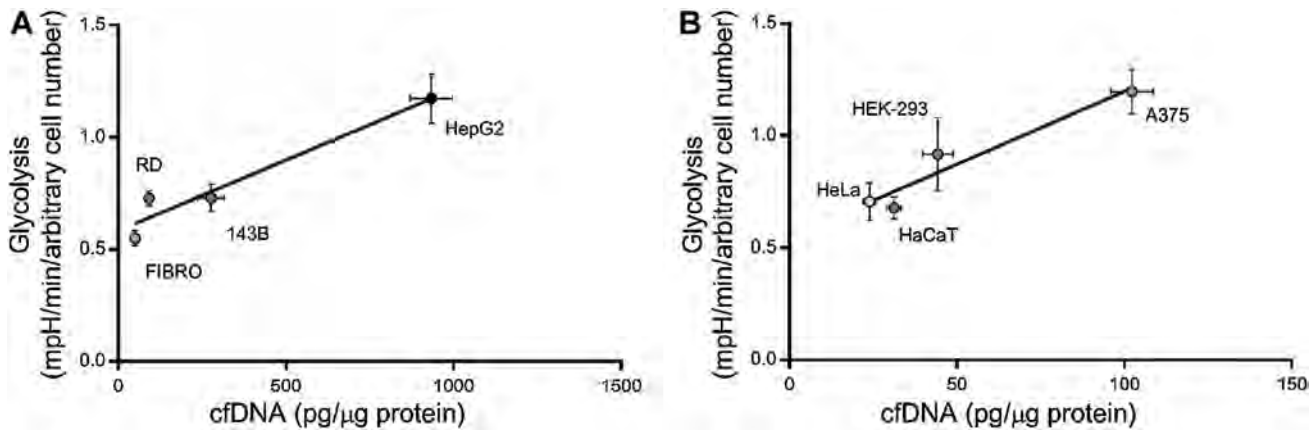

Fig. 11 Scatter plots depicting the correlation between glycolysis and cfDNA release for **a** group 1 (HEPG2, FIBRO, RD, and 143B) and **b** group 2 (HeLa, HaCaT, A375, and HEK-293). Values indicate the mean ± standard deviation

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.

Acknowledgements This work was supported by the National Research Foundation (NRF), South Africa [Grant Numbers SFH14061869958, SFH13092447078]. The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the authors and are not to be attributed to the NRF.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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CHAPTER 5

LATERAL TRANSFER OF PHARMACEUTICALLY-INDUCED EFFECTS *IN VITRO*: CLINICAL IMPLICATIONS OF THE PUTATIVE MESSAGING FUNCTIONS OF CELL-FREE DNA

As mentioned, studies have shown that newly synthesised, actively released nucleic acids (i) can translocate to nearby and remote areas of the body, enter cells and genetically or epigenetically alter their biology and (ii) have been implicated in oncogenesis, genometastasis, the bystander effect, inhibiting tumour growth and the spread of resistance to pharmacological treatment and pathogen virulence (Aucamp *et al.*, 2016a; Avery *et al.*, 1944; Berry & Dedrick, 1936; Bulicheva *et al.*, 2008; Catlin, 1960; Chen *et al.*, 2014; Ermakov *et al.*, 2011; Garcia-Arranz *et al.*, 2016; Garcia-Olmo *et al.*, 2015; García-Olmo *et al.*, 2010). Since cirDNA can transmit genetic or epigenetic information, does this imply that cirDNA can end up transferring genetic or epigenetic alterations induced by environmental effects and/or xenobiotic exposure? If so, this implies that cirDNA will spread these induced changes across cells, tissues and organs similar to the bystander effect transmitting irradiation-induced damage to adjacent non-irradiated systems, in turn supporting the concept of cirDNA serving as homeostatic entities or messengers that can promote synchrony of adaptation in tissues and organs (paragraph 3.4 (Aucamp *et al.*, 2016b)).

However, this also implies that cirDNA can be capable of transferring these induced changes to patients during blood transfusions, implying that the presence of cirDNA in biofluids can have biochemical or physiological and, therefore, clinical implications that requires urgent attention.

5.1 Circulating DNA and blood donation

Blood can be transfused without prior modification (whole blood transfusion) or be divided into erythrocyte, plasma, cryo- and platelet precipitates and sometimes granulocytes. The DNA of a donor can, therefore, be transferred to a recipient via (i) cirDNA freely circulating in the plasma fluid, (ii) cirDNA bound to the surfaces of blood components, such as erythrocytes and platelets, and/or (iii) via the DNA of the blood components themselves, such as the platelets and granulocytes. The latter refers to the transfer of genomic DNA and is, therefore, not applicable to this study, though it may be just as capable of transferring xenobiotic-induced effects from donors to recipients if the xenobiotics can genetically or epigenetically affect genomic DNA.

CirDNA loss after collection will likely be limited: (i) blood plasma, the most likely source of cirDNA, is frozen for storage that will inhibit extracellular DNase activity that can degrade the DNA; (ii) whether DNases are present in precipitated blood components is not known. However, cell-

surface-bound cirDNA is effectively protected from DNases (paragraph 3.2); (iii) CirDNA can pass through blood transfusion filters (which is normally 170 – 200 microns, but can be reduced to finer filters, e.g. 60 microns, with additional filters) that protects recipients from potentially harmful microaggregates that form during blood storage (National-blood-users-group). García-Olmo *et al.* (2010) showed that cirDNA from human plasma can pass through 0.4 micron filters of Corning Transwell plates and there were no significant differences between the effects of human plasma administered to cell cultures indirectly through the Transwell plates and plasma administered directly to the cells, indicating that no DNA was lost due to the filter. Additionally, studies by Fuchs *et al.* (2013) showed that neutrophil extracellular traps (NETs) can also pass through blood transfusion filters and induce effects associated with NET functions. As mentioned in the article of paragraph 3.2, NETs form high molecular weight cirDNA fragments similar to necrosis, indicating that all putative fractions of cirDNA (including, but not necessarily limited to, that of cellular breakdown and active DNA release mechanisms) are equally capable of being transferred to recipients.

Since blood transfusion processes can, therefore, not prevent the transfer of cirDNA to transfusion recipients, the next logical step would be to look into blood donation guidelines regarding medication use. To protect both blood donors and recipients, guidelines are in place that allows, defers or disqualifies blood donations by donors taking medication. These guidelines are primarily based on (i) the status of the underlying disease, (ii) serious pharmacological complications in recipients, especially teratogenic effects, and (iii) whether the medication can affect blood characteristics, e.g. coagulation and platelet number or activity (Becker *et al.*, 2009). Deferral periods do not, however, take pharmacokinetics into consideration and, in some cases, also neglect to take into account the plasma content of the blood products and the dilution of medication concentrations upon transfusion, resulting in many unnecessary deferrals. Compromises can, however, be made when medication only affects certain blood products, e.g. the platelets and plasma of donors taking plasma-bound teratogenic medication may not be used, but the erythrocytes can be donated. Pharmacological mechanisms that limits blood product donations or result in donation deferral or disqualification are listed in Table 5-1 (aaBB, 2016; American-Red-Cross, 2017).

Table 5-1: Pharmacological mechanisms of medication that affects eligibility of blood donation

Pharmacological mechanism	No deferral required	Deferral until a certain period after medication use was stopped	Disqualification as a blood donor
Over-the-counter oral homeopathic medications, herbal remedies and nutritional supplements	Acceptable to donate blood products		
Anti-platelet agents (affects platelet function)	Whole blood may be donated	2 – 14 day deferral before platelets may be donated	
Anticoagulants (may cause excessive bruising or bleeding in donors)		2 – 7 day deferral for the benefit of the donor	
Teratogenic medication (can cause birth defects if transfused to a pregnant woman)		Deferral period of several months to years depending on the medication	Certain medications, e.g. etretinate for psoriasis treatment, can disqualify donors
Growth hormone from human pituitary glands (may cause the development of a rare nervous system condition, Creutzfeldt-Jakob disease)			May not donate blood products
Bovine insulin (due to risk of transmitting “mad cow disease”)			May not donate blood products
Hepatitis B immune globulin (hepatitis prophylaxis)		1 year deferral to prevent possible transmission of hepatitis should the prophylaxis treatment not have prevented disease	
Experimental or unlicensed medication and vaccines		1 year deferral or as indicated by medical director	
Antibiotics (deferrals are primarily to prevent transmission of infection than due to the medication)		Oral antibiotics: on day of last dose; Intravenous antibiotics: 10 day deferral	
Vaccines		Deferral period depends on which vaccine was received	

Deferral guidelines are primarily in place to prevent donor injury and the direct transfusion of harmful medications to the recipient. What can be concluded from these deferral guidelines is that, other than critical and obvious pharmacological complications to the recipient, little further attention is paid to the pharmacological effects and adverse effects of non-deferred medication. Whether pharmacological effects can also be transferred indirectly to recipients via biochemical changes in the blood products and, should this be the case, whether these biochemical changes

are persistent well after the discontinuation of treatment to allow transferral after deferral periods have not yet been determined or considered. There have been reports of persistent adverse effects in patients after discontinuing medication, such as fluoroquinolone antibiotics (ISMP, 2016) and discontinuation syndromes resulting from medication withdrawal reactions, e.g. from selective serotonin reuptake inhibitors (Gandhimathi & Balammal, 2013). In terms of the transfer of information from blood donor to recipient via cirDNA, epigenetic effects can have a particularly significant impact. Many common non-epigenetic pharmaceutical compounds have been found to induce alterations in DNA methylation patterns or histone confirmations, including celecoxib (COX-2 inhibitor for pain and inflammation), hydralazine (calcium ion balancer in vascular smooth muscle for hypertension treatment) and valproate (short chain fatty acid anti-epileptic) (Lötsch *et al.*, 2013).

5.2 Demonstrating, *in vitro*, whether circulating DNA can be affected by and transfer *in vivo* pharmaceutically-induced effects by using valproic acid

Valproic acid (VPA) is widely used as an anticonvulsant, mood stabilizer and migraine prophylaxis (Phiel *et al.*, 2001; Spina & Perugi, 2004) and has been found to directly target and inhibit histone deacetylase (HDAC) (Phiel *et al.*, 2001) and induce replication-independent active DNA demethylation by stimulating the accessibility of a demethylase enzyme (Detich *et al.*, 2003), resulting in widespread epigenetic reprogramming (Milutinovic *et al.*, 2007). VPA can, therefore, result in the induction of DNA methylation-regulated gene expression, which has been theorized to be the cause for VPA's teratogenic effects (Massa *et al.*, 2005). Furthermore, pre-clinical and clinical trials have also been performed with VPA as an anti-tumour agent (Blaheta *et al.*, 2005; Braiteh *et al.*, 2008), as HDAC inhibitors have been found to, for example, inhibit tumour cell proliferation (Blaheta *et al.*, 2005) and induce the expression of MICA or MICB that sensitizes cells to NK cell-mediated killing (Zhang *et al.*, 2009). Altered gene expression via epigenetic processes have been shown to regulate metabolic-related genes and to modulate metabolic dysfunctions (Xu *et al.*, 2016). VPA and its metabolites also impair oxidative phosphorylation (OXPHOS) playing a central role in valproate-induced steatosis and steatohepatitis (Luís *et al.*, 2007). The inhibition of mitochondrial pyruvate oxidation (Silva *et al.*, 1997) and 2-oxoglutarate- and glutamate-driven OXPHOS through the inhibition of dihydrolipoyl dehydrogenase activity (Luís *et al.*, 2007) was identified as the mechanisms behind this. However, the epigenetic effects of VPA on OXPHOS were not determined and may also have played a role.

Blood donation of epilepsy patients is prohibited by most institutions, due to the teratogenic effects of the medication taken, including VPA. Little information, however, is supplied regarding VPA use for other indications, such as migraine prophylaxis and mood disorders, but there are cases where only the erythrocytes of donors taking VPA are donated. Though not necessarily a pharmacological compound eligible for blood donation, VPA does have many potential

biochemical effects that can be targeted experimentally in order to determine whether or not pharmaceutically-induced effects can be transferred from treated systems to untreated ones via cirDNA. In this thesis, particular focus was given to the metabolic and DNA methylation effects of VPA.

5.2.1 Lateral transfer of valproic acid-induced changes in cell-free DNA characteristics and cellular metabolism

In order to determine the effects of VPA-treatment on cfDNA release, HepG2 cells were therapeutically treated (in other words a treatment that elicit measurable biochemical effects without killing the cells by the time that samples are collected) with two 24 hour doses of 5 mM of VPA (refer to article in paragraph 5.2.3). This resulted in a significant increase in cfDNA release. When the collected cfDNA was administered to untreated cells, it appeared that the cfDNA derived from the untreated cells had a more significant effect than that of the VPA-treated cells, resulting in a nearly two-fold increase in subsequent cfDNA release, whereas the cfDNA derived from the VPA-treated cells resulted in subsequent cfDNA release similar to that of untreated control cells.

Due to the OXPHOS-inhibiting effects of VPA, the Seahorse XFe96 analyser (Seahorse Biosciences, USA) and two kits, a Seahorse XF Cell Mito Stress Test Kit and Seahorse XF Glycolysis Stress Test Kit, were used to determine whether cfDNA can transfer these OXPHOS effects to untreated cells.

From these bioenergetics analyses it was determined that (for more detailed results and discussions, please refer to article in paragraph 5.2.3):

1. The effects induced by the cfDNA of untreated and VPA-treated cells occur nearly immediately.
2. The effects are of relatively short duration.
3. These effects are primarily changes in glycolytic activity, correlating with previous findings (paragraphs 4.3.3 – 4.3.6).
4. Multiple doses (three doses) of cfDNA produced different glycolytic effects compared to single doses.
5. In both single and multiple doses, the effects of the cfDNA derived from VPA-treated cells were more significant than that of the cfDNA derived from untreated cells.
6. Most importantly, these effects induced by cfDNA could not be replicated by fragmented genomic DNA (gDNA) controls from both untreated and VPA-treated cells.
7. Though the metabolic effects of cfDNA diminished after 24 hours, the effects of cfDNA treatment on the cells' subsequent cfDNA release patterns reveal that these treatments do

still affect cells after 24 hours, perhaps at lower levels that no longer can affect cells at metabolic levels.

The mechanism or reason behind the metabolic effects of the cfDNA has not yet been determined. A likely explanation is that the widespread epigenetic reprogramming induced by VPA (Milutinovic *et al.*, 2007), which may include the HDAC inhibiting activity induced by 5 mM VPA, could have resulted in changes in cellular metabolic activity. The potential of cfDNA to transfer epigenetic changes from VPA-treated to untreated cells was, therefore, assessed in paragraphs 5.2.4 – 5.2.6.

5.2.2 Guidelines for authors – Biochimie

(https://www.elsevier.com/journals/biochimie/0300-9084/guide-for-authors. Date of access: 26 January 2017)

The following article (paragraph 5.2.3) was submitted as a regular research paper. This Elsevier paper has a "your paper your way" policy, allowing authors to submit manuscripts without specific formatting requirements. Once accepted for publication, however, the guidelines listed in paragraph 4.1.1 are employed. The abstract has a limit of 300 words.

5.2.3 Article published in Biochimie

Aucamp, J., Van Dyk, H.C., Bronkhorst, A.J., Pretorius, P.J. 2017. Valproic acid alters the content and function of the cell-free DNA released by hepatocellular carcinoma (HepG2) cells *in vitro*. *Biochimie*, 140(2017):93-105.

I performed the treatment of cell cultures, cfDNA and gDNA extractions, quantifications and fragment size analysis and drafting of the manuscript. H.C. van Dyk performed the bioenergetics analysis and assisted in the drafting of the manuscript and figures. Prof Pretorius critically reviewed the manuscript.

Biochimie 140 (2017) 93–105

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Valproic acid alters the content and function of the cell-free DNA released by hepatocellular carcinoma (HepG2) cells *in vitro*

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ARTICLE INFO

Article history:
Received 12 February 2017
Accepted 27 June 2017
Available online 28 June 2017

Keywords:
Cell-free DNA
Intercellular messenger
Glycolysis
Valproic acid

ABSTRACT

Background: It has long been believed that cell-free DNA (cfDNA) actively released into circulation can serve as intercellular messengers, and their involvement in processes such as the bystander effect strongly support this. However, this intercellular messaging function of cfDNA may have clinical implications that have not yet been considered.
Methods: CfDNA was isolated from the growth medium of HepG2 cells treated with valproic acid (VPA). This cfDNA was then administered to untreated cells and cellular metabolic activity was measured.
Results: VPA altered the characteristics of cfDNA released by treated HepG2 cells *in vitro*. When administered to untreated cells, the cfDNA from cells treated with VPA resulted in the dose-dependent induction of glycolytic activity within 36 min of administration, but little to no alterations in oxidative phosphorylation. The glycolytic activity lasted for 4–6 h, whereas changes in subsequent cfDNA release and characteristics were found to remain persistent after two 24 h treatments. Fragmented genomic DNA from VPA-treated cells did not induce the effects observed for cfDNA obtained VPA-treated cells.
Conclusions: It is possible for cfDNA to, under *in vitro* conditions, transfer pharmaceutically-induced effects to untreated recipient cells. Further investigation regarding this occurrence under *in vivo* conditions is, therefore, strongly encouraged.
General significance: The intercellular messaging functions of cfDNA present in donated biological fluids has potential clinical implications that require urgent attention. These implications may, however, also have potential as new forms of treatment that can circumvent pharmacological barriers.

1. Introduction

In 1928, Fred Griffith discovered that infectious heat-killed bacteria could transfer pathogenic characteristics to live non-pathogenic strains via an interchange of culture medium [1]. Subsequent experiments showed that the acquisition of pathogenic characteristics of the non-pathogenic strains could be accredited to the transmission of genetic material [2], the first evidence for the existence of DNA outside cells. The presence of DNA in an extracellular environment (cell-free DNA (cfDNA)) of organisms from all kingdoms (reviewed in Ref. [3]), including humans [4] and the many endogenous and exogenous sources of extracellular DNA in humans (apoptosis, necrosis, bacterial and viral DNA, for example) [5] have since been identified (for a comprehensive review of cfDNA research history and development, refer to [6]).

Elevated cfDNA levels have been associated with many diseases and physiological conditions and their structures and DNA sequences very often reflect characteristics of the tissue from which they originate [7–9]. The utilization of cfDNA as non-invasive diagnostic and prognostic markers, therefore, became a great interest and current cfDNA-related research is predominantly focused on translating these diagnostic and prognostic potentials into clinical practice. However, a critical factor that has not yet been considered by researchers is the clinical implications that the proposed messaging functions of cfDNA may have. Actively released nucleic acids can translocate to neighbouring or remote parts of the body, enter target cells and genetically or epigenetically alter their biology [10–15] and has been implicated in oncogenesis, the bystander effect, the blocking of tumor growth, and the spread of bacterial and viral virulence and antibiotic- and chemoresistance [2,6,12,13,15–19].

The lateral transfer of xenobiotic-induced cfDNA changes is,

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http://dx.doi.org/10.1016/j.biochi.2017.06.016
0300-9084/

Abbreviations

CfDNA	Cell-free DNA
ECAR	Extracellular acidification rate
gDNA	Genomic DNA
HDAC	Histone deacetylase
OXPHOS	Oxidative phosphorylation
OCR	Oxygen consumption rate
VPA	Valproic acid

therefore, a real possibility with significant clinical implications. The objective of this study is the utilization of *in vitro* means to evaluate whether or not cfDNA has the capacity to transmit messages, in the form of drug-induced effects, between cells. To do this, we determined whether the pharmacological compound, valproic acid (VPA), can affect the release and nature of cfDNA and whether these changes can be transferred to other untreated cells. VPA, a short-chain fatty acid, is widely used as an anti-epileptic or anti-convulsant, mood stabilizer and migraine prophylaxis [20,21]. It has also been found to directly target and inhibit histone deacetylase (HDAC) [20] and induce replication-independent active DNA demethylation by stimulating the accessibility of a demethylase enzyme [22], resulting in widespread epigenetic reprogramming [23]. VPA and its metabolites also impair oxidative phosphorylation (OXPHOS) through the inhibition of mitochondrial pyruvate oxidation [24] and 2-oxoglutarate- and glutamate-driven OXPHOS through the inhibition of dihydrolipoyl dehydrogenase activity [25], playing a central role in valproate-induced steatosis and steatohepatitis [25]. To determine whether VPA can induce changes in cfDNA characteristics and can subsequently alter a recipient cell's metabolism, human hepatocellular carcinoma (HepG2) cells were treated with VPA and the resulting cfDNA collected. Untreated HepG2 cells were then treated with this cfDNA and screened for changes in OXPHOS and glycolytic activity.

2. Materials and methods

2.1. Cell culture and sample collection

HepG2 cells (ATCC® HB-8065™) were cultured 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) (Gibco), 1% penicillin streptomycin (Lonza), 1% L-glutamine (Lonza), 1% non-essential amino acids (Lonza) and 1% amphotericin B (Biochrom). The cells were seeded into 75 cm^2 flasks (TPP) at 15% confluence with a final volume of 10 mL and incubated in a humidified atmosphere at 37 °C and 5% CO_2 for 12 h. The growth medium was then replenished with either growth medium (untreated cells), VPA (Sigma Aldrich) or cfDNA, respectively, and incubated for 24 h, hereafter referred to as a 24 h treatment. The growth medium was then collected in 15 mL tubes (TPP) and the cells collected by trypsinization in 15 mL tubes. This process was repeated for a second incubation period of 24 h and is hereafter referred to as a 48 h treatment. After this second incubation period the growth medium and cells were, again, collected in 15 mL tubes. Following each collection, the growth medium was centrifuged at 5000*g* for 10 min, transferred to fresh 15 mL tubes and stored at −20 °C until use. The cells were 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.

2.2. Extraction and quantification of cell-free DNA and cellular protein

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. The individual samples were added to binding buffer NTI, 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 subsequent cell treatment analyses, bulk extraction of the cfDNA of HepG2 growth medium 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 total volume of collected growth medium (10 mL per flask) was used during the extractions. The total volume of growth medium-NTB mixture (30 mL) was divided into four spin columns to prevent loss of cfDNA. 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 manufacturer's instructions.

The frozen cell pellets were suspended in 3 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, 30sec on/30sec 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 manufacturer's instructions.

2.3. Fragment size evaluation of cell-free DNA

Capillary electrophoresis was performed to analyse the size distribution of the extracted cfDNA. 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 represents 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.

2.4. Histone deacetylase activity assay

Duplicate flasks of HepG2 cells received a 24 and 48 h VPA treatment, respectively. The cells were trypsinized, the duplicate flasks' cells collected and pooled into 15 mL tubes and pelleted at 500*g* for 5 min. The packed cell volume of each sample was measured as approximately 25 μL. Nuclear proteins were then extracted from the cells using the NucBuster™ Protein Extraction Kit (Novagen) according to the manufacturer's instructions. The nuclear extracts were stored at − 80 °C until used. The histone deacetylase activity (HDAC) assay was performed using the HDAC

assay kit for fluorometric detection (Upstate Cell Signalling Solutions) according to the manufacturer's instructions using HepG2 nuclear extract as nuclear protein controls.

2.5. Bioenergetic analysis

The Seahorse XF^{e}96 analyser (Seahorse Biosciences, USA) is an instrument used to measure bioenergetics in cells. It assesses OXPHOS and glycolysis by simultaneously measuring the OCR and ECAR, respectively, using optic fibres which excite fluorophores that are embedded within the probes of an XF cartridge plate. Each probe is surrounded by four injection ports which allows up to four compounds to be sequentially injected into the wells of the cartridge plate during the XF analysis. Two different tests were carried out according to manufacturer's instructions, namely the Seahorse XF Cell Mito Stress Test Kit and Seahorse XF Glycolysis Stress Test Kit (Seahorse Biosciences, USA) [26]. The Mito Stress Test, however, did not show any significant results, thus, only the Glycolysis Stress Test will be described in more detail. HepG2 cells were counted using the Scepter 2.0 handheld cell counter (Merck Millipore, Billerica, USA) and seeded in 96 well XF cell culture microplates at 14 000 cells/well. Following the incubation period (23 h), the growth media was replaced with XF assay media (modified DMEM supplemented with 2 mM L-glutamine, pH 7.4) and the plate was incubated for 1 h in a non-CO_2 incubator. The instrument protocol varied for each of the different bioenergetic analyses, as described in Section 3.3, where the metabolic effect (if any) of cfDNA and gDNA obtained from control and VPA-treated cells was assessed. The DNA was eluted in elution buffer and diluted in nuclease-free water, and since the elution buffer was known to alter the pH of the media, a vehicle control was included for all analyses where the same volume of elution buffer and nuclease-free water was added. XF assay media was also used as a negative control for all treatments.

The Glycolysis Stress Test included the following cartridge injections: 10 mM glucose, 1 μM oligomycin and 50 mM 2-deoxyglucose (a glucose analogue). Four glycolytic parameters can be calculated using this kit, namely: Non-glycolytic acidification (last measurement prior to glucose injection), glycolysis (maximum ECAR measurement following glucose injection), glycolytic capacity (maximum ECAR measurement following oligomycin injection) and glycolytic reserve (glycolytic capacity minus glycolysis). Version 2.3 of the Seahorse Wave software was used to process and analyse all results.

2.6. Statistics

All Seahorse graphs were drawn using GraphPad Prism 5 (Version 5.00) and all bar graphs were drawn in Microsoft Excel 2010. Version 23 of SPSS Statistics (IBM) was used to perform all one-way ANOVAs and Tukey's HSD post-hoc test, where a p-value of less than 0.05 was regarded as statistically significant.

3. Results and discussion

For the purpose of this study 2, 5 and 8 mM VPA doses were used to treat the HepG2 cells instead of the proposed dosages for epileptic seizures, as treating this cancerous cell line with doses higher than 8 mM resulted in significant cell death. The choice of VPA concentration is, therefore, in order to induce measurable biochemical changes without inducing cell death due to the anticancer effects of the compound [27].

3.1. Valproic acid induces detectable increases in actively released cell-free DNA levels

Screening of the cfDNA release levels of HepG2 cells treated with one 24 h dose of 2, 5 and 8 mM VPA (Fig. 1) showed a slight increase in cfDNA levels in all three doses compared to the untreated control cells. The 48 (2 × 24) hour treatment resulted in significantly higher cfDNA levels compared to single doses, with a slight increase in the amounts of cfDNA released at the three concentrations. All three concentrations of VPA do, therefore, elicit measurable effects on HepG2 cfDNA and the sustained increases in cfDNA release as the VPA concentration increases, though not statistically significant, do demonstrate a tendency towards a dose-dependent increase in cfDNA released with a larger amount of DNA released after 48 h.

The HDAC activity at 2, 5 and 8 mM VPA was determined to identify therapeutic concentrations required for the HepG2 cells. Changes in cell morphology were detected for the 5 mM and 8 mM VPA concentrations after 24 and 48 h of treatment (supplementary figure). Cells became thinner and elongated with cytoplasmic extensions compared to the untreated cells, observations similar to cells treated with the histone deacetylase inhibitor, Trichostatin A [28,29]. These morphological changes were barely detectable in cells treated with 2 mM VPA. Screening of the HepG2 HDAC activity of the 48 h treatments of 2, 5 and 8 mM VPA (Fig. 2) correlated with the morphological changes observed during treatment, revealing that 2 mM VPA inhibited HDAC activity to 72.8%, whereas 5 and 8 mM VPA inhibited HDAC activity to 56% and 53%, respectively. This suggests that 5 and 8 mM may serve as more efficient therapeutic doses.

Elucidation of the cfDNA fragment sizes with capillary electrophoresis revealed that untreated HepG2 cells primarily released cfDNA fragments at ~2000 bp (78% of the cfDNA sample), a peak area of the electropherograms that has been shown to not be associated with apoptosis or necrosis and is suggested to be associated with actively released DNA [30], with very low levels of nucleosomal fragment patterns (19% of the cfDNA sample) visible after 48 h (Table 1). The 48 h treatment with 2 mM VPA resulted in the formation of a peak at 150–200 bp and a smaller peak at 300–400 bp, which have been shown to correlate with the presence of apoptosis [30], and a smear of DNA fragments that appear to form from the first peak to ~3000 bp, which is likely to be necrosis as random necrotic DNA fragmentation forms smears during

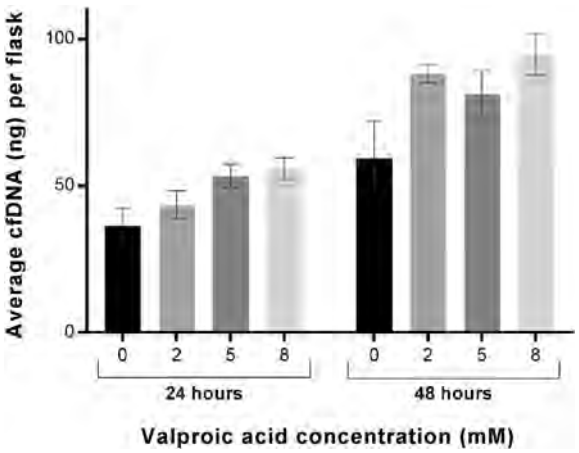


Fig. 1. Average amount of cfDNA of HepG2 cells released into growth medium after 24–48 h of 2, 5 and 8 mM VPA treatment. The value of each bar represents the average (±SD, n = 6) amount of cfDNA released in 10 mL of growth medium.

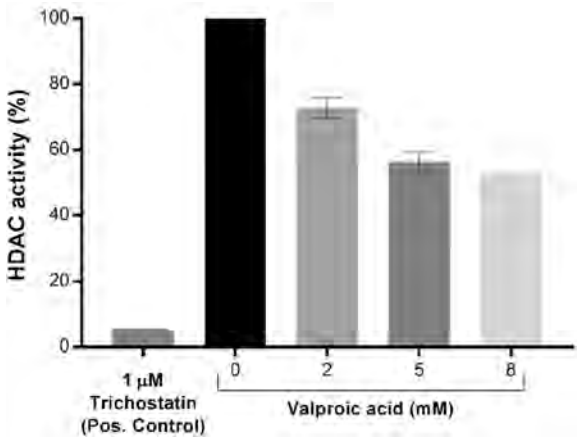


Fig. 2. HDAC assay of HepG2 cells treated with VPA for 48 h with Trichostatin A as positive control (±SD, n = 2).

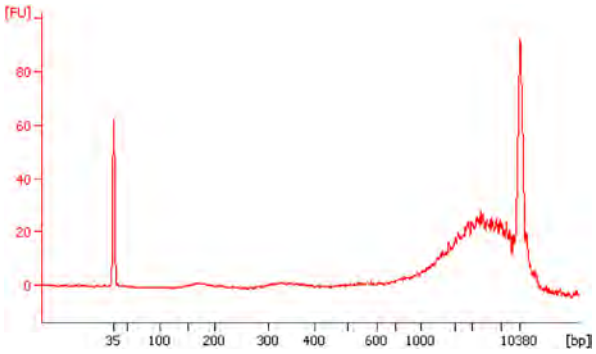
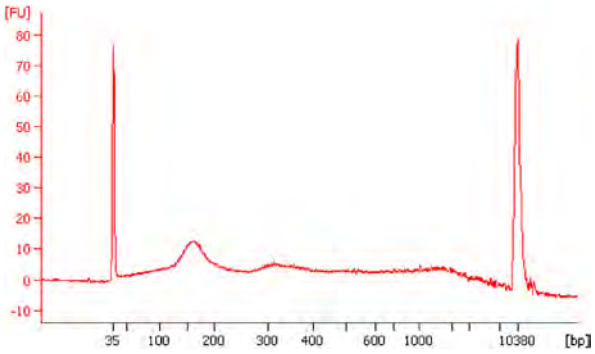
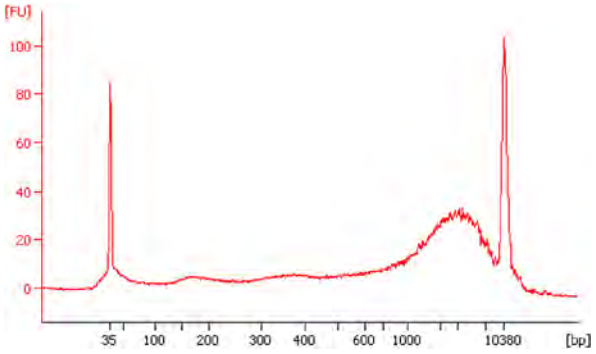
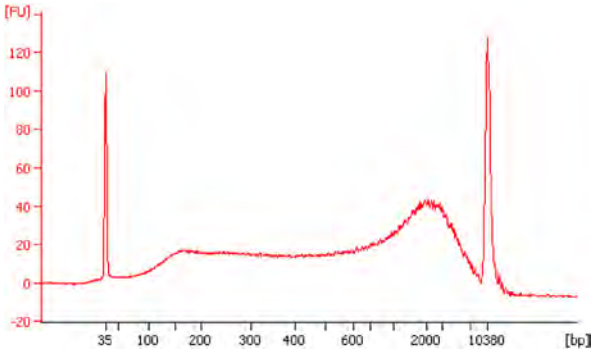
normal gel electrophoresis [31]. These apoptotic and/or necrotic DNA fragments represent 82% of the tested cfDNA sample. There is also a near absence in the ~2000 bp peak (16% of the cfDNA sample), that may also form part of the necrotic DNA smear. The 48 h treatment with 5 mM VPA resulted in cfDNA fragment patterns similar to that of the untreated cells, but with nucleosomal DNA fragments (at 150–200 bp and 300–400 bp) and a possible 39 bp peak. A nucleosomal fragment-to-actively released DNA ratio indicates that the increased levels of cfDNA release of the VPA-treated cells are due to a relatively equal increase in both the amount of the actively released ~2000 bp peak and nucleosomal fragment formation via apoptosis. The cfDNA of cells after 48 h of treatment with 8 mM VPA contained necrotic DNA at higher levels than that of 2 mM VPA with the smear starting at ~150 bp and continuing until the formation of a ~2000 bp peak, which may indicate that the 33% fraction of the ~2000 bp peak size in the cfDNA sample (in fluorescent units) is a combination of actively released DNA and the necrotic DNA smear. The increased VPA dosage may, therefore, result in a decrease of the actively released ~2000 bp peak and consists primarily of necrotic DNA fragments.

Due to the similarities in HDAC activity and cfDNA release levels at 5 and 8 mM VPA and the primarily necrotic DNA content of the 8 mM VPA cfDNA compared to the higher levels of actively released DNA content of the 5 mM VPA cfDNA, which is suggested to serve as intercellular messengers [32–34], all subsequent experiments was performed using 5 mM VPA-treated cells.

3.2. The effect of cell-free DNA treatment on the subsequent cell-free DNA release of HepG2 cells

The cfDNA of HepG2 cells treated with growth medium (control cells) and 5 mM VPA, respectively, for 48 h were administered to native HepG2 cells as a 48 h treatment of 1.7 ng/mL (the total average concentration of cfDNA obtained from biological replicate flasks of untreated control cells, serving as the minimum concentration of cfDNA to which HepG2 cells are exposed to in 75 cm^2

Table 1
Capillary electropherograms showing the fragment sizes of cfDNA isolated from HepG2 cells after a 48 (2 × 24) hour treatment.

Untreated cells		2 mM VPA	
			
42–914 bp (other DNA fragments)	19%	42–914 bp (other DNA fragments)	82%
914–9100 bp (actively released DNA)	78%	914–9100 bp (actively released DNA)	16%
5 mM VPA		**8 mM VPA**	
			
42–914 bp (other DNA fragments)	50%	42–914 bp (other DNA fragments)	66%
914–9100 bp (actively released DNA)	47%	914–9100 bp (actively released DNA)	32%

flasks). Interestingly, the 48 h treatment of HepG2 cells with control cell cfDNA resulted in a nearly two-fold increase in subsequent cfDNA release (Fig. 3). No changes in cell morphology were detected in the treated cells, suggesting that cfDNA from VPA-treated cells do not elicit HDAC-dependent morphological effects as was seen when the cells were treated with VPA (supplementary figure).

Subsequent fragment size analysis reveals that this change in the control-treated cells' cfDNA level is primarily changes in the amount of the fragment quantities (relative peak heights) and not changes in fragment length (Table 2). The electropherograms showed increased levels of actively released DNA fragments at ~2000 bp and an increase in small and nucleosomal DNA fragments that results in an equal ratio of small and nucleosomal fragments-to-actively released DNA in the sample, indicating that the cfDNA induced apoptotic effects. CfDNA of HepG2 cells treated with 5 mM VPA, on the other hand, did not result in a significant change in cfDNA release after treating HepG2 cells for 48 h (Fig. 3). This is possibly due to (i) the effect of 5 mM VPA cfDNA being of short duration and ending after 24 h of exposure to cells or (ii) 5 mM VPA cfDNA not effectively triggering the effects induced by normal cfDNA after 48 h treatment, causing the decreased release levels of the cfDNA. The electropherograms revealed a small and nucleosomal fragments-to-actively released DNA ratio very similar to that of the control-treated cells (Table 2) with lower ~2000 bp levels and disturbances in the baseline indicative of low levels of small and/or nucleosomal DNA fragment formation. This similarity in small and nucleosomal fragments-to-actively released DNA ratio combined with the lack of significant nucleosomal fragment peaks in the treated cell cfDNA may indicate that the small and nucleosomal DNA fraction of the 5 mM VPA cfDNA contains higher levels of small DNA fragments than nucleosomal fragments, implying the possible presence of necrosis.

3.3. *The effect of cell-free DNA treatment on the metabolic activity of HepG2 cells*

For all subsequent analyses, the cfDNA and gDNA obtained from cells treated with their own cfDNA will be referred to as control cfDNA and control gDNA, respectively, and the cfDNA and gDNA obtained from cells that were treated with 5 mM VPA will be referred to as VPA cfDNA and VPA gDNA, respectively. Focus was

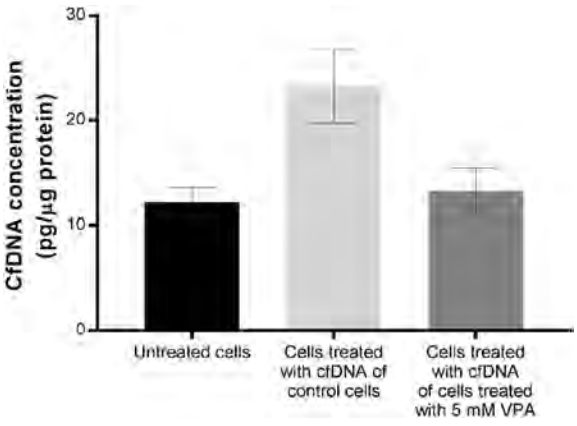


Fig. 3. Average cfDNA release of HepG2 cells after a 48 h treatment of growth medium (untreated cells) and 1.7 ng/mL cfDNA extracted from the growth medium of cells after 48 h of growth medium (control cells) and 5 mM VPA treatment, respectively. The value of each bar represents the average (±SD, n = 6) amount of cfDNA released in 10 mL of growth medium normalized in terms of the total cellular protein present in each culture flask.

Table 2
Capillary electropherograms showing the sizes of cfDNA isolated from HepG2 cells after a 48 (2 × 24) hour treatment.[a]

Untreated cells		Control-treated cells		Treated cells	
42–914 bp (small + nucleosomal DNA fragments)	19%	42–914 bp (small + nucleosomal DNA fragments)	53%	42–914 bp (small + nucleosomal DNA fragments)	53%
914–9100 bp (actively released DNA)	78%	914–9100 bp (actively released DNA)	42%	914–9100 bp (actively released DNA)	41%

[a] Control-treated cells were treated with 1.7 ng/mL cfDNA extracted from the growth medium of cells after 48 h of incubation in growth medium without VPA. Treated cells were treated with 1.7 ng/mL cfDNA extracted from the growth medium of cells treated with 5 mM VPA for 48 h.

initially on the OXPHOS analyses and thereafter it was shifted to the glycolytic activity in the HepG2 cells. Two different strategies were also followed for administering the cfDNA and gDNA. The first strategy involved the pre-treatment of the cells with cfDNA or gDNA by adding the DNA to the growth medium and incubating the cells for a set period of time prior to isolation. The second strategy utilized the injection ports of the XF analyser by loading the cfDNA or gDNA into these ports and injecting them into the plate wells during the XF analysis.

3.3.1. OXPHOS analyses

Cells were incubated for 15 h following seeding, after which the cells were pre-treated with three concentrations of control and VPA cfDNA, respectively. As mentioned in section 3.2, the minimum concentration of cfDNA to which HepG2 cells are exposed to in 75 cm^2 flasks is ~1.7 ng/mL. With this in mind 2 ng/mL was used as the minimum concentration along with 6 and 10 ng/mL doses, but the volume used was downscaled from 10 mL (75 cm^2 flask) to 0.1 mL (96 well cartridge plate), resulting in amounts of 0.2, 0.6 and 1 ng/well in order to compensate for the significant difference in cell yield between a 75 cm^2 flask and 96 well cartridge plate. These treatments were administered at two doses, each followed by a 24 h incubation. A positive control was also included where cells were treated with 5 mM VPA. The XF Mito Stress Test was used that includes cartridge injections of oligomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), rotenone and antimycin A in XF assay media containing 5 mM glucose and 1 mM pyruvate. This 48 h incubation with control and VPA cfDNA did not reveal any significant differences in the oxygen consumption rate (OCR) values (results not shown) for any of the OXPHOS parameters that can be determined using the XF Mito Stress Test kit (Seahorse Biosciences, USA). No differences could be seen in the extracellular acidification rate (ECAR) values either. A reduction in basal respiration and ATP production was seen in the positive control group, as expected [25].

3.3.2. Basal OCR and ECAR readings for injections of cell-free DNA

Since the prolonged 48 h treatment with 2, 6 and 10 ng/mL doses of cfDNA did not appear to have any effect on the HepG2 cells, it was decided to assess the acute response of the cells to increased doses of cfDNA. This was done by loading the injection ports of the XF cartridge plate with 10, 30 and 100 ng/mL (1, 3 and 10 ng/well) of the VPA cfDNA and then analysing the basal OCR and ECAR readings in XF assay media (containing 5 mM glucose and 1 mM pyruvate). The effect of two and three sequential cartridge injections of the same amounts of cfDNA was also assessed. Each injection was set 36 min apart as previous research by Mittra and colleagues [35] has shown that the maximum level of cfDNA uptake and integration into cell nuclei occurs within 30 min of cfDNA treatment. For the three sequential cartridge injections, the final content of cfDNA in each well was thus 3, 9 and 30 ng. For the two sequential cartridge injections, the final content of cfDNA in each well was thus 2, 6 and 20 ng. Due to the practical limitations of the XF analyser, the two sequential cartridge injections group had to receive a third injection volume, and the third cartridge injection administered thus contained nuclease-free water. The negative control group received three sequential cartridge injections of XF assay media. The Seahorse graphs for both the ECAR and OCR readings are shown in Fig. 4.

A marked difference could be seen between the ECAR of three cartridge injections of 10 ng VPA cfDNA (30 ng in total), and the 1 ng and 3 ng doses and controls of Fig. 4A and in Fig. 4B, the same increase could be seen with two cartridge injections (20 ng in total) of VPA cfDNA, but the difference was not as pronounced as the three cartridge injections. In Fig. 4A and B a spike followed by a drop in ECAR could be seen in all the groups, except the negative control,

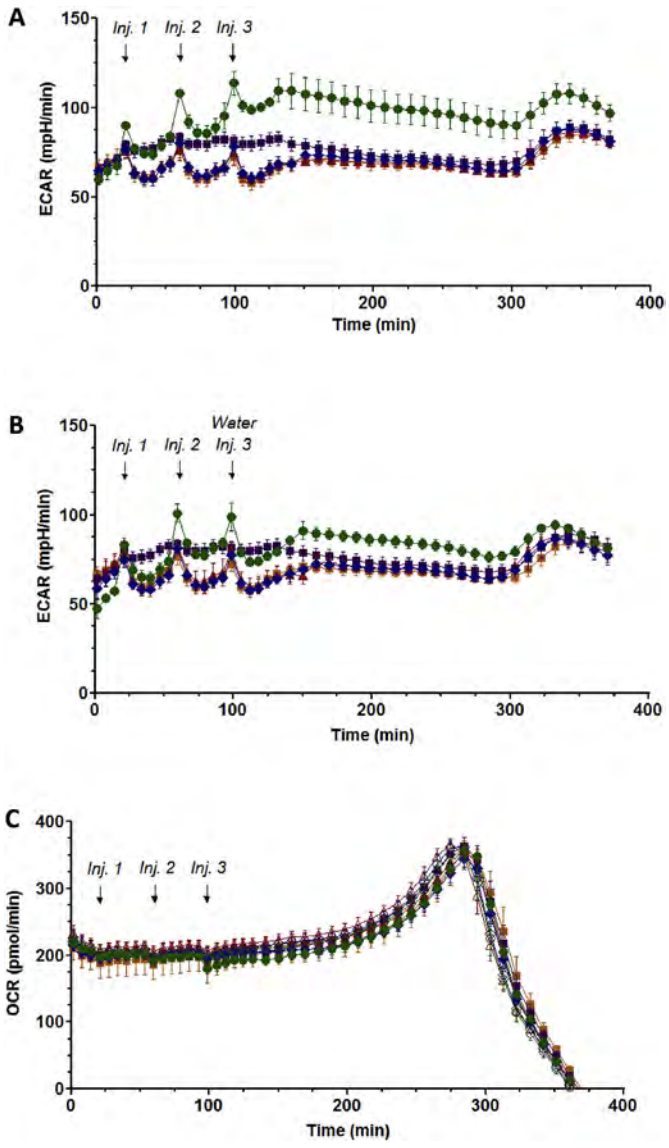


Fig. 4. Seahorse graphs depicting the basal ECAR **(A and B)** and OCR **(C)** readings versus time for three different concentrations (1, 3 and 10 ng) of VPA cfDNA that was injected into the cartridge plate **(A)** three sequential times and **(B)** two sequential times (with a third injection of water) (Inj = Injection, Closed square: vehicle and negative control, Closed triangle: 1 ng VPA cfDNA, Closed diamond: 3 ng VPA cfDNA, Closed circle: 10 ng VPA cfDNA). **(C)** The OCR readings for both two and three cartridge injections of the VPA cfDNA. Each data point represents the mean OCR or ECAR readings (N = 3–8) ± SD (Closed square: vehicle and negative control, Closed triangle: 1 ng VPA cfDNA (3 inj), Open triangle: 1 ng VPA cfDNA (2 inj), Closed diamond: 3 ng VPA cfDNA (3 inj), Open diamond: 3 ng VPA cfDNA (2 inj), Closed circle: 10 ng VPA cfDNA (3 inj), Open circle: 10 ng VPA cfDNA (2 inj)).

following each injection. Since this also occurs in the vehicle control, it was concluded that the nuclease-free water was the cause of this pattern seen in these groups. The ECAR appears to return to a similar level as the negative control following each injection, and eventually stabilize. Similar to that seen when performing the XF Mito Stress Test (section 3.3.1), Fig. 4C shows no marked differences between the OCR readings for any of the groups, thus indicating that the VPA cfDNA has a more evident effect on glycolysis than on OXPHOS (contrary to the direct treatment with VPA which was previously shown to negatively impact OXPHOS [25]). The focus was thus hereafter shifted to glycolysis. The profiles seen in these graphs at approximately 300 min are believed to be caused by cell

proliferation within each well during the XF analysis thus leading to more cells and increased oxygen consumption. The limited oxygen availability, depleted substrates in the media and over-confluency of the cells are believed to have led to a complete switch to anaerobic glycolysis and to cell death.

3.3.3. Glycolytic parameters for the pre-treatment and injection of cell-free DNA

Due to the marked effect that the VPA cfDNA appeared to have on the ECAR, the focus was thereafter shifted to the XF Glycolysis Stress Test, as described in section 2.5. A link between glycolysis and cfDNA was also seen in our previous research [26], without any correlation to OXPHOS activity. In order to assess the effect of control and VPA cfDNA, as well as the effect of pre-treatment versus acute cartridge injection, cells were incubated with both 10 ng control and VPA cfDNA for 6 and 24 h prior to the XF analysis. The first injection port of the XF cartridge plate was also loaded with 10 ng control and VPA cfDNA for the cartridge injection group. The first injection port for all the other wells not receiving cfDNA or the cfDNA vehicle control, were loaded with XF assay media. The cartridge injection of the XF Glycolysis Stress Test compounds commenced 30 min after this first injection. The glycolytic parameters that were calculated from these results are shown below in Fig. 5. A one-way ANOVA was performed with a Tukey HSD post-hoc test in order to determine any statistically significant differences between groups.

Similar to the 48 h pre-treatment OXPHOS analyses, in Fig. 5 the 24 h incubation also did not appear to show any significant differences to the vehicle controls in any of the glycolytic parameters, with the only difference seen in the glycolytic reserve of the control cfDNA (Fig. 5C). This difference was, however, less significant ($p < 0.05$) than all the other differences observed ($p < 0.001$). For the 6 h incubation, a significant decrease was seen in glycolysis for both the control and VPA cfDNA groups compared to the vehicle control (Fig. 5). However, the 6 h VPA cfDNA group had a significantly increased glycolytic reserve (Fig. 5C) compared to the vehicle control and the 6 h control cfDNA group.

The most pronounced effect could, however, clearly be seen in the cartridge injections with both the control and VPA cfDNA resulting in significantly increased glycolysis (Fig. 5A) and non-glycolytic acidification (Fig. 5D) compared to the vehicle controls. Only the control cfDNA cartridge injection showed a significant increase in glycolytic capacity (Fig. 5B), while both control and VPA cfDNA cartridge injections showed decreased glycolytic reserve compared to the vehicle controls (Fig. 5C). Interestingly, the VPA cfDNA cartridge injection resulted in significantly greater glycolysis and significantly decreased glycolytic reserve compared to the control cfDNA cartridge injection group, while the control cfDNA group had significantly greater glycolytic capacity.

These experiments thus revealed that the cells are capable of metabolizing cfDNA within 30 min and that both the control and the VPA cfDNA had an altered effect on numerous glycolytic parameters, but the way in which they affected these parameters differ. After 6 h this effect appears to be reversed in both groups, and after 24 h the cfDNA no longer appears to have any effect on any of the glycolytic parameters. It was thus decided to focus all further analyses on the acute exposure of cells to cfDNA via cartridge injection, rather than prolonged incubation.

3.3.4. Glycolytic parameters for a concentration range of cell-free DNA and genomic DNA

Correlations between increased cfDNA concentrations and glycolytic parameters were investigated using one cartridge injection of three concentrations (5, 10 and 15 ng) of control and VPA cfDNA and gDNA. No statistically significant differences were seen between DNA treatment groups and the respective vehicle controls for glycolytic capacity or non-glycolytic acidification (Fig. 6B and D). For both glycolysis (Fig. 6A) and glycolytic reserve (Fig. 6C) a statistically significant increase and decrease (respectively) in ECAR values was seen in the 10 ng VPA cfDNA group compared to the vehicle control. For glycolysis, this 10 ng VPA cfDNA group was also significantly greater than the 10 ng control cfDNA, 10 ng control gDNA and 10 ng VPA gDNA groups. The exact opposite was seen for the glycolytic reserve where the 10 ng VPA cfDNA group was significantly decreased compared to these other three 10 ng groups. This difference between the control and VPA cfDNA groups correlates with the results seen in Fig. 5A and C. This concentration range also showed 10 ng cfDNA to be the optimal concentration since no significant differences were seen at higher or lower concentrations. The VPA gDNA did not appear to have the same effect as the cfDNA.

3.3.5. Glycolytic parameters for multiple injections of cell-free DNA and genomic DNA

It was lastly decided to combine all of the above experiments so that the effect of 1 and 3 cartridge injections of both control and VPA cfDNA and gDNA could be assessed. The assay was furthermore adapted by removing the last cartridge injection of 2-deoxyglucose (2-DG), so that (i) the cfDNA/gDNA/vehicle control was injected into the plate wells, (ii) 30 min later glucose was injected and (iii) oligomycin was injected 30 min after that. The glycolytic parameters were still able to be calculated despite the lack of 2-DG. Following the oligomycin injection, the instrument continued to measure for approximately 4 h, similar to Fig. 4 (the cells in this combined analysis, however, were in XF assay media that did not contain glucose or pyruvate). For the 1 injection group of cells, cfDNA and gDNA was injected into plate wells using the XF analyser, while for the 3 injection group only the third injection was administered using the XF analyser injection ports and the first two injections were pipetted manually (30 min apart) into the XF assay media within each well prior to inserting the plate into the instrument. The glycolytic parameters are shown in Fig. 7 and the Seahorse graphs for the 3 cartridge injections results are shown in Fig. 8.

No statistically significant differences were seen between the DNA and vehicle control groups for glycolysis (Fig. 7A) or non-glycolytic acidification (Fig. 7D), but for glycolytic capacity (Fig. 7B) and glycolytic reserve (Fig. 7C), however, 3 cartridge injections of VPA cfDNA was significantly greater than its vehicle control. For both of these glycolytic parameters, 3 cartridge injections of VPA cfDNA yielded significantly greater values than 3 cartridge injections of control cfDNA, control gDNA and VPA gDNA. This differed to the results seen in Figs. 5 and 6 where the VPA cfDNA group had significantly greater glycolysis and decreased glycolytic reserve compared to control cfDNA/gDNA and VPA gDNA (with no difference in glycolytic capacity).

In Fig. 8A and C it can be seen that, following the oligomycin injection, the VPA cfDNA's resulting ECAR remained markedly higher than that of the control cfDNA or VPA gDNA groups and the vehicle controls for approximately 250 min, where it then slowly returned to levels similar to the other groups. The ECAR of the control cfDNA appeared to be slightly greater than its vehicle control following the oligomycin injection, but the overall profile was similar to all the other control and gDNA groups and none of the differences seen were statistically significant. The VPA gDNA was again seen to not have the same effect as the VPA cfDNA. This profile was similar to that seen in Fig. 4A, but the peak seen at approximately 325 min in Fig. 4A was not seen in Fig. 8. This may be due to the differences in cellular stress induced by the Glycolysis Stress Kit versus the Cell Mito Stress Test Kit (due to additional glucose and oligomycin injections), or possibly since this analysis

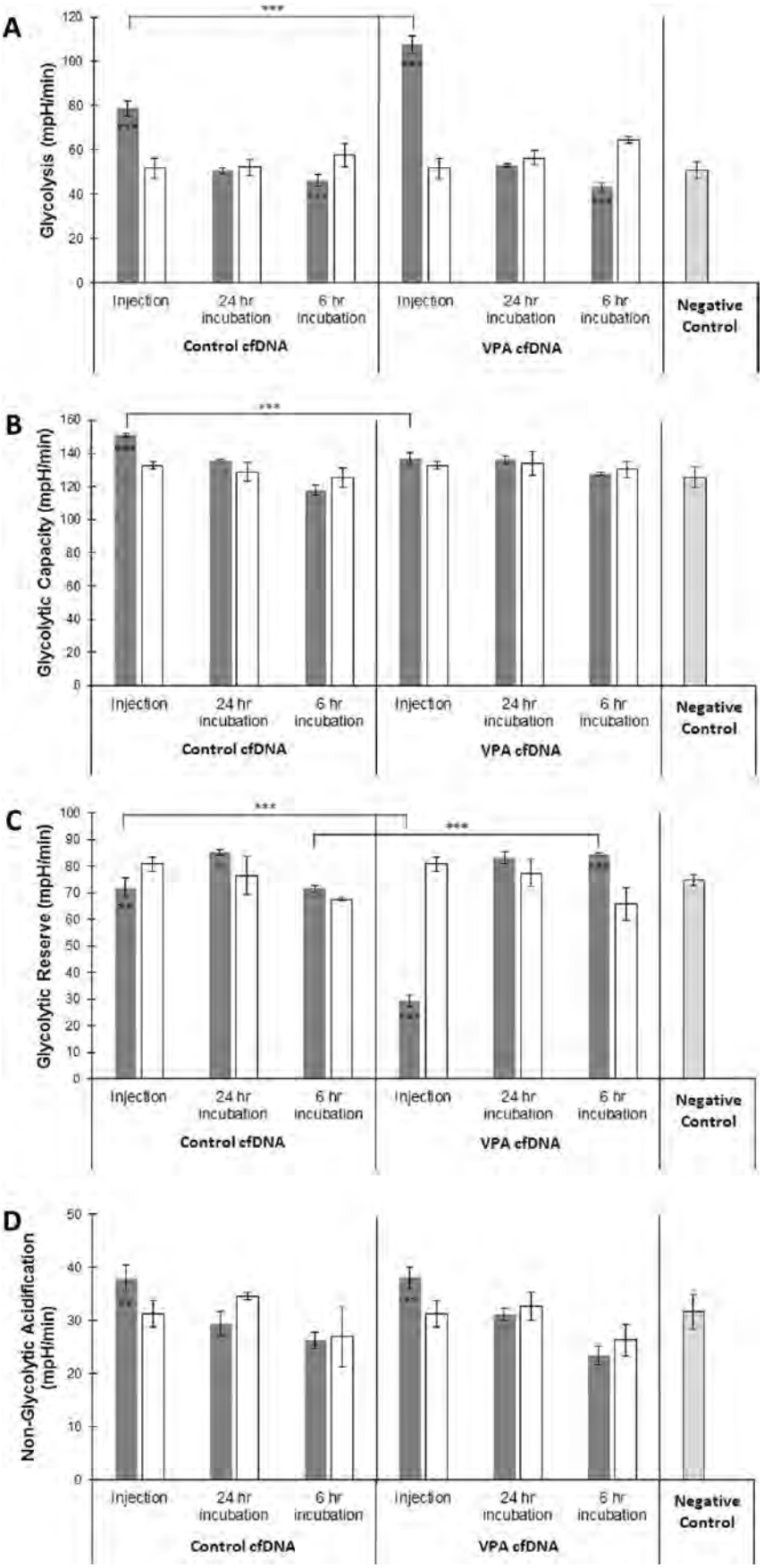


Fig. 5. **(A)** Glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification of HepG2 cells pre-treated for 6 and 24 h and acutely exposed (via cartridge injection) to 10 ng control and VPA cfDNA (grey bars: DNA injections, white bars: vehicle controls). Asterisks within the DNA bar indicate a statistically significant difference between the cfDNA treated group and its vehicle control. Asterisks outside of the bars indicate differences between control cfDNA and VPA cfDNA. All values represent the ECAR mean values ± SD (N = 4). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

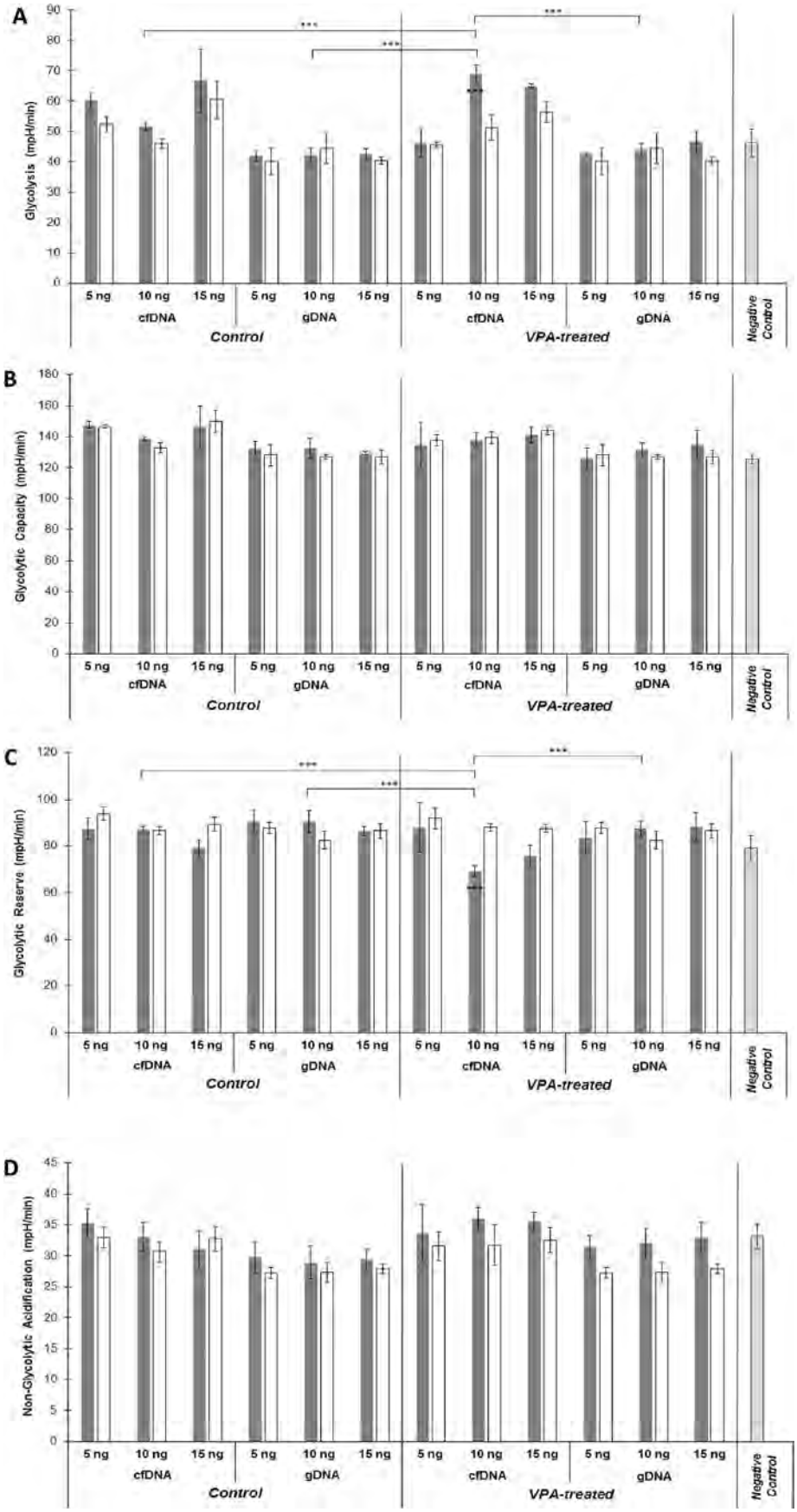


Fig. 6. **(A)** Glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification of HepG2 cells exposed to control and VPA cfDNA and gDNA at three different concentrations (5, 10 and 15 ng) (grey bars: DNA injections, white bars: vehicle controls). Asterisks within the DNA bar indicate a statistically significant difference between the cfDNA or gDNA treated group and its vehicle control. Asterisks outside of the bars indicate differences between the control cfDNA or gDNA and VPA cfDNA or gDNA groups. All values represent the ECAR mean values ± SD (N = 2–6). ***$p < 0.001$.

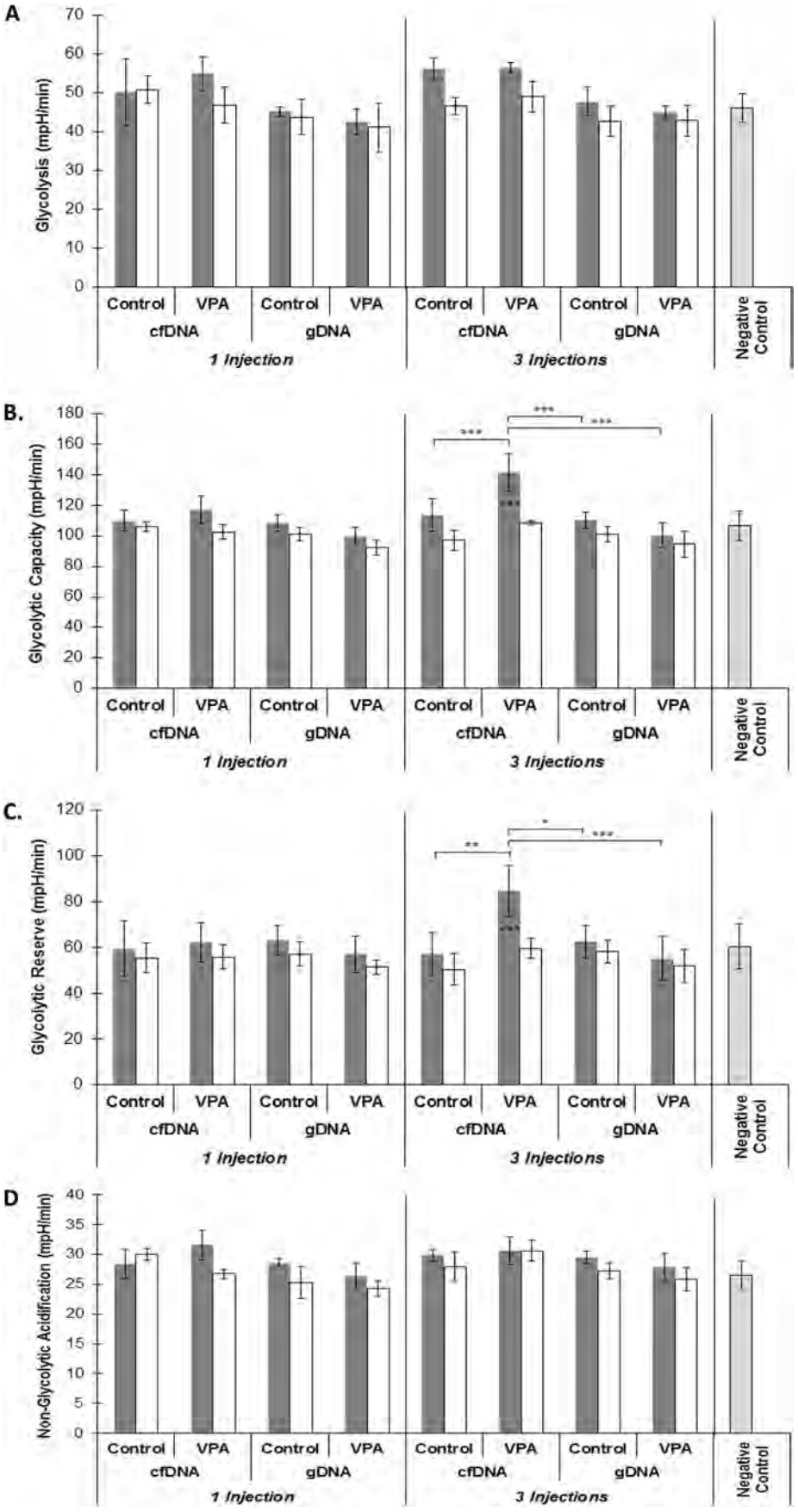


Fig. 7. **(A)** Glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification of HepG2 cells acutely exposed once or three times to control and VPA-treated cells cfDNA and gDNA via cartridge injection (grey bars: DNA injections, white bars: vehicle controls). Asterisks within the DNA bar indicate a statistically significant difference between the cfDNA/gDNA treated group and its vehicle control. Asterisks outside of the bars indicate differences between the control cfDNA/gDNA and VPA-treated cells cfDNA/gDNA treatment groups. All values represent the ECAR mean values ± SD (N = 4). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

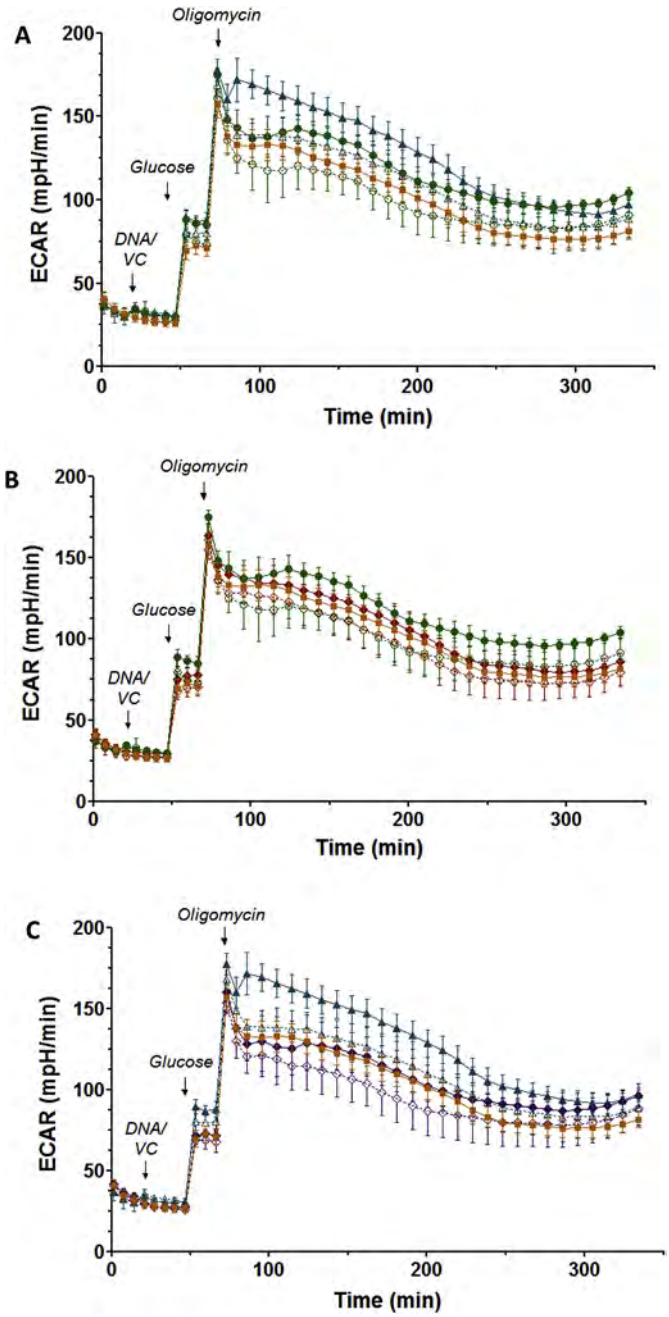


Fig. 8. Seahorse graphs depicting the ECAR readings versus time for cells acutely exposed three times to **(A)** control and VPA cfDNA (Closed square: negative control, Closed circle: Control cfDNA (3 inj), Open circle: control cfDNA vehicle control (3 inj), Closed triangle: VPA cfDNA (3 inj), Open triangle: VPA cfDNA vehicle control (3 inj)), **(B)** control cfDNA and gDNA (Closed square: negative control, Closed circle: Control cfDNA (3 inj), Open circle: control cfDNA vehicle control, Closed diamond: Control gDNA (3 inj), Open diamond: Control gDNA vehicle control (3 inj)) and **(C)** VPA cfDNA and gDNA (Closed square: negative control, Closed triangle: VPA cfDNA (3 inj), Open triangle: VPA cfDNA vehicle control (3 inj), Closed diamond: VPA gDNA (3 inj), Open diamond: VPA gDNA (3 inj)) via cartridge injections, followed by cartridge injections of glucose and oligomycin. The DNA/VC injection shown here indicate the third cartridge injection as the first two treatments were administered prior to inserting the plate into the XF analyser. Each data point represents the mean ECAR readings (N = 4) ± SD. VC = Vehicle control.

was not allowed to run for as long.

The OCR readings during this analysis were also investigated, and a similar profile was obtained to that seen in Fig. 4C where each group appeared to reach a peak, after which there was a steady decline in OCR until 0 pmol/min was reached. In this analysis, however, it was found that the different groups reached different OCR peak values at different measurement numbers, i.e. some groups peaked earlier or later than others. The measurement number at which each group peaked was recorded in the bar graph below (Fig. 9), while the actual peak OCR value at this measurement number was indicated as a line graph on the secondary axis.

Surprisingly, the differences seen between the measurement numbers did not appear to be caused by the DNA treatments themselves since the vehicle control and DNA treatment group appeared to peak at very similar measurement numbers. The 3 cartridge injection VPA cfDNA peak OCR value did, however, also appear to be markedly increased compared to its vehicle control. The 3 cartridge injection control cfDNA group peaked much earlier than all the other groups and reached a very similar value to the corresponding 3 cartridge injection VPA cfDNA group. The other OCR data did not reveal any differences between groups other than for these peaks that began to form after approximately 100 min (>25 measurement numbers).

4. Conclusions

To summarize, the treatment of HepG2 cells with 5 mM VPA resulted in increased levels of both nucleosomal fragment and actively released cfDNA fractions. In turn, treating HepG2 cells with the cfDNA of untreated and VPA-treated cells induced dose-dependent, primarily glycolytic (with very little OXPHOS) effects as seen in our previous research [26]. These effects were found to occur within 36 min of treatment and lasts for 4–6 h, correlating well with Mittra and colleagues' findings regarding the duration of nuclear cfDNA incorporation [35]. The following was observed:

i. Acute single doses of control cfDNA and VPA-treated cfDNA resulted in a significant increase in glycolysis levels and non-glycolytic acidification, a significant decrease in glycolytic reserve (VPA-cfDNA effect > control cfDNA effect) and an increase in glycolytic capacity of only the control cfDNA-treated cells.
ii. After 6 h the opposite occurs with decreases in glycolysis in both VPA-treated cfDNA- and control cfDNA-treated cells and an increase in glycolytic reserve in cells treated with VPA-treated cfdNA.
iii. Except for a very slight increase in glycolytic reserve in cells treated with control cfDNA, there were no metabolic changes detected after 24 h of treatment.
iv. Multiple doses of cfDNA at 30 min intervals produced different effects compared to single doses, resulting in no differences in glycolysis and non-glycolytic acidification levels and increased glycolytic capacity and glycolytic reserve (VPA-cfDNA > control cfDNA)
v. Single cfDNA doses did not induce any changes in OXPHOS activity, but multiple cartridge injections did show indications of increased OXPHOS activity in VPA-treated cfDNA- (the opposite of that observed in VPA-treated cells) and control cfDNA-treated cells, with the peak in OCR of the control cfDNA-treated cells occurring earlier than that of the cells treated with VPA-treated cfDNA. CfDNA of cells treated with the pharmacological compound does, therefore, not necessarily induce the exact same effects as that of the compound itself.
vi. In both single and multiple doses of cfDNA, the effects induced by the VPA-treated cfDNA doses could not be replicated by its gDNA control counterpart, indicating that recipient cells do not respond to or utilize cfDNA and gDNA fragments of VPA-treated cells in the same manner.

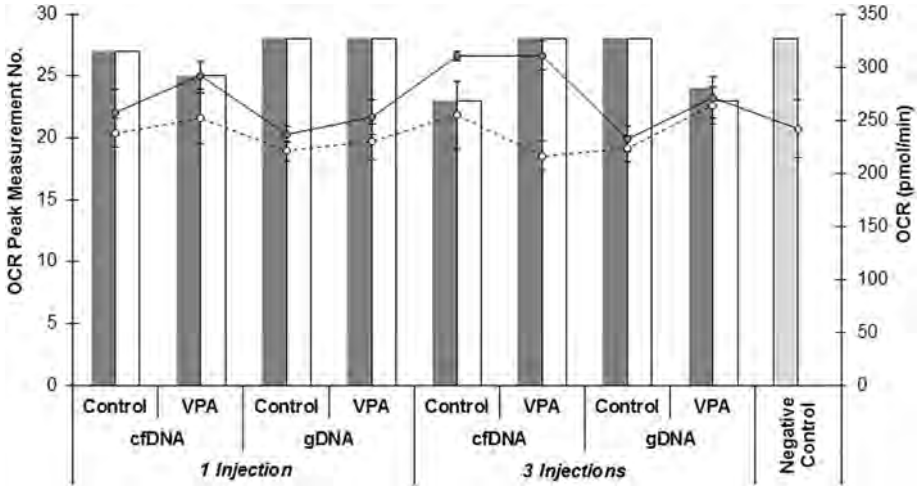


Fig. 9. A bar graph displaying the measurement number at which the OCR reading peaked and a line graph indicating the respective OCR value at the peak measurement number for HepG2 cells treated with 1 and 3 acute exposures to control and VPA cfDNA and gDNA via cartridge injection (grey bar: DNA peak measurement number, white bar: vehicle control peak measurement number). The line graph represents the OCR mean values ± SD (N = 4) (Closed circle: DNA samples OCR, Open circle: vehicle control OCR).

vii. Treatment of HepG2 cells with two 24 h doses of cfDNA did not elicit any metabolic effects, but it did result in (a) increased levels of nucleosomal fragments in the subsequent cfDNA release by both the cells treated with control cfDNA and cells treated with VPA-treated cfDNA and (b) higher levels of cfDNA release by control cfDNA-treated cells compared to untreated cells and cells treated with VPA-treated cfDNA. The effects of cfDNA treatment may, therefore, last longer than indicated by cell metabolism effects and Mittra and colleagues' nuclear incorporation monitoring [35].

viii. The mechanism of the induced metabolic effects of the cfDNA is not yet determined. The widespread epigenetic reprogramming induced by VPA [23] is, however, suspected to be one of the mechanisms involved.

This *in vitro* demonstration of the lateral transfer of pharmaceutically-induced effects may indicate that there is a potential risk that the cfDNA in patients using medication can contain pharmaceutically-induced alterations that can be transferred to other individuals, e.g. during blood transfusions and organ transplantations and result in the induction of adverse drug reactions in the recipient in the absence of the actual drug. The transfer of cfDNA from donor to patient during the transfusion of whole blood, plasma, platelets or erythrocytes is very likely, as (i) cfDNA bound to platelets and erythrocytes are protected from DNAse activity [36], (ii) storage conditions can protect cfDNA in plasma and (iii) cfDNA of both high (e.g. neutrophil extracellular traps) and low molecular weight can pass through protective blood transfusion filters (170–200 μm) [13,37,38]. Moreover, guidelines that restrict medication use in blood donors focus predominantly on disease status, serious pharmacological effects (particularly teratogenic effects) and the effect of medication on blood coagulation [39]. Other than critical and obvious pharmacological complications to the recipient, little further attention is, therefore, paid to the pharmacological and adverse effects of medication. Compromises can also be made when medication only affects certain blood products, e.g. erythrocytes can be donated by donors taking plasma-bound teratogenic medication [40], of which VPA is an example.

Whether cfDNA transferred from donors to recipients can result in the transfer or induction of significant biochemical effects has not yet been determined. However, *in vitro* genometastasis experiments [13] have shown that cfDNA can indeed transfer information that can potentiate adverse effects. Colon cancer cfDNA was capable of transferring K-ras mutations to NIH-3T3 cells, but without inducing carcinogenesis. However, administering these treated cells into mice resulted in cancer development and K-ras mutations in the liver and plasma DNA. The cfDNA, therefore, gave the treated cells the potential to elicit oncogenic activity. Epigenetic inheritance is another example of a means by which cfDNA can transfer pharmaceutically-induced effects. Many commonly used pharmaceutical drugs have been found to induce epigenetic alterations [41,42]. Conventional targets of pharmaceutical drugs can affect the epigenome and the drugs themselves may have epigenetic targets, both of which are able to increase the probability of adverse drug reactions [43]. These epigenetic changes also become heritable, resulting in phenotypic changes that continues for several generations [44], e.g. autism via fetal VPA exposure [45], possibly implying that cfDNA-transferred epigenetic effects can be persistent. Investigations regarding the epigenetic alterations in the cfDNA of HepG2 cells treated with VPA is currently underway and preliminary results have shown that both VPA and VPA-treated cfDNA does indeed alter both the percentage of DNA methylation and the amount of methylated and unmethylated DNA released by treated cells (unpublished data). These results, therefore, show that it is possible for cfDNA to, under *in vitro* conditions, transfer pharmaceutically-induced epigenetic effects to untreated recipient cells. Whether these transferred effects can induce significant and persistent biochemical effects remains an open question. Further investigation regarding the lateral transfer of pharmaceutically-induced effects via cfDNA under *in vivo* conditions is, therefore, strongly encouraged.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Research Foundation (NRF), South Africa [grant number SFH14061869958]. The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not to

be attributed to the NRF.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2017.06.016.

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5.2.4 Pharmaceutically-induced epigenetic effects in cell-free DNA *in vitro* and the risk of clinical implications *in vivo*: A pilot study

As mentioned, VPA can induce widespread epigenetic reprogramming (Milutinovic *et al.*, 2007) by inducing or inhibiting epigenetic mechanisms, including DNA methylation (Detich *et al.*, 2003). Differences between the DNA methylation status of (i) cirDNA from different tissues (Lehmann-Werman *et al.*, 2016; Snyder *et al.*, 2016) and (ii) cirDNA of healthy subjects and cancer patients (Warton *et al.*, 2016; Warton & Samimi, 2015; Wen *et al.*, 2015) have been studied, but can xenobiotic-induced changes in DNA methylation influence cirDNA characteristics along with that of gDNA and will these changes be similar or different? In this paragraph, the effects of VPA treatment on the DNA methylation status of four genes in both gDNA and cfDNA were compared.

The tumour suppressor and DNA damage response genes, RASSF1A (Ras association domain family 1) and GSTP1 (glutathione-S-transferase-pi gene), were chosen as potential targets for VPA-induced DNA demethylation. These genes are involved in cell-cycle checkpoints, DNA repair, apoptosis, detoxification and signal transduction and are mainly inactivated in cancers via promoter hypermethylation (methylation-mediated gene silencing) thereby promoting tumorigenesis (Li *et al.*, 2012). Hepatocellular carcinoma (HCC) cells, in particular, present with significant RASSF1A and GSTP hypermethylation (Li *et al.*, 2012; Schagdarsurengin *et al.*, 2003) and it has been shown that decreasing the DNA methylation status of RASSF1A in HCC results in the re-expression of the gene, a concomitant decrease in tumour cell proliferation and induced apoptosis (Wang *et al.*, 2015). VPA has indeed been shown to decrease RASSF1A methylation levels (Gu *et al.*, 2012) making RASSF1A and GSTP1 suitable target genes for measuring changes in DNA methylation status due to exposure to both VPA treatment and cfDNA derived from VPA-treated cells.

Additionally, two repetitive genetic elements, LINE-1 and ALU1, were also screened for changes in methylation status. These elements can serve as useful proxies for global DNA methylation due to the fact that they are commonly heavily methylated in normal tissue, hypomethylated in tumour tissue, and are spread ubiquitously throughout the genome (Pearce *et al.*, 2012). Furthermore, results from an article currently being prepared for journal submission (I serve as a co-author) determined that actively released cfDNA from 143B cells consisted primarily (88 %) of repetitive DNA, which exceeds any value predicted for the human genome, and that both LINE-1 and ALU elements were significantly overrepresented. LINE-1 and ALU1 will, therefore, provide a broader overview of the DNA methylation status of gDNA and the majority contents of cfDNA.

An *in vitro* pilot study was prepared in order to demonstrate whether gDNA and cfDNA methylation changes occur upon both pharmacological treatment and cfDNA exposure (paragraph 5.2.5). Cells were treated with VPA in the same manner described in the article of paragraph 5.2.3 (two

24 hour doses of 5 mM). Additionally, untreated cells were exposed to two 24 hour doses of cfDNA derived from untreated cells and VPA-treated cells, respectively. To determine whether DNA methylation changes could be of short duration, as observed for metabolic activity induced by cfDNA in paragraph 5.2.3, DNA methylation status was also screened after 30 minutes and 4 hours of cfDNA exposure.

The results revealed only slight changes in DNA methylation in both gDNA and cfDNA that were not statistically significant. However, the following must be kept in mind:

1. The pilot study involved only two 24 hour doses and, therefore, represents acute treatment and not chronic treatment. Studies have shown that environmentally-induced DNA methylation alterations, which can be modulated by hormones and other cellular messengers, undergo a form of a "kindling-like" phenomenon, where successive stimulations and binding of transcription factors to gene regulatory regions can change nucleosome structure (Abdolmaleky *et al.*, 2015). Subsequent exposure to such factors or modulators will then be accompanied by facilitated DNA methylation and gene expression alterations, resulting in the threshold for alteration of DNA methylation level decreasing upon repeated exposures. Episodic exposure to chronic medication with DNA methylation effects may, therefore, imply that the effects of every new exposure will have a stronger effect on the epigenome.
2. The VPA treatment regimen followed during the experiment served as therapeutic treatment of the cell line, i.e. was not intended to cause cell death, but elicit measurable biochemical effects. VPA's HDAC inhibition and DNA demethylation properties make it an effective anticancer agent (Blaheta *et al.*, 2005), implying that the current dose used may not have been sufficient enough to induce more significant DNA methylation effects and that using a higher dose in order to induce stronger effects would have likely resulted in cell death.
3. Most importantly, although the observed effects of both VPA and cfDNA treatments were not statistically significant, these effects may still affect cell phenotype. Changes as low as 5 % in specific genes can still result in a significant inverse correlation (> 60 %) between differentially methylated and expressed genes (Ozer & Sezerman, 2017), which can significantly affect gene transcription regulation.

The pilot study observations, therefore, could suggest that pharmaceutically-induced epigenetic adverse effects may be transferable to adjacent cells. Whether such pharmaceutically-induced epigenetic changes can occur *in vivo* and be transferred between organisms

5.2.5 Prepared pilot study manuscript

This manuscript was prepared in article format to summarise the data obtained during the pilot study in the form of a short communication for the sake of completeness of the thesis. This manuscript was not published.

Pharmaceutically-induced epigenetic effects in cell-free DNA in vitro and the risk of clinical implications in vivo: A pilot study

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

Abstract

Objectives: A pilot study was conducted to investigate the possibility that pharmaceutically induced epigenetic effects can be transferred to untreated cells via cell-free DNA (cfDNA). First, it was determined whether valproic acid (VPA)-induced DNA methylation can be detected in cfDNA, and second, whether these effects can be transferred to untreated cells via spontaneous transfection.

Design and methods: CfDNA and genomic DNA was isolated from untreated and VPA-treated HepG2 cells, respectively, and amplified with real-time PCR using methylation-specific GSTP, RASSF1A, LINE-1 and ALU1 primers. The isolated cfDNA was administered to untreated cells and changes in LINE-1 methylation in genomic DNA were measured.

Results and conclusions: VPA-induced methylation of genomic DNA was detected in cfDNA, and cfDNA derived from VPA-treated cells induced similar methylation changes in recipient cells that were not treated with VPA. These observations support the hypothesis that cfDNA molecules possess the capacity to act as an intercellular messenger.

Introduction

The possibility that DNA released into biofluids (cell-free DNA (cfDNA)) may act as an intercellular messenger (Aucamp *et al.*, 2016; Gahan & Stroun, 2010; Thakur *et al.*, 2014) has many potential biochemical and genetic implications. It has been observed that cfDNA fragments often contain cancer mutations originating from the parent tissue (Kahlert *et al.*, 2014), can induce metastasis in susceptible cells (García-Olmo *et al.*, 2010), and transfer damage (Ermakov *et al.*, 2013). The cfDNA derived from non-differentiating cells has also been shown to block tumor growth (Garcia-Olmo *et al*., 2015). In this pilot study we tested whether the intercellular messaging functions and selective form and characteristics of cfDNA release may indicate that pharmaceutically-induced epigenetic effects can be transferred from treated to untreated cells *in vitro*. The study determined (i) whether exposing cells to the pharmacological compound, valproic acid (VPA), can influence the DNA methylation levels of cfDNA and (ii) whether these changes can be transferred, via cfDNA, to untreated cells. The transfer of epigenetic changes between cells via cfDNA can implicate cfDNA as a risk factor in a clinical setting and, therefore, warrants further investigation.

Materials and methods

HepG2 cells (ATCC® HB-8065™) were cultured, according to ATCC guidelines, in growth medium fortified with 1 % of penicillin-streptomycin, L-glutamine, non-essential amino acids (Lonza) and amphotericin B (Biochrom). Cells were seeded at 15 % confluence in 75 cm^2 flasks (TPP) and 6 x 10^4 cells/well in a 12 well plate (Corning) at a final volume of 10 and 1 mL, respectively, and incubated in a humidified atmosphere at 37 °C and 5 % CO_2 for 12 hours. The growth medium was then replenished with growth medium (negative control) and either 5 mM VPA (Sigma Aldrich) or 17 ng cfDNA, respectively, and incubated for 30 min and 4 hours (12 well plates) or 24 hours (75 cm^2 flasks). For the 75 cm^2 flasks, this process was repeated for a 48 hour treatment. The growth medium and cells were collected as described previously (Bronkhorst *et al*., 2016).

CfDNA was extracted from growth medium using the NucleoSpin Gel and PCR Clean-up kit using buffer NTB instead of NTI. For the extraction of genomic DNA (gDNA), cells were suspended in lysis buffer (100 mM TrisHCl, 0.5 M EDTA, 10 % SDS, 5 M NaCl and 20 mg/ml proteinase K) and incubated at 37 °C for 2 hours. A 1:1 ratio of propan-2-ol was added and mixed until DNA precipitation was completed, centrifuged at 10 000 x g for 3 minutes, rinsed with 70 % ethanol. The centrifugation step was then repeated, and the pellet dried and dissolved in the supplied elution buffer. The cfDNA and gDNA were quantified with the Qubit® dsDNA High Sensitivity Assay kit and Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies).

VPA-induced DNA methylation changes in gDNA and cfDNA, bisulfite converted with the EpiTect bisulfite kit (Qiagen), were evaluated using the methylation-specific primers of tumor suppressor genes, GSTP1 and RASSF1A (Li *et al*., 2012), and repetitive elements, LINE-1 (Wang *et al*., 2013) and Alu1 (Graff *et al*., 1997). PCR-amplification was performed using HotStarTaq® Plus DNA Polymerase and EpiTect HRM PCR Buffer with EvaGreen® dye (Qiagen). EvaGreen® has similarities to SYBR® Green and equal binding affinity for GC-rich and AT-rich regions with no apparent sequence preference. PCR amplification conditions were: 95 °C for 5 minutes, followed by 40 cycles. of 95 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 15 seconds.

Results and discussion

VPA-treatment did not significantly alter the methylation status of RASSF1A cfDNA (Fig. 1A), GSTP1 cfDNA and gDNA (Fig. 1B) and the gDNA of ALU1 and LINE-1 (Fig. 1C). However, there is a tendency torwards an increase in the methylated fraction of RASSF1A gDNA and ALU1 cfDNA and both the unmethylated and methylated fractions of the LINE-1 cfDNA. Exposing cells for 48 hours to the cfDNA of untreated cells resulted in a tendency towards an increase in gDNA methylated LINE-1 fractions, whereas the cfDNA derived from VPA-treated cells resulted in a

decrease in gDNA unmethylated LINE-1 fractions. To see whether DNA methylation could occur earlier than 24 – 48 hours, cells were also treated for 30 minutes, which showed no differences between control and VPA-treated cell cfDNA treated cells, and 4 hours, which showed that the gDNA methylation status of cells treated with cfDNA derived from VPA-treated cells remained similar to that of 30 minutes, while that of the cells treated with control cell cfDNA significantly decreased in both unmethylated and methylated LINE-1 fractions. VPA treatment, therefore, may affect cfDNA characteristics and result in altered epigenetic effects in recipient cells compared to that of the cfDNA of untreated control cells. Whether the epigenetic changes of the VPA treatment resulted in these subsequent changes induced by the cfDNA treatments requires further investigation.

Environmentally-induced DNA methylation alterations, which can be modulated by hormones and other cellular messengers, are said to result in a "kindling-like phenomenon" in which nucleosome structures are changed via successive stimulations and binding of transcription factors to gene regulatory regions (Abdolmaleky *et al*., 2015). Subsequent exposure to similar stimulation will, in effect, be accompanied by facilitated DNA methylation and gene expression alterations, resulting in decreasing threshold levels for DNA methylation alterations upon repeated exposures. Episodic exposure to medication with DNA methylation effects (in this case the chronic use of medication such as VPA) may, therefore, result in a gradual increase in epigenomic effect with every new exposure. It is, therefore, possible that continuing treatment of cells with VPA and cfDNA, respectively, would have resulted in increased DNA methylation changes. This also implies that patients taking chronic DNA methylation-altering medication may gradually develop increased epigenetic effects that may alter cells' responsiveness to environmental exposure, gene expression and response to therapy, thereby resulting in adverse effects (Kacevska *et al*., 2011). LINE-1 demethylation and activation, for example, has been shown to drive the transcription of neighboring sequences originally silenced by DNA methylation, resulting in illegitimate transcription (Weber *et al*., 2010). DNA methylation is also an important mechanism in the regulation of retroelements and DNA hypomethylation can lead to the activation and transposition of transposable elements, which in turn can induce the illegitimate transcription of neighboring genes once incorporated within or near genes.

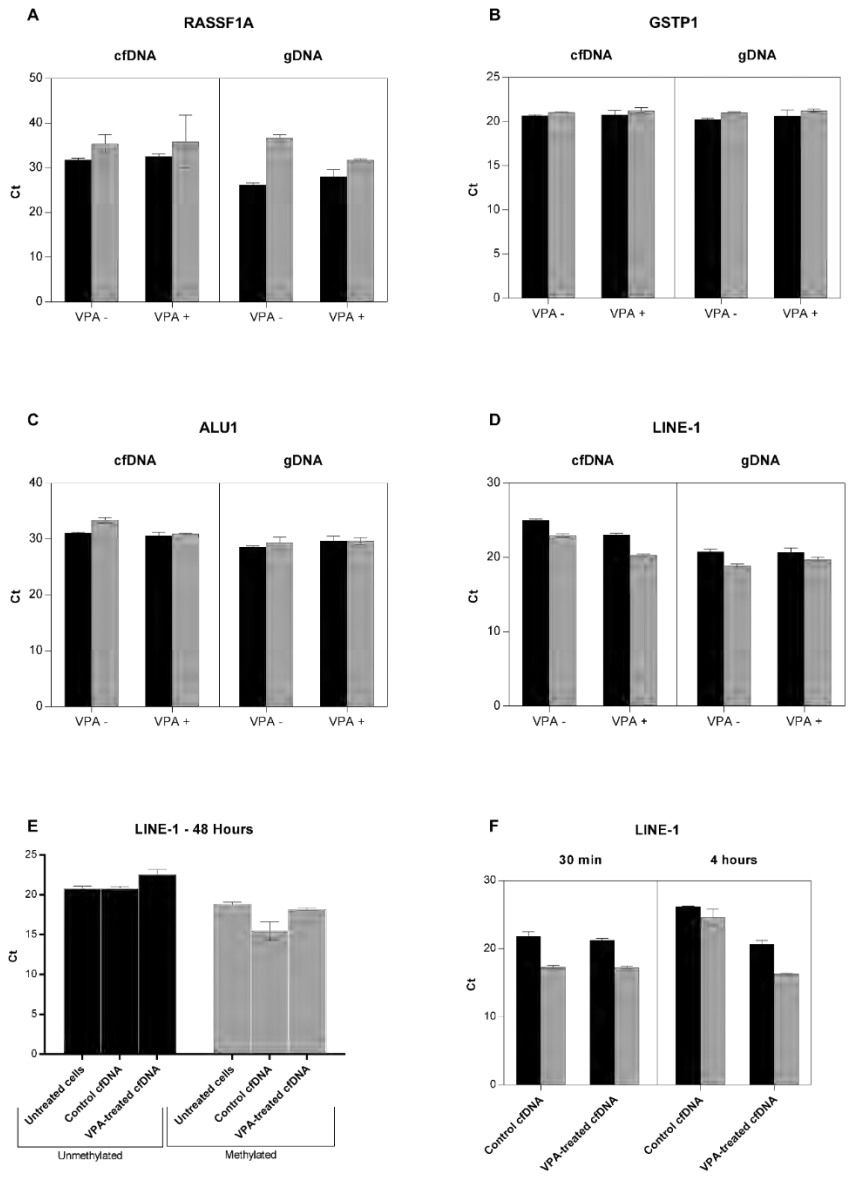

Figure 5-1: **Ct values of cfDNA and gDNA methylation.** (a) GSTP, (b) RASSF1A, (c) ALU1 and (d) LINE-1. CfDNA and gDNA was collected from 75 cm^2 flasks of untreated and VPA-treated HepG2 cells (± SD, n = 4). (e) Ct values of gDNA methylation of LINE-1. The gDNA was collected from 75 cm^2 flasks of HepG2 cells treated with two 24 hour doses of 17 ng cfDNA derived from untreated cells and VPA-treated cells, respectively (± SD, n = 4). (f) Ct values of gDNA methylation of LINE-1. The gDNA was collected from a 12 well plate of HepG2 cells treated for 30 minutes and 4 hours, respectively, with 17 ng cfDNA derived from untreated cells and VPA-treated cells, respectively (± SD, n = 4). Black bars = unmethylated, white bars = methylated. Low Ct values indicate higher levels of initial PCR template and earlier cycle amplification.

Conclusion

Although the epigenetic changes induced by the VPA and subsequent cfDNA treatments were not particularly significant, there are indications of a tendency towards induced changes in DNA

methylation. Furthermore, LINE-1 DNA methylation changes induced by subsequent treatment of cells with cfDNA derived from VPA-treated cells support the hypothesis that pharmaceutically-induced epigenetic adverse effects may be transferable to adjacent cells. Regardless, although the induced epigenetic effects of both acute treatments of VPA and cfDNA are not significant, these effects may, however, still affect cell phenotype. Ozer and Sezerman (2017) has recently presented a valid level change threshold for DNA methylation changes and showed that DNA methylation changes as low as 5 % in specific genes can still result in an inverse correlation of more than 60 % between differentially methylated and expressed genes, which can significantly affect gene transcription regulation. Whether or not DNA methylation changes will affect cell phenotype will, therefore, depend on whether the changes occurred in important genomic regions (e.g. regions that can affect the accessibility of gene promoter regions for transcription factor binding).

Author contributions

A.J. Bronkhorst and I contributed equally to the drafting of this pilot study manuscript. I performed the treatment of cell cultures, cfDNA and gDNA extractions. A.J. Bronkhorst performed the PCR assays of the VPA-treated cells and I performed the PCR assays of the cfDNA-treated cells. Prof Pretorius critically reviewed the manuscript.

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CHAPTER 6

INTRODUCTION OF THREE-DIMENSIONAL CELL CULTURES TO CIRCULATING DNA RESEARCH:

To this point it was demonstrated that the utilisation of *in vitro* 2D cell cultures:

1. Can limit the origins of cfDNA to primarily the tissue origin, diseased cells (in this case tumour cells), cell type and morphology in question.
2. Can produce cfDNA samples consisting of single origin fractions (nucleosomal fragments (apoptotic) and ~2 000 bp fragments (actively released)).
3. Provides a simpler physiological environment, disconnected from the circulation and all other physiological systems or occurrences, that simplifies not only the identification of cfDNA origins, but may also simplify biomarker detection as there are no outside impacts or background noise.
4. Can produce cfDNA release patterns that correlate with that of human plasma samples (Applied-Biosystems, 2015).
5. Provides a highly flexible model that can be used to screen for changes in cfDNA release and characteristics during exposure to stress, infection, xenobiotics.

However, for many researchers 2D cell cultures do not sufficiently represent physiological environments as they cannot recreate cell-cell and cell-extracellular matrix interactions established in an *in vivo* environment (Christakou *et al.*, 2015; Howes *et al.*, 2014; Lee *et al.*, 2016; Pampaloni *et al.*, 2007). Despite this limitation, the abovementioned benefits can still aid in (i) the discovery of disease-specific biomarkers, as the 2D environment will not result in the disappearance of critical biomarkers or mutations, and (ii) the elucidation of the biological functions of cirDNA, as the 2D environment should not affect the function itself, but may affect the effect elicited. One can, therefore, initially use *in vitro* means to aid in the identification of a target, followed by *in vivo* means to confirm the target and elucidate its physiological occurrence, characteristics and/or implications. Or, perhaps, simply upscale to a more complex *in vitro* model.

The utilisation of 3D cell cultures can greatly benefit cirDNA research in the sense that these *in vitro* models can re-establish the biochemical and mechanical cues that form *in vivo* communication networks, thereby allowing them to mimic the specificity of real tissues (Pampaloni *et al.*, 2007). Two specific forms of 3D cultures, in particular, can provide the unique opportunity of observing cfDNA characteristics in a physiologically relevant environment. The first 3D cell culture method involves the utilisation of Fibercell systems, a small-scale cell and co-culture system that can be used for large-scale cell product (e.g. proteins) harvesting. This 3D culture system was created to mimic tissue circulation, utilising hollow fibres laid out in bioreactors that

serve as artificial capillaries. As mentioned in the article of paragraph 3.2, these models have been used for (i) *in vivo* drug analysis, (ii) protein and exosome production (Whitford *et al.*, 2015), (iii) cell secretome analysis (Chang *et al.*, 2009) and (iv) infection models (Cadwell, 2015), including the development of a tuberculosis model that has been approved by the European Medicines Agency for the development of drugs and treatment regimens for clinical trials (Gumbo *et al.*, 2015; Hughes, 2015). This particular model has a forecasting efficiency within 94 % of clinical trial outcomes, making this *in vitro* model as efficient as a clinical trial. The utilisation of Fibercell system will, therefore, have two added benefits to those mentioned for 2D cell cultures:

1. It is a model originally intended for large-scale cell product harvesting
2. It can simulate *in vivo* conditions as efficiently as a clinical trial.

The second 3D cell culture method is developed to mimic tissue biopsies rather than tissue circulation, utilising the development of cellular aggregates, named spheroids, which can be produced from single and/or co-cultures in rotating microgravity bioreactors (Wrzesinski & Fey, 2013). This particular form requires 21 days of spheroid development, resulting in spherical cell structures (consisting of a proliferating outer layer and quiescent, hypoxic, apoptotic and necrotic core cells (Edmondson *et al.*, 2014)) with re-established physiological functions that became suppressed during the trypsinisation process (Wrzesinski & Fey, 2013). The utilisation of this spheroid culture method will, therefore, have several added benefits to those mentioned for 2D cell cultures:

1. It allows the cultured cells to overcome physiological function suppression induced by trypsinisation.
2. It produces cellular systems consisting of cells in various stages, relating them more to *in vivo* tissues.
3. No synthetic scaffolds or matrices are involved that could affect the biological characteristics of the cell culture and cfDNA.
4. Most of the physiological and metabolic functions of the spheroids can remain stable for up to three weeks, allowing one to perform longer term or chronic treatment studies.
5. It, therefore, combines the benefit of using models restricted to only the area or cells in question with the benefit of using a physiologically relevant cell model.

In this chapter, the concept of utilising the cfDNA from *in vitro* 3D cell cultures to study the *in vivo* biological function of cirDNA and aid in the discovery of physiologically- and/or disease-specific biomarkers is introduced.

6.1 *In vitro* analysis of cell-free DNA using a three-dimensional cell culture model

The spheroid culture method of Wrzesinski and Fey (2013) was used to compare the release patterns and characteristics of cfDNA produced from HepG2/C3A spheroids to that of 2D cell

cultures and human plasma samples (refer to article in paragraph 6.2). Growth medium was collected every 48 – 72 hours during the 21 day developmental stage and every 48 hours during 336 hours of treatment with paracetamol (100 mg/kg). CfDNA was extracted, quantified and fragment analysis performed and compared to spheroid growth, necrotic core formation and glucose consumption.

After 20 days of spheroid development, the bioreactors contained a third more cells than that which a standard 75 cm^2 flask can maintain, producing microgram levels of cfDNA that is of great benefit for subsequent experiments, such as sequencing and mutation analysis. The cfDNA release and fragment patterns of both the development stage and subsequent treatment stage effectively mirrored brief and/or minor changes in spheroid growth and glucose consumption. The fragment patterns of the spheroid cfDNA also correlated with that of both 2D cell cultures (Bronkhorst *et al.*, 2016d) and human plasma samples (Applied-Biosystems, 2015), with the exception that there is a continuous presence of nucleosomal fragment DNA throughout both the development and treatment stages. This is likely due to hypoxia-induced apoptosis in the deeper cell layers of the growing spheroids. Increased spheroid growth causes the compaction of cells and reduced availability of nutrients and oxygen, resulting in increased necrotic core formation. Hypoxia in the core can also induce apoptosis, a process that HepG2/C3A cells cannot circumvent compared to other cancer cells, as these cells do not appear to have p53 mutations (Bain *et al.*, 2007; Soussi, 2012) that can inactivate TP53 tumour suppressor function (Hanahan & Weinberg, 2011; Lowe & Lin, 2000). The continuous presence of nucleosomal DNA fragments in this cell line can, however, be prevented through the utilization of more apoptosis-resistant cell lines and this 3D culture method can still serve as an effective “closed-circuit” model despite the nucleosomal DNA fragments as the model still restricts the putative sources and causes of cfDNA to that of the target cell type.

6.2 Guidelines for authors – The International Journal of Biochemistry and Cell Biology

(https://www.elsevier.com/journals/the-international-journal-of-biochemistry-and-cell-biology/1357-2725/guide-for-authors. Date of access: 26 January 2017)

The following article (paragraph 6.3) was submitted as a regular research article. This Elsevier paper has guidelines identical to those listed in paragraph 4.1.1, except for the following: regular research articles have a limit of 5 000 words. In addition to an abstract and a reference list, regular research articles should contain an introduction, materials and methods, results, discussion, conclusions, acknowledgements and reference section. The abstract should not exceed 250 words. The following checklist is provided for illustrations:

- Molecular weight markers are included in all immunoblot and gel figures.
- For comparative studies, all the protein bands must originate from the same gel.

- Bar graphs must include standard errors or standard deviations. Avoid over-complicated coding patterns for bars. Micrographs must include scale bars.
- Appropriately sized numbers, letters, and symbols should be used so they are no smaller than 2.4 mm (10 pt) in size after reduction to a single column width (7.84 cm or 3.085 inches), or a 2-column width (16.5 cm or 6.5 inches). Superscript and subscript characters are included in this rule. Numbers, letters, and symbols used in multi-panelled figures must be consistent.
- Figures should preferably be drawn to occupy a single column width (7.84 cm or 3.085 inches). Occasionally, the figures may occupy a double column width if such size enhances presentation. Produce images near to the desired size of the printed version.
- Make sure that the lettering is such that when the figure is in print, the size of the font is the equivalent to, or slightly larger than the text of the journal.
- Axis labels should be printed with an initial capital letter followed by lower case lettering with units in open brackets. Axis line width should be uniform and 1 pt in thickness.
- Column and line diagrams should not be boxed or include gridlines.
- Use a logical naming convention for your artwork files, and supply a separate listing of the files and the software used.

A journal reference template is available for EndNote. Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown'. References should be arranged first alphabetically and then further sorted chronologically, if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication. E.g. Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

6.3 Article published in The International Journal of Biochemistry and Cell Biology

Aucamp, J., Calitz, C., Bronkhorst, A.J., Gouws, C., Hamman, S., Pretorius, P.J. 2017. Cell-free DNA in a three-dimensional spheroid cell culture model: A preliminary study. *International journal of biochemistry and cell biology*, 89(2017):182-192.

C. Calitz and I contributed equally to the drafting of this article. I performed the cfDNA extractions, quantifications, fragment size analysis and LDH assay. C. Calitz performed the 3D cultures, paracetamol treatments, planimetry and glucose consumption assays. Dr C. Gouws, Prof S. Hamman and Prof Pretorius critically reviewed the manuscript.

International Journal of Biochemistry and Cell Biology 89 (2017) 182–192

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Cell-free DNA in a three-dimensional spheroid cell culture model: A preliminary study

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ARTICLE INFO

Keywords:
Cell-free DNA
3D cell culture
Spheroid
Capillary electrophoresis
Rotating bioreactors

ABSTRACT

Background: Investigating the biological functions of cell-free DNA (cfDNA) is limited by the interference of vast numbers of putative sources and causes of DNA release into circulation. Utilization of three-dimensional (3D) spheroid cell cultures, models with characteristics closer to the *in vivo* state, may be of significant benefit for cfDNA research.
Methods: CfDNA was isolated from the growth medium of C3A spheroid cultures in rotating bioreactors during both normal growth and treatment with acetaminophen. Spheroid growth was monitored via planimetry, lactate dehydrogenase activity and glucose consumption and was related to isolated cfDNA characteristics.
Results: Changes in spheroid growth and stability were effectively mirrored by cfDNA characteristics. CfDNA characteristics correlated with that of previous two-dimensional (2D) cell culture and human plasma research.
Conclusions: 3D spheroid cultures can serve as effective, simplified *in vivo*-simulating "closed-circuit" models since putative sources of cfDNA are limited to only the targeted cells. In addition, cfDNA can also serve as an alternative or auxiliary marker for tracking spheroid growth, development and culture stability.
Biological significance: 3D cell cultures can be used to translate "closed-circuit" *in vitro* model research into data that is relevant for *in vivo* studies and clinical applications. In turn, the utilization of cfDNA during 3D culture research can optimize sample collection without affecting the stability of the growth environment. Combining 3D culture and cfDNA research could, therefore, optimize both research fields.

1. Introduction

Since the discovery of cell-free DNA (cfDNA) in human plasma and other biological fluids (Fleischhacker and Schmidt, 2007; Peters and Pretorius, 2011), investigation of these peculiar cfDNA fragments has become a fast-growing research field due to its immense potential as a non-invasive diagnostic and prognostic marker for both disease and normal physiological conditions. Intercellular and inter-organ messaging functions have also been proposed for cfDNA in the form of newly synthesized, spontaneously released DNA/RNA-lipoprotein complexes (Aucamp et al., 2016; Gahan, 2006; Gahan and Stroun, 2010). Apart from the active release of cfDNA (Gahan and Stroun, 2010; Van der Vaart and Pretorius, 2007), practically any physiological source or process that can result in the release of DNA into biological fluids can contribute to cfDNA content (refer to Thierry et al. (2016) as an example of the multiple putative cfDNA sources in cancer). From an *in vivo* perspective this multitude of putative cfDNA sources results in background noise and false results. This interference, in concurrence with a lack of knowledge regarding the biological functions of cfDNA, are factors that prevent the effective translation of cfDNA research into clinical practice.

The study of the biological characteristics and functions of cfDNA through the utilization of *in vitro* cell cultures has been proposed. Our previous research showed that an osteosarcoma cell culture model has a distinct pattern of apoptotic and actively released DNA levels (Bronkhorst et al., 2016). However, two-dimensional (2D) cell cultures have their own set of difficulties regarding cfDNA research, one being whether the obtained information sufficiently reflects that of *in vivo* conditions. To date it has been well acknowledged that 2D cell cultures are not representative of the cellular environment found in organisms

Abbreviations: cfDNA, cell-free DNA; 2D, two-dimensional; 3D, three-dimensional; LDH, lactate dehydrogenase; CE, capillary electrophoresis; APAP, acetaminophen

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http://dx.doi.org/10.1016/j.biocel.2017.06.014
Received 16 January 2017; Received in revised form 7 June 2017; Accepted 22 June 2017
Available online 24 June 2017
1357-2725/

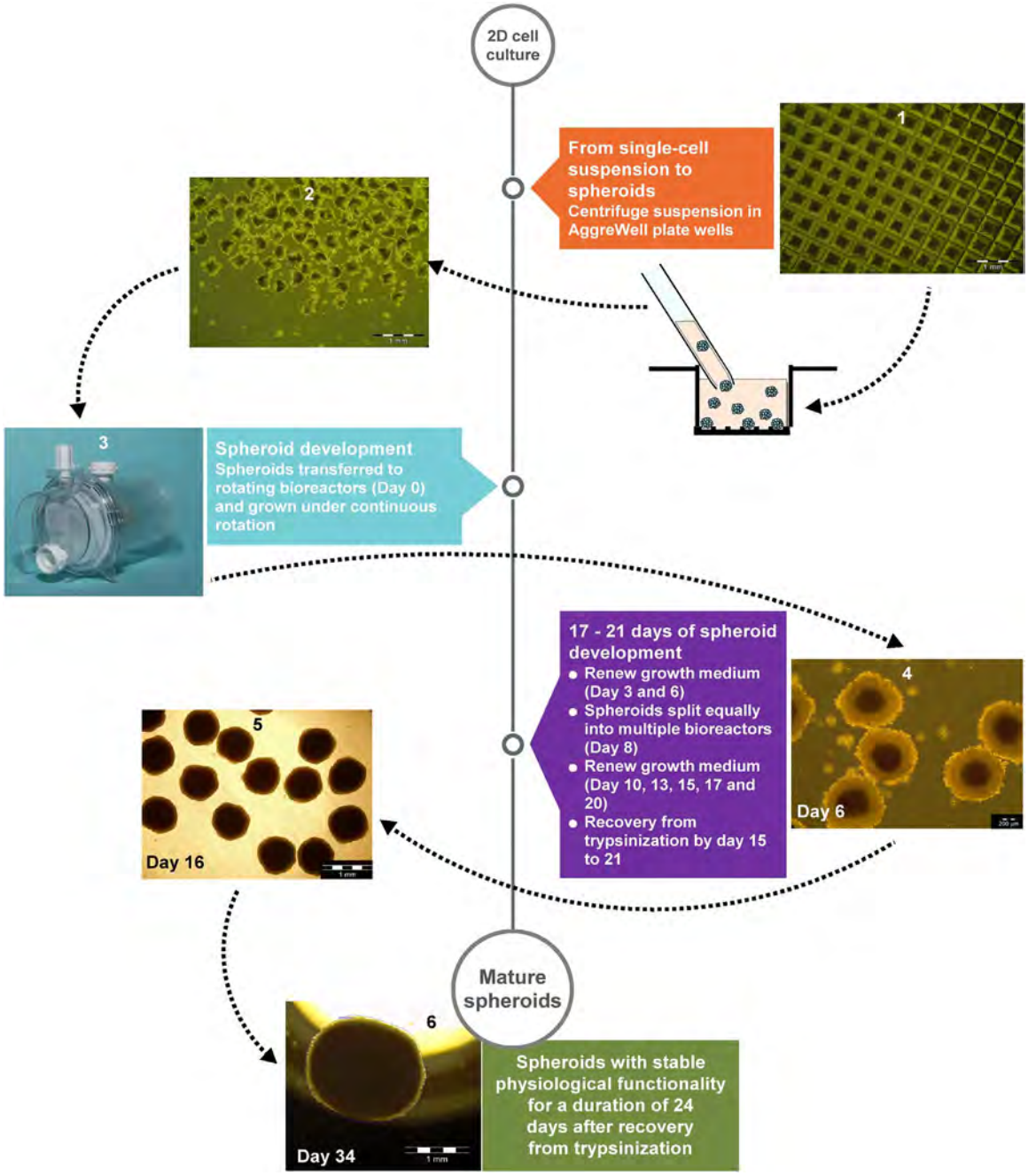


Fig 1. The development of HepG2/C3A spheroids via the rotating bioreactor technique (Wrzesinski and Fey, 2013; Wrzesinski et al., 2014). (1) AggreWell™400 with cells after centrifugation of the single cell suspension and 24 h of incubation; (2) Spheroids collected from the AggreWell™400; (3) Rotating bioreactor (MC2 Biotek) (Fey and Wrzesinski, 2012a); (4) Light microscopy of 6 day old HepG2/C3A spheroids; (5) Light microscopy of 16 day old HepG2/C3A spheroids; (6) Light microscopy of a HepG2/C3A spheroid 14 days after development (34 days old).

as they cannot effectively simulate tissue-specific architecture, mechanical and biochemical cues and cell–cell communication (Edmondson et al., 2014; Pampaloni et al., 2007). The use of living organisms, on the other hand, has its own unique scientific, ethical and social challenges (Edmondson et al., 2014). 2D cultures serve as "closed-circuit" models that promote the restriction of putative DNA sources to only that of the cell type in question. One will no longer have a "closed-circuit" model when using living models and, therefore, no control over the targeted physiological environment, resulting in background noise due to unforeseen biological influences. Moreover, the use of living organisms over *in vitro* methods also contravenes the 3Rs principle (Flecknell, 2002).

Comparison of the size profile of cfDNA from 2D cell cultures (Bronkhorst et al., 2016) to that of plasma samples (Applied-Biosystems, 2015) showed striking similarities, as both samples contained nucleosomal fragment patterns, as well as DNA fragments with a size of 2 000 bp, which was determined to not be of apoptotic or necrotic origin (Bronkhorst et al., 2016). This observation bridges the gap between the utilization of cell cultures and biological fluids in researching the biological functions of cfDNA. To further benefit from the simple, highly flexible, morally acceptable and less invasive approach of cell cultures, while maintaining the physiologically relevant cellular behavior of living organism models, the utilization of three-dimensional (3D) cell cultures has been investigated.

1.1. Three-dimensional cell culturing

Traditional 2D cell culture models often lack tissue −specific properties found within *in vivo* systems. Cells within whole organisms (*in vivo*) form part of an intricate system having interactions with both neighbouring cells as well as the extracellular matrix (ECM). These interactions between cells and the ECM result in a complex communication network made possible by both biochemical and mechanical signals (Fey and Wrzesinski, 2012b; Lin and Chang, 2008; Page et al., 2013). The overall 3D architecture of a tissue is essential to its function. Due to the fact that 2D cell models are lacking several advanced physiological functions necessary to correlate *in vitro* conditions with those present *in vivo*, cells grown in 2D cannot be seen as equivalent to those present in intact organs (Fey and Wrzesinski, 2012b; Page et al., 2013; Wrzesinski and Fey, 2013; Wrzesinski et al., 2014). 3D cell culture models attempt to bridge the gap between cell-based experimental approaches, *in vivo* animal models and humans (Antoni et al., 2015; Fey and Wrzesinski, 2012b; Lin and Chang, 2008; Wojdyla et al., 2016; Wrzesinski and Fey, 2015).

A variety of 3D cell culture techniques are currently being explored, each offering various advantages and disadvantages. Multi-cellular 3D spheroid cell culture systems, in particular, have the possibility of overcoming the difficulties presented by both *in vivo* animal models and 2D cell culture models (Antoni et al., 2015; Breslin and O'Deiscoll, 2013; Lin and Chang, 2008). These spheroids have multi-cellular arrangements that mimic the 3D architecture of tissues, with sizable cell–cell interactions like that of tight junctions, and diffusion limits mimicking *in vivo* physiological barriers found during drug transport (Fey and Wrzesinski, 2012a, 2012b; Metha et al., 2012; Tvardovskiy et al., 2015; Wojdyla et al., 2016; Wrzesinski and Fey, 2013, 2015; Wrzesinski et al., 2013, 2014). Different multi-cellular spheroid systems are currently under investigation, including hanging drop cultures, non-adhesive surfaces, spinner flasks, NASA rotary system, micro-molding, 3D scaffolds, PNIPAAm cell sheets, primaria dishes, galactosylated substrates, pellet cultures, monoclonal growth and external force enhancement (Breslin and O'Deiscoll, 2013; Lin and Chang, 2008).

1.2. Dynamic micro-tissue spheroid cultures

The dynamic micro-tissue spheroid culturing technique utilizes rotating bioreactors that is revolutionizing mainstream *in vitro* cell culture work, providing better *in vivo* correlation than traditional 2D cell culturing models, and its application in drug toxicity studies has great potential (Fey and Wrzesinski, 2012a; Fey and Wrzesinski, 2012b; Wojdyla et al., 2016; Wrzesinski and Fey, 2015). Wrzesinski and colleagues developed, characterized and established this rotating bioreactor spheroid cell culturing system using the immortal hepatic HepG2/C3A cell line (Fey and Wrzesinski, 2012a, 2012b; Wojdyla et al., 2016; Wrzesinski and Fey, 2013, 2015; Wrzesinski et al., 2013, 2014) Cell suspensions are centrifuged in AggreWell® 400 plates (Fey and Wrzesinski, 2012b; Razian et al., 2013) to create spheroids, which are transferred to resealable bioreactors (Fey and Wrzesinski, 2012a) and continuously rotated in order to prevent the spheroids from adhering to one another and the bioreactor surface (Fig. 1). Whereas other spheroid culturing methods quickly produce spheroids via self-aggregation or scaffolding support systems, this rotating bioreactor technique involves three weeks of development within the rotating reactors, resulting in:

(1) An *in vitro* cell culture that provides physiologically relevant toxicity data

Fey and Wrzesinski (2012b) investigated the median lethal dose (LD_{50}) of six commonly used drugs (acetaminophen (APAP), amiodarone, diclofenac, metformin and valproic acid) in toxicity studies, by means of rotating bioreactors and the HepG2/C3A cell line, as well as comparing that to 2D cell culturing conditions and available *in vivo* observations. To circumvent uncertainty experienced regarding cell numbers and population size, the spheroid data was normalized to amount of protein (μg) present within the spheroids. The latter allowed for dosages administered to spheroids to correlate with dosages administered to animal models during *in vivo* toxicity studies (mg/kg). Microscopy, planimetry and protein content were measured and it was found that the comparison of planimetric area and protein content of the spheroids demonstrated a clear correlation with a relative standard deviation of 21%. Intracellular ATP content, measured to determine cell viability for each of the drug treatments, correlated well with published *in vivo* data, with noted differences in LD_{50} values obtained in 2D cultures compared to 3D spheroid cultures, indicating the usefulness of this *in vitro* technique for determination of LD_{50} values.

(2) The recovery of cultured cells from trypsinization

Trypsinization of cells during sub-culturing is a process wherein the protease trypsin proteolytically degrades ECM proteins resulting in a single cell suspension. Trypsinization at regular intervals such as is needed for continuous 2D cell culturing will result in a disruption of advanced cellular functions, signal transduction, gene expression as well as influencing ECM repair processes and natural cell structure (Page et al., 2013; Wrzesinski and Fey, 2013). Wrzesinski and Fey (2013) found that cells recover after trypsinization in both 2D and 3D cultures. In 2D cultures this typically takes place after 5 days, however cells then again have to be trypsinated, placing 2D cells in a continuous "wound healing" cycle. For the 3D spheroids, on the other hand, this recovery of physiological functions and ultra-structural traits continues up until 15–18 days, as suggested by changes in adenylate kinase, ATP, urea, and cholesterol production. This corresponded well with published literature as several other cell lines namely: Caco-2, HT 29, MDCK, MCF-10A and HepG2, also reported similar changes between 15 and 21 days. Wrzesinski and Fey thus proposed that this is a pervasive recovery process rather than differentiation, which may explain the physiological capabilities that more closely resemble *in vivo* conditions within 3D spheroid culturing.

(3) The development of structures with tissue-like qualities and stable physiological functionality

Cells grown in 3D constructs such as spheroids at dynamic equilibrium are focused on functionality effectively mimicking the *in vivo* condition. It was determined that the HepG2/C3A 3D spheroids exhibited stable physiological functionality for a duration of 24 days after recovery from trypsinization (Wrzesinski et al., 2013). The 3D spheroid cultures provided a metabolically competent homeostatic cell model that remains within equilibrium with the culturing environment for a period of 24 days. Such a stable system permits it to be used as a means to a determine drug toxicity and mode of drug action, evaluation of biomarkers as well as the study of system biology all of which requires metabolic functions to be stable over a long term period (Antoni et al., 2015; Justice et al., 2009; Wrzesinski et al., 2013). Furthermore, it was concluded that the spheroids developed significant changes within every aspect of cellular metabolism, oxidation, transport, growth and morphology that serves as the foundation of architectural, functional and physiological differences within cells (Wrzesinski et al., 2014) (Fig. 2). The spheroids develop cholesterol, urea and ATP levels close to the physiological levels of liver tissue. A significant increase in haemoglobin levels compensates for oxygen transport throughout the spheroid and results in a reduction in protein oxidation. DNA content decreases with a concomitant increase in DNA repair enzymes, indicating increased DNA function and repair. The bioreactor culturing technique also results in a "pressurized" system that promotes the penetration of growth medium, nutrients and oxygen into spheroids (Wrzesinski and Fey, 2017), preventing the formation of the so-called necrotic core observed in other spheroid culture techniques due to a lack of access to nutrients and clearance of waste products (Alvarez-Pérez et al., 2005; Gong et al., 2015; Lin and Chang, 2008). Moreover, electron microscopy has revealed that spheroids develop microscopic structures similar to that of *in vivo* liver tissues, including bile canaliculi, sinusoid channels with microvilli and glycogen granules

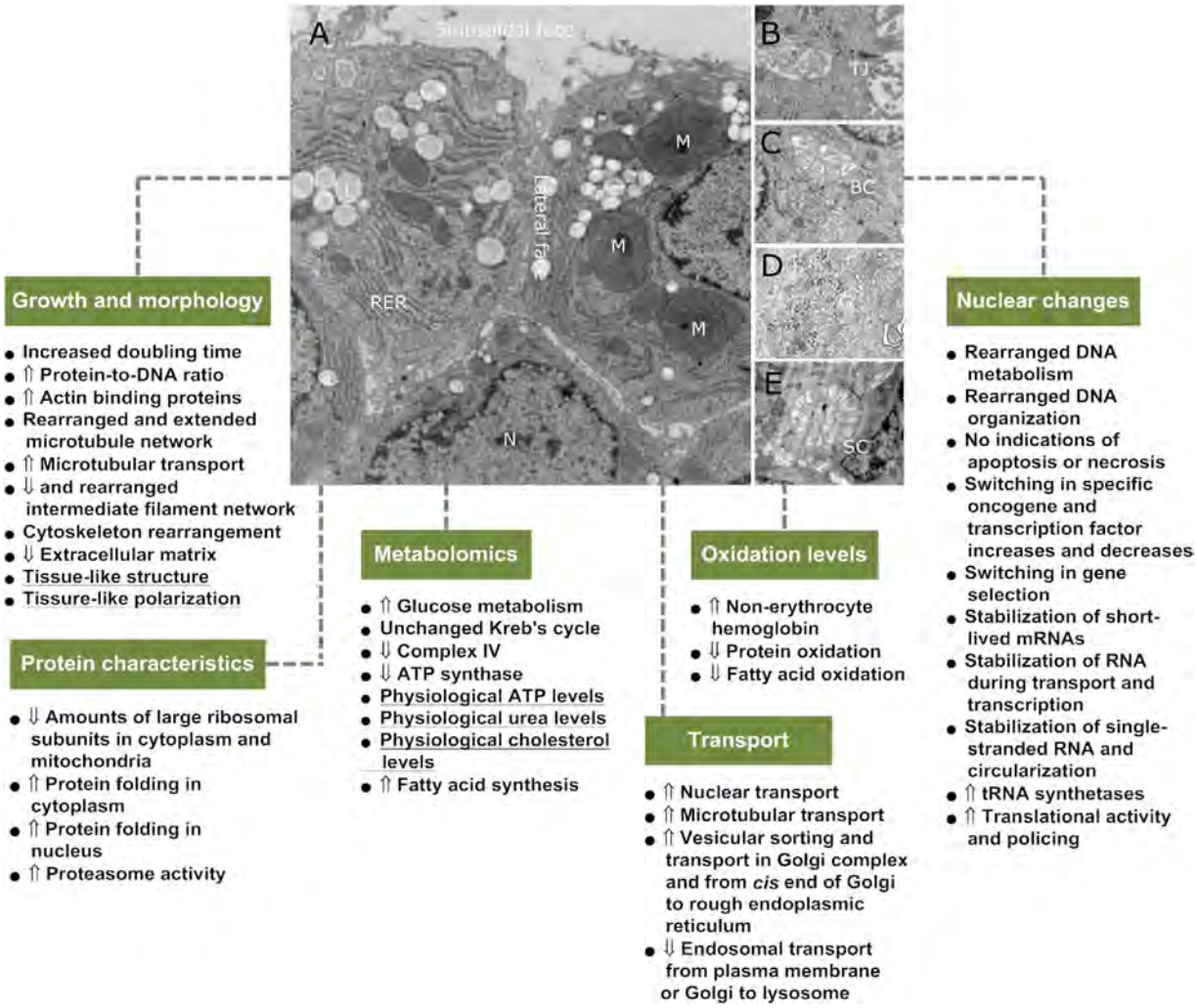


Fig. 2. Summary of the morphological and biochemical characteristics of mature HepG2/C3A spheroids (Wrzesinski and Fey, 2013; Wrzesinski et al., 2014). Electron microscopy of five 21 day old HepG2/C3A spheroids (A, B, C, D and E) with lipid droplets (L), mitochondria (M), rough endoplasmic reticulum (RER), nucleus (N), tight junctions (TJ), bile canaliculi (BC), glycogen granules (G) and sinusoidal channels (SC) (Wrzesinski and Fey, 2013).

(Wrzesinski and Fey, 2013) (Fig. 2).

2. Materials and methods

2.1. Two-dimensional cell culture conditions

Hepatocellular carcinoma derivative cells of the HepG2 cell line, HepG2/C3A (ATCC® CRL-10741™), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 1 g glucose/l (Gibco) fortified with 1% non-essential amino acids (Lonza), 10% fetal bovine serum (Gibco), 1% penicillin streptomycin (Lonza), 1% L-glutamine (Lonza) and 1% amphotericin B (Biochrom). Cells were grown in 75 cm^2 flasks (Corning) at 37 °C and 5% CO_2 in humidified atmosphere.

2.2. Three-dimensional cell culture preparation

2.2.1. Spheroid preparation using AggreWell™400 plates

Cell spheroids were prepared using an AggreWell™400 plate (Stemcell Technologies) according to manufacturer's specifications and previously published data (Fey and Wrzesinski, 2012b; Razian et al., 2013; Wrzesinski and Fey, 2013; Wrzesinski et al., 2013; Wrzesinski et al., 2014). HepG2/C3A cells were seeded into each of the wells of the AggreWell™ plate at a seeding density of 1.2×10^6, and centrifuged in a Sigma 3-16KL centrifuge (Sigma, Germany) for 3 min at 120 x *g*. Following seeding, the cells were incubated in the AggreWell™ plate overnight to ensure aggregation.

2.2.2. Spheroid culture in bioreactors and growth medium collection

Spheroids were detached from the AggreWell™ by gently washing the wells with pre-warmed growth medium (Razian et al., 2013). Detached spheroids were then collected into Petri dishes and the spheroid quality was determined using a light microscope. Spheroids were selected based on visual quality, showing similarities in areas of compactness, as well as equal size and roundness. Selected spheroids were then placed into bioreactors (MC2 Biotek, Hørsholm) and cultivated at 37 °C, 5% CO_2, 95% air in a humidified Galaxy 170R incubator (Eppendorf Company, Stevenage, United Kingdom) for 21 days (Fey and Wrzesinski, 2012a; Fey and Wrzesinski, 2012b; Wrzesinski and Fey, 2013; Wrzesinski et al., 2013; Wrzesinski et al., 2014). The growth medium of each reactor was regularly renewed (Fig. 1) and the spent growth medium was collected for cfDNA extraction at each round of medium change for 21 days during the development of the spheroids (prior to the initiation of the experiment) and thereafter from mature spheroids before each dosage of APAP. The collected medium was centrifuged (EBA 21 Hettich Zentrifugen, Turttlingen, Germany) at $5000 \times g$ for 5 min and the supernatant frozen at −20 °C.

2.2.3. Drug treatment and experimental group setup

APAP was purchased from Sri Krishna Pharmaceuticals. Two experimental groups were prepared in new bioreactors on day 20 by

placing 115 spheroids into each, with two biological replicates (bioreactors) prepared for each experimental group. Spheroids were treated with APAP (100 mg/kg) and growth medium (negative control) every 2 days for a period of 15 days. Dosages were based on the protein mass, spheroid size and the amount of spheroids at each specific day of treatment (Fey and Wrzesinski, 2012b). For all experimental groups, microscopy and planimetry was performed in six-fold with three technical replicates each, resulting in an average of 18 measurements. Growth medium (200 µL) was collected every 2 days and centrifuged for 5 min at 140 × *g*. The supernatant was centrifuged at 15 000 × *g* for 15 min, 130 µL supernatant was collected, snap frozen and stored at −150 °C for lactate dehydrogenase (LDH) analysis. All other spent medium was collected and processed for cfDNA analysis as described in Section 2.2.2.

2.2.4. Glucose quantification

Glucose consumption was measured every 2 days in spent medium using the One Touch Select™ blood glucose monitoring system (Life Scan, Sug, Switzerland) and One Touch Select™ glucose measurement strips (Life Scan, Sug, Switzerland) ($n = 6$).

2.2.5. Microscopy and planimetry

Photomicrographs of the spheroids were taken using a Nikon Eclipse TS100 light microscope (Nikon Instruments, Tokyo, Japan) and a DFK 72AUC02 USB 2.0 color industrial camera (The Imaging Source, Bremen, Germany). Images were analysed by measuring the "shadow area" of the spheroids using the ImageJ software (open-source, Java-based imaging program) to calculate the planar surface in μm^2.

2.2.6. Lactate dehydrogenase assay

An LDH activity assay kit was purchased from Sigma-Aldrich (Sigma, Germany) and prepared according to the manufacturer's specifications in a 96 well plate (TPP Company).

2.3. Cell-free DNA extraction and quantification

CfDNA was extracted with 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, vortexed and centrifuged briefly. For each biological replicate the cfDNA was extracted in triplicate. Extraction samples were prepared by mixing growth medium 1:2 with binding buffer NTI. The samples were vortexed, centrifuged briefly, the entire volume added to spin columns in three 0.6 mL regiments, and centrifuged at 11 000 x *g* for 1 min. Wash buffer was used to clean the spin columns and the cfDNA eluted into 20 µL of elution buffer. The cfDNA was quantified using the Qubit® dsDNA High Sensitivity Assay kit and Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) according to the manufacturer's instructions. The concentrations were then normalized with reference to the amount of protein present (calculated from data of established spheroid growth rate and protein concentrations during the 21 day development period), or to the planimetry data where established protein data was not available (see Method as described in Fey and Wrzesinski 2012b).

2.4. Capillary gel electrophoresis

Capillary electrophoresis (CE) was performed to analyse the size distribution of cfDNA extracted at the different time intervals. The High Sensitivity DNA kit (Agilent Technologies) was used according to the manufacturer's instructions and the analysis performed with the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. CfDNA (0.8 ng) was separated analogously to CE, normalized to a ladder and two DNA markers, the band sizes calculated automatically by the software and the final results were represented as electropherograms. To better observe the ratios of the nucleosomal to larger fragments of APAP-treated spheroids, the amount of DNA used during CE was doubled for the day 8–14 samples.

2.5. Statistics

Statistical analysis of the results was performed with the Tukey multiple comparisons test via GraphPad software. The results of the APAP-treated samples were statistically compared with that of the negative control.

3. Results and discussion

Tissue cells interact with one another and with the ECM through biochemical and mechanical cues and these interactions establish communication networks that maintain tissue specificity and homeostasis. Experimental 3D cell culture systems that re-establish such physiological interactions can mimic the specificity of real tissues (Pampaloni et al., 2007). 3D cell cultures can serve as simplified and flexible models in the form of cellular aggregates called spheroids, which can be produced from single and/or co-cultures via scaffolding/matrix or scaffold-free culturing methods (Wrzesinski and Fey, 2015). Spheroid cultures consist of cells in various stages (proliferating outer layers and quiescent, apoptotic, hypoxic and necrotic core cells) relating them more to *in vivo* tissues compared to 2D cultures (Edmondson et al., 2014). Their growth conditions affect cellular growth rate, spatial organization of cell surface receptors, signal transduction from the outside to the inside of cells, gene and protein expression, cell proliferation, differentiation and cellular behaviour.

For this study, a scaffold- and matrix-free 3D spheroid culture method that requires development over 21 days in rotating bioreactors is of particular interest (Wrzesinski and Fey, 2013). Wrzesinski and Fey (2013) showed that the trypsinization process required to dislodge cells from culture flasks immediately suppresses several important physiological functions. Normal 2D cell cultures could not successfully recover these functions due to required trypsinization when the cells reach confluence, while the 3D spheroids re-establish these physiological conditions after ~18 days. Most morphological, physiological and metabolic functions were shown to reach physiological levels and remain stable for a further 24 days. Wrzesinski and Fey (2013)'s method for 3D spheroid development, therefore, efficiently overcomes the physiological restrictions of *in vitro* cell culture methods and does not contain synthetic matrices and scaffolds to form the spheroids. The release patterns and characteristics of cfDNA from these 3D cultures were elucidated during their development (first 21 days) and treatment (after 21 days) with a medicinal compound, APAP, in order to determine whether (i) 3D spheroids produce similar patterns of cfDNA contents as 2D cultures and plasma samples, (ii) the cfDNA yield can be improved through the use of 3D cultures and (iii) cfDNA release and fragment patterns can provide useful information regarding the stability of the cultures and therapeutic and/or toxicological effects of treatments. APAP was chosen as model compound as this study formed part of a pilot hepatotoxicity study, and APAP is a well-known and characterized hepatotoxic drug.

3.1. Developing spheroids

Fig. 3A depicts the amount of cells and protein in the bioreactors during 3D spheroid development, which, after 20 days, is a third more than that which can be maintained in a standard 75 cm^2 flask. From these cultures almost microgram amounts of cfDNA could be isolated from the growth medium by the end of spheroid development, compared to nanogram amounts from the growth medium of 2D cell cultures (Aucamp et al., 2017), which is of great benefit for subsequent cfDNA-related research, e.g. mutation screening or sequencing. Quantification of cfDNA over time (Fig. 3B) revealed patterns that correlate well with the growth conditions in the bioreactors. Initially each

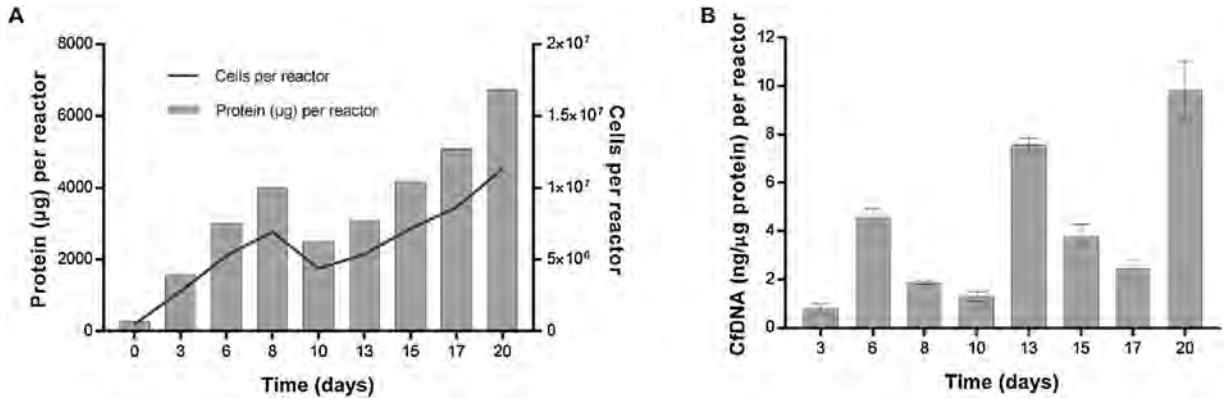


Fig. 3. 3D spheroid development over three weeks. (A) The calculated bioreactor protein and cell content of 3D bioreactors over time. (B) CfDNA release per protein content per bioreactor over time ± SD, n = 6 (three replicates from two bioreactors). Growth medium was exchanged every 2–3 days and the amount of spheroids per bioreactor reduced on day 8 and 20 after sample collection.

bioreactor contained 300 HepG2/C3A cell aggregates produced from a quarter of a 75 cm^2 flask, correlating to the low cfDNA yield for the first three days. The growth medium was replaced with fresh medium every 2 days with the exception of day 6, 13 and 20, which were replaced after 3 days when an increase in cfDNA yield was observed at these time intervals. On day 8 the spheroids were divided into more bioreactors with each bioreactor containing 150 spheroids, resulting in the slight reduction of cfDNA levels in day 10 compared to day 8.

Electropherograms of the cfDNA collected during spheroid development (Table 1) revealed fragment patterns similar to that of 2D cultures (Bronkhorst et al., 2016) and plasma samples (Applied-Biosystems, 2015). The presence of small DNA fragments (in small amounts at 42–60 bp in length), nucleosomal DNA fragment patterns (associated with apoptosis (Bronkhorst et al., 2016)) and a fragment peak at ~2000 bp (associated with actively released DNA (Bronkhorst et al., 2016)) were detected during each time interval. The ratio (percentage) between the amount of 2000 bp DNA fragments and the amount of DNA fragments from 45 to 950 bp, including nucleosomal fragments, in the cfDNA samples indicates that the four nucleosomal fragment peaks are predominant in the bioreactors during spheroid development. The 2 000 bp fragment peak levels (in fluorescent units) were found to increase by day 3 with a concomitant decrease in the nucleosomal fragments. The 2000 bp fragments continued to increase up to day 8 with a concomitant increase in nucleosomal fragments, causing the nucleosomal fragment-to-actively released fragment ratio to increase. As this ratio increases, the fourth nucleosomal peak combines with the 2000 bp peak, causing the peak value to shift to 1000–2000 bp rather than 2000–4000 bp. After sample collection on day 8 in order to ensure balanced culture conditions the number of spheroids per bioreactor was reduced from 300 to 150, resulting in the slight increase of the nucleosomal fragment-to-actively released fragment ratio by day 10 due to induced stress on the spheroids during the spheroid removal process (which requires that bioreactor rotation must be suspended and the spheroids be allowed to sink to the bottom of the bioreactor to remove growth medium without removing any spheroids).

After 3 days of incubation, both the nucleosomal and larger DNA fragments slightly increased on day 13, but the nucleosomal fragment-to-actively released fragment ratio decreased. By day 15, 17 and 20 the nucleosomal fragment-to-actively released fragment ratio significantly increased as the four nucleosomal fragment peak levels increased with a concomitant decrease in the 2 000 bp peak levels. This occurrence could be due to: (1) the spheroids reaching maturity, thereby switching from proliferative cells to a functional cellular form, implying that the actively released cfDNA is connected to cell proliferation and decreases due to the switch in cellular functionality; (2) Increased stress on cells due to the overcrowding of the larger spheroids in the bioreactors. For the subsequent treatment experiments spheroids were transferred to the necessary amount of bioreactors. In this case the electropherogram data may indicate that the amount of spheroids should be reduced earlier to prevent unnecessary stress on spheroids during the last days of development. The cfDNA in bioreactor growth medium may, therefore, assist in the monitoring of spheroid growth and development.

3.2. Viability assays

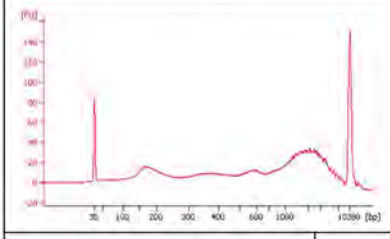
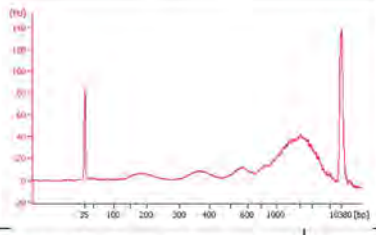
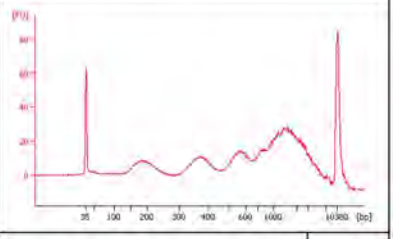
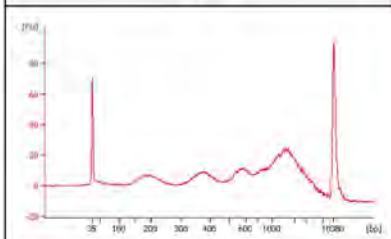
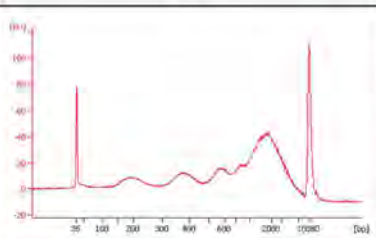
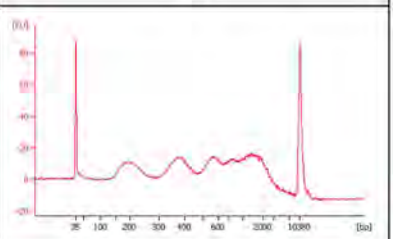
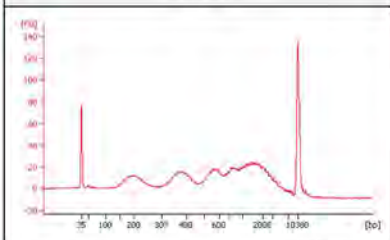
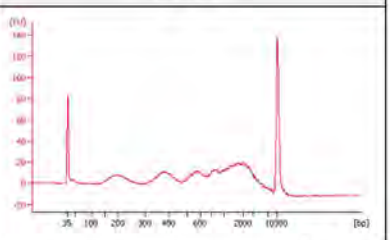
3.2.1. Spheroid growth and glucose consumption

Planimetry was used to determine the average size of the spheroids (Fig. 4A) and the LDH activity used to monitor the viability of the spheroids (Fig. 4B) in each bioreactor. The glucose consumption was also used to determine cell viability or activity (Fig. 4C). The untreated (negative control) and APAP-treated spheroids showed similar high levels of growth with a slight, statistically significant decrease in spheroid growth for APAP at day 10, correlating well with decreased viability (LDH activity) and glucose consumption. The reduction in APAP-treated spheroid growth at 10–12 days resulted in a decrease in LDH activity and a concomitant increase in glucose consumption per spheroid area. Though not statistically significant, the results show a tendency towards a positive correlation between spheroid size and LDH activity (decreased viability). Bigger or older, spheroids develop a significantly slower proliferation rate, resulting in a decrease in glucose consumption in relation to total spheroid mass, thereby explaining the possible inverse correlation observed between spheroid size and glucose consumption at 10–12 days.

3.2.2. Cell-free DNA release per spheroid area

CfDNA quantification in the case of the control spheroids revealed an initial increase in cfDNA release at day 2, followed by a decrease and eventual stable level of further cfDNA release by day 6 (Fig. 5). The increase is likely from stressed cells due to the transfer of the spheroids to multiple bioreactors. The total amount of cfDNA per bioreactor increased from 2.79 to 3.89 µg during day 0–6 and decreased steadily to 3.17 µg by day 14. Electropherogram data (Table 2) showed initially high levels of both nucleosomal fragments and the longer DNA fragments with more nucleosomal fragment DNA than actively released DNA, likely due to the transfer of the spheroids to respective bioreactors. Increasing levels of these peaks from day 2–12 correlate with the amount of cfDNA released per bioreactor, with the nucleosomal fragment-to-actively released fragment ratio gradually decreasing over time possibly indicating stabilization of the spheroids. By day 14 it appears that both nucleosomal fragment and actively released fragment peak levels increased with a slight nucleosomal fragment-to-actively released fragment ratio increase indicating that the increased nucleosomal fragment levels are more than that of the larger DNA fragments. This could be due to the beginning of a proliferative response to the

Table 1
Capillary electropherograms of the cfDNA released during 3D spheroid development and the percentage of the ~2000 bp actively released fragments and the other DNA fractions, including nucleosomal DNA fragments and a smaller fragment, present in 800 pg of cfDNA sample.

DAY 3		DAY 6		DAY 8	
					
42 – 60 bp (small fragments)	1 %	42 – 60 bp (small fragments)	1 %	42 – 60 bp (small fragments)	2 %
42 – 950 bp (small + nucleosomal DNA fragments)	62 %	42 – 883 bp (small + nucleosomal DNA fragments)	48 %	42 – 883 bp (small + nucleosomal DNA fragments)	60 %
950 – 8 900 bp (actively released DNA)	35 %	883 – 8 900 bp (actively released DNA)	49 %	883 – 8 900 bp (actively released DNA)	37 %
DAY 10		**DAY 13**		**DAY 15**	
					
42 – 60 bp (small fragments)	2 %	42 – 60 bp (small fragments)	2 %	42 – 60 bp (small fragments)	2 %
42 – 900 bp (small + nucleosomal DNA fragments)	63 %	42 – 910 bp (small + nucleosomal DNA fragments)	57 %	42 – 900 bp (small + nucleosomal DNA fragments)	73 %
900 – 8 900 bp (actively released DNA)	33 %	910 – 8 900 bp (actively released DNA)	40 %	900 – 8 900 bp (actively released DNA)	23 %
DAY 17		**DAY 20**			
					
42 – 60 bp (small fragments)	1 %	42 – 60 bp (small fragments)	2 %		
42 – 900 bp (small + nucleosomal DNA fragments)	67 %	42 – 910 bp (small + nucleosomal DNA fragments)	64 %		
900 – 8 900 bp (actively released DNA)	29 %	910 – 8 900 bp (actively released DNA)	33 %		

increased availability of nutrients due to the removal of spheroids during previous sampling.

Spheroids treated with 100 mg/kg APAP released significantly less cfDNA per bioreactor and per spheroid area compared to that of the negative control (Fig. 5) at day 0, likely due to less stressed cells due to the transfer of the spheroids to multiple bioreactors compared to that of the negative control. However, by day 2 the amount of cfDNA per bioreactor and per spheroid area became comparable to that of the negative control. At day 10 the amount of cfDNA released increased significantly, followed by a decrease at day 12–14, to levels closer to

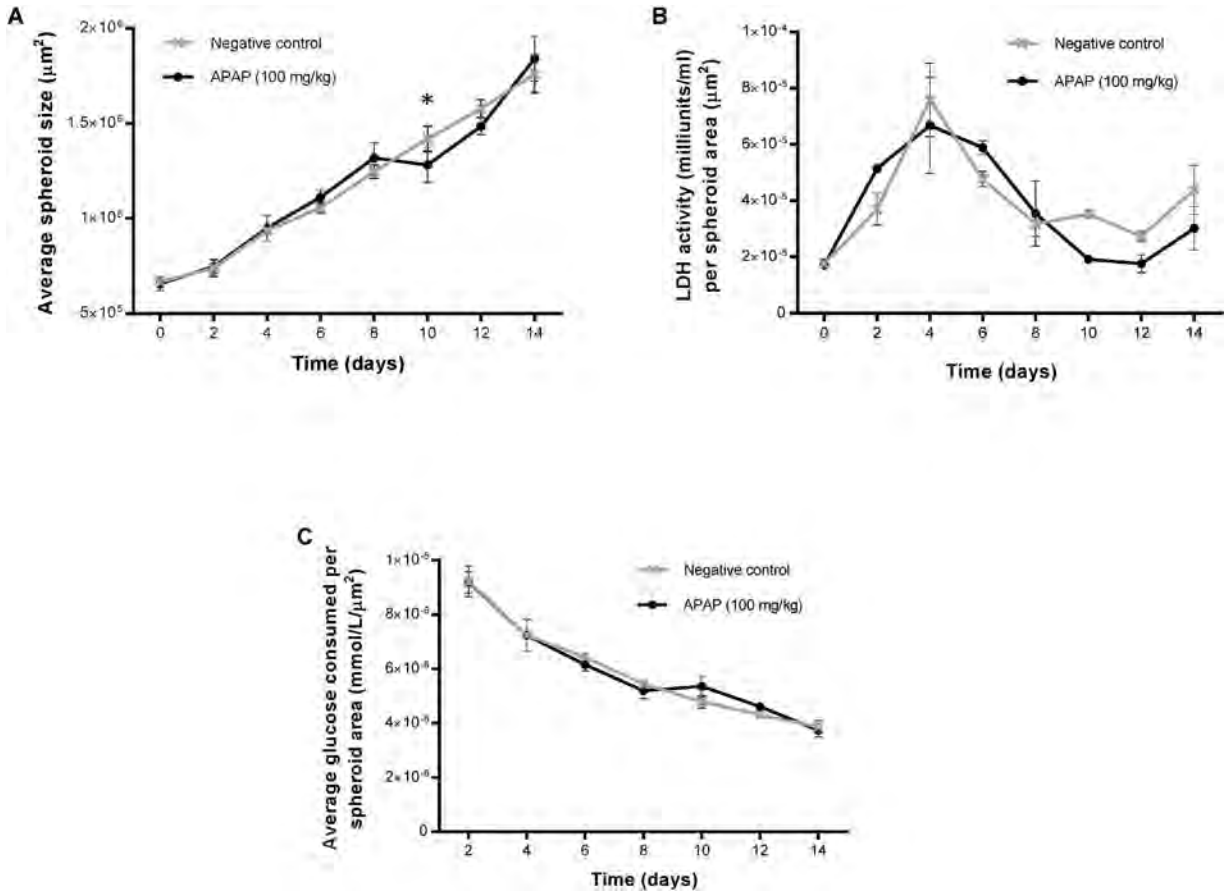


Fig. 4. Average growth rate and glucose consumption of 3D spheroids. (A) Average spheroid size ± SD, n = 6 (six replicates from one bioreactor), *p < 0.05 versus negative control), (B) LDH activity ± SD, n = 2 (two replicates from one bioreactor) and (C) glucose consumption per spheroid area ± SD, n = 6 (six replicates from one bioreactor) over time. Growth medium was exchanged and 6 spheroids removed from each reactor every 2 days.

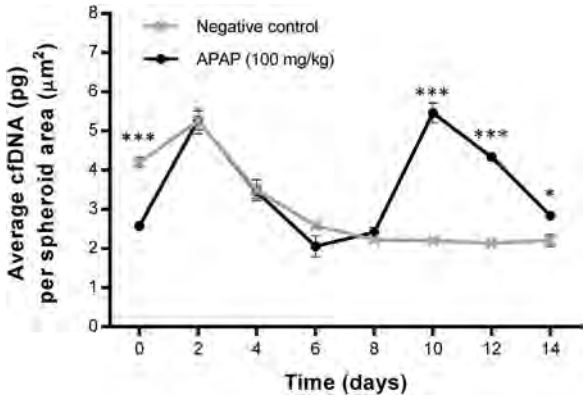


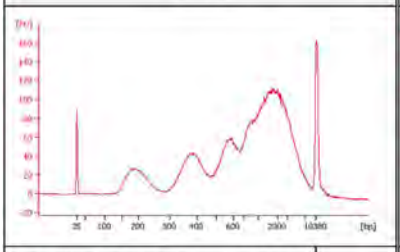
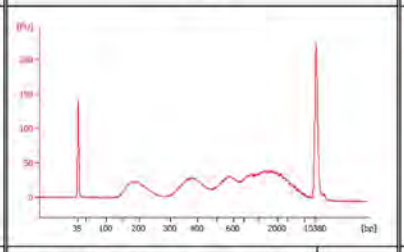
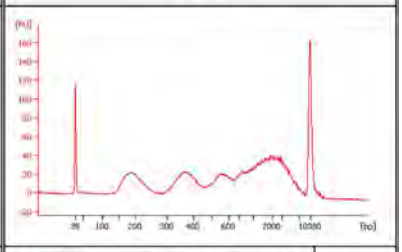
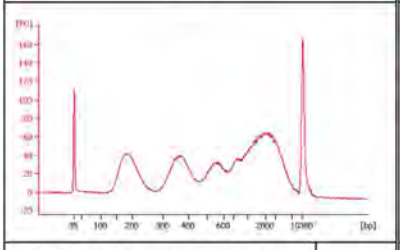
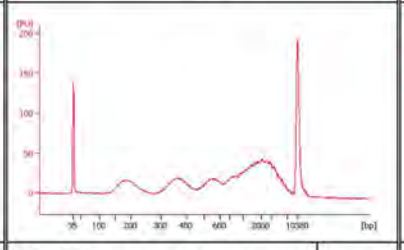
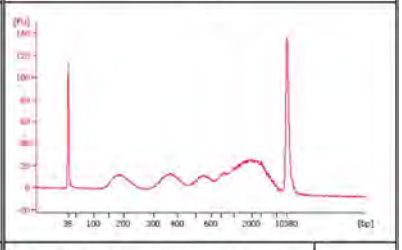
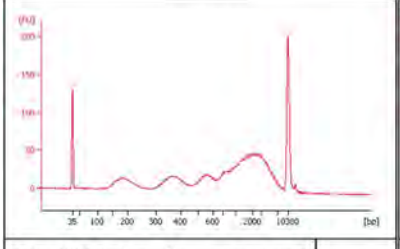
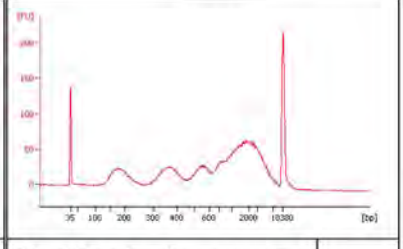
Fig. 5. Average cfDNA release per spheroid area in the presence of APAP over time (Error bars represent SD, n = 3) (three replicates from one bioreactors and repeated twice). Growth medium was exchanged and 6 spheroids removed from each reactor every 2 days. ***p < 0.001 and *p < 0.05 versus negative control.

that of the negative control spheroids. These changes correlate with the time at which changes were observed in spheroid growth, glucose consumption and LDH activity compared to that of the negative control (Fig. 4). Therefore, the significant changes in the amount of cfDNA from day 10 indicates that the changes observed in Fig. 4, though not statistically significant, does indeed indicate that a biological reaction occurred in response to the APAP treatment. Electropherograms (Table 3) showed an increase in nucleosomal fragment-to-actively released fragment ratio from day 0–2, followed by a gradual decrease from day 2–8 as the 2000 bp fragment peak levels gradually increased. As observed in section 3.1, an increase in this ratio results in a shift of the 2000 bp peak to 1000–2 000 bp. To better observe the nucleosomal fragment-to-actively released fragment ratio, the amount of DNA used for CE was doubled for the day 8–14 samples. A significant decrease in nucleosomal fragment-to-actively released fragment ratio followed by day 10 as the 2 000 bp fragment peak levels reached a significantly high level, which exceeded the amount of nucleosomal fragment fraction in the sample by 31%. A subsequent significant increase in nucleosomal fragment-to-actively released fragment ratio from day 10–14 may indicate that the last optimal therapeutic level for 100 mg/kg APAP could have been reached at day 10 and that subsequent dosages started to induce hepatotoxicity commonly associated with APAP overdosage (Kon et al., 2007; McGill et al., 2012). This may be corroborated by the initial decreased growth and increased energy consumption observed at day 10 in Fig. 4. Investigation regarding further subsequent dosages of APAP is encouraged to substantiate this.

3.2.3. *Amount of nucleosomal DNA fragments in cell-free DNA samples*

The fragment patterns of the spheroid cfDNA correlates with that of both 2D cell cultures (Bronkhorst et al., 2016) and human plasma samples (Applied-Biosystems, 2015), with the exception that there is a continuous presence of nucleosomal fragment DNA, proposed to represent apoptotic cfDNA (Bronkhorst et al., 2016), throughout spheroid development and in both the untreated control and treated bioreactors during the toxicological studies. The promotion of growth medium, nutrient and oxygen penetration into spheroids due to the "pressurized" system of the bioreactors (Section 1.2) negates the possibility of increased cell death due to increased spheroid size, decreased access to nutrition, respiration and waste clearance. The continuous presence of nucleosomal DNA fragments, particularly in the samples of the spheroids that completed 21 day development and were used for the

Table 2
Capillary electropherograms of cfDNA from the negative control bioreactors over time and the percentage of the ~2000 bp actively released fragments and nucleosomal DNA fragments present in 800 pg of cfDNA sample.

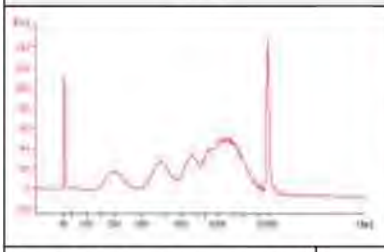
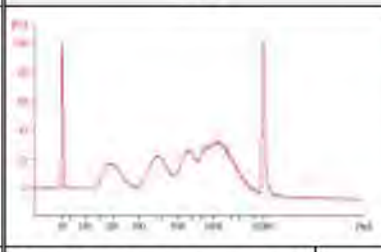
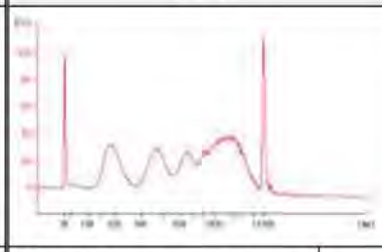
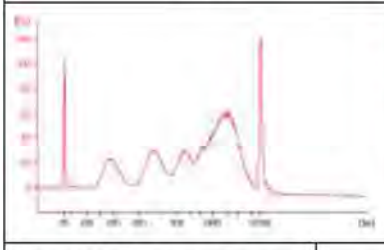
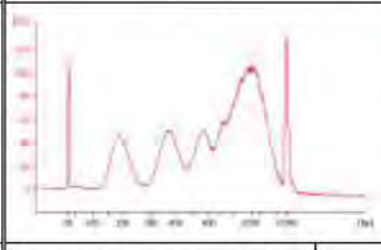
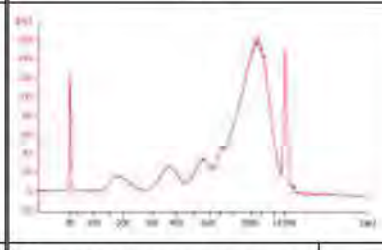
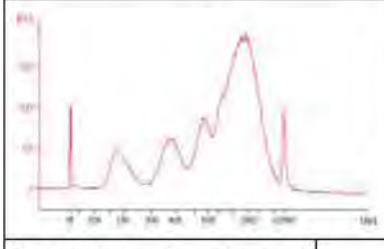
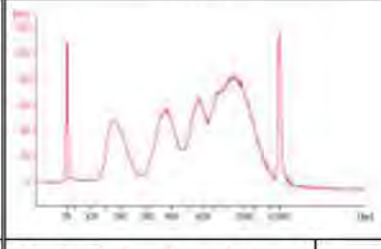
DAY 0		DAY 2		DAY 4	
					
42 – 900 bp (small + nucleosomal DNA fragments)	55 %	42 – 910 bp (small + nucleosomal DNA fragments)	68 %	42 – 900 bp (small + nucleosomal DNA fragments)	62 %
900 – 8 900 bp (actively released DNA)	44%	910 – 8 900 bp (actively released DNA)	31 %	900 – 8 900 bp (actively released DNA)	37 %
DAY 6		**DAY 8**		**DAY 10**	
					
42 – 900 bp (small + nucleosomal DNA fragments)	64 %	42 – 900 bp (small + nucleosomal DNA fragments)	58 %	42 – 900 bp (small + nucleosomal DNA fragments)	59 %
900 – 8 900 bp (actively released DNA)	35 %	900 – 8 900 bp (actively released DNA)	41 %	900 – 8 900 bp (actively released DNA)	40 %
DAY 12		**DAY 14**			
					
42 – 883 bp (small + nucleosomal DNA fragments)	52 %	42 – 883 bp (small + nucleosomal DNA fragments)	56 %		
883 – 8 900 bp (actively released DNA)	46 %	883 – 8 900 bp (actively released DNA)	43 %		

toxicological studies, may be a likely indication of equilibrium between cell proliferation and cell death, a homeostatic trait of *in vivo* tissue (Fulda et al., 2010). This 3D culture method can, however, still serve as an effective "closed-circuit" model despite the nucleosomal DNA fragments as the model still restricts the putative sources and causes of cfDNA to that of the target cell type.

3.2.4. Utilizing cell-free DNA in three-dimensional cell culture monitoring

Two significant specificities of this 3D spheroid development method are (i) the duration of spheroid development, depending on the cell line's ability to stabilize growth under rotating conditions (in the case of C3A's, 18 days are required), and (ii) the sensitivity of the spheroid cultures to sampling. The average 2D culture flask of 75 cm^2 can contain up to 1×10^5 cells/cm^2. The 3D spheroids of a single bioreactor, on the other hand, is developed from 3×10^5 individual cells with an initial doubling time similar to that of 2D cell cultures that decreases slightly as time progresses (Wrzesinski and Fey, 2013), resulting in ~1.1×10^7 cells per reactor by day 20 (1.5 times higher than the yield of a 75 cm^2 flask). This is only the development phase of the spheroids and can, therefore, result in even larger cell numbers per bioreactor when grown for experimental purposes since spheroid cultures can remain stable for up to a further 24 days after completion of

Table 3
Capillary electropherograms of cfDNA from bioreactors treated with 100 mg/kg acetaminophen over time and the percentage of the ~2000 bp actively released fragments and nucleosomal DNA fragments present in 800 pg (day 0–6) and 1.6 ng (day 8–14) of cfDNA sample.

DAY 0		DAY 2		DAY 4	
					
42 – 850 bp (small + nucleosomal DNA fragments)	61 %	42 – 883 bp (small + nucleosomal DNA fragments)	70 %	42 – 883 bp (small + nucleosomal DNA fragments)	67 %
850 – 8 900 bp (actively released DNA)	40 %	883 – 8 900 bp (actively released DNA)	29 %	883 – 8 900 bp (actively released DNA)	32 %
DAY 6		**DAY 8**		**DAY 10**	
					
42 – 883 bp (small + nucleosomal DNA fragments)	59 %	42 – 833 bp (small + nucleosomal DNA fragments)	57 %	42 – 833 bp (small + nucleosomal DNA fragments)	34 %
883 – 8 900 bp (actively released DNA)	40 %	833 – 8 900 bp (actively released DNA)	42 %	833 – 8 900 bp (actively released DNA)	65 %
DAY 12		**DAY 14**			
					
42 – 833 bp (small + nucleosomal DNA fragments)	51 %	42 – 833 bp (small + nucleosomal DNA fragments)	67 %		
833 – 8 900 bp (actively released DNA)	48 %	833 – 8 900 bp (actively released DNA)	31 %		

their 21 day development. A 3D culture bioreactor can, therefore, easily surpass the amount of cells that a 2D culture flask can maintain and as the size of the spheroids become larger, the availability of nutrients among the spheroids reduces. In effect, removing an amount of spheroids from bioreactors for analytic purposes can have a large impact on the growth environment of the remaining spheroids, resulting in altered growth rate and size due to sudden availability of more nutrients. The maintenance of stable growth environments becomes difficult when spheroids have to be removed from reactors for cell growth and development monitoring and/or long term experiment sampling. Methods that can monitor cell growth, development and other processes using growth medium instead of the cells themselves can, therefore, be a great advantage during spheroid culture experiments.

The cfDNA characteristics observed during both spheroid development and treatment effectively mirrored minor changes in growth rate and glucose consumption and correlated well with previous observations of spheroidal growth and development (Wrzesinski and Fey, 2013, 2017; Wrzesinski et al., 2013), indicating that cfDNA can serve as an effective marker to monitor spheroid development, growth rate and bioreactor environment stability. For the quantification of cfDNA in bioreactors only 1.8 mL of bioreactor growth medium was used. Due to the significantly larger amount of cells in bioreactors and longer

incubation periods compared to standard 2D cell cultures the bioreactors provide significantly larger yields of cfDNA (in micrograms), indicating that the amount of growth medium used for quantification can be significantly reduced. The replacement of spheroid-requiring monitoring methods with low volume, growth medium-requiring cfDNA quantifying methods and CE can, therefore, serve as a new and efficient approach to monitoring spheroid cultures without affecting the growth environment of the spheroids.

The cfDNA results appear to reveal a positive correlation between cfDNA release and spheroid glucose consumption, and a negative correlation between cfDNA release and spheroid growth. Further investigation is required to confirm whether these correlations are common occurrences. Furthermore, the data provided more insight regarding the effect of the length of time between doses of compounds for toxicology testing, as there was little evidence of increased apoptosis and necrosis indicative of increased toxicity. This implies that (i) the samples were collected after the spheroids recovered from the toxic effects of the compounds and (ii) that the dosage interval may, therefore, be too long to be considered as a therapeutically representative dosage regimen. This regimen, however, is normally effective for 2D cell cultures and, therefore, illustrates the adaptation of these 3D cultures to more physiologically relevant conditions.

4. Conclusions

In this study it was determined that cfDNA release and fragment patterns mirror the brief and/or minor changes in the growth and glucose consumption of spheroids developed in rotating bioreactors during both the development phase and toxicological studies. The fragment patterns of the spheroid cfDNA correlates with that of both 2D cell cultures (Bronkhorst et al., 2016) and human plasma samples (Applied-Biosystems, 2015), The microgram yields of cfDNA compared to that of 2D cell cultures is highly beneficial for subsequent studies of the origin, purpose and biological functions of the cfDNA. Moreover, cfDNA data obtained from 3D cultures will likely be more relatable to *in vivo* conditions due to their re-establishment of tissue cell communication networks that maintain tissue specificity and homeostasis. This study, therefore proposes (i) the utilization of cfDNA in 3D spheroid-utilizing studies as a marker for 3D spheroid growth and development to spare precious spheroids and maintain a stable physiological environment and (ii) the utilization of Wrzesinski and Fey (2013)'smethod of spheroid development in rotating bioreactors as a "closed-circuit" model for cfDNA research to study the biological functions and origins of cfDNA as replacement for or for use in conjunction with *in vivo* models in order to aid the discovery of novel biomarkers for clinical non-invasive diagnostic, prognostic and theranostic applications.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Research Foundation (NRF), South Africa [SFH14061869958, RCA13091339132]. The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

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Wrzesinski, K., Rogowska-Wrzesinska, A., Kanlaya, R., Borkowski, K., Scwämmle, V., Dai, J., Joensen, K.E., Wojdyla, K., Carvalho, V.B., Fey, S.J., 2014. Cultural divide: exponential growth in classical 2D and metabolic equilibrium in 3D environments. PLoS One 9 (9), e106973. http://dx.doi.org/10.1371/journal.pone.0106973.

6.4 Pilot study: Utilising three-dimensional cell cultures to screen for changes in cell-free DNA characteristics induced by a natural plant product

During the spheroid culture treatments of paragraph 6.3, bioreactors were also treated with *Xysmalobium undulatum*, also known as Uzara, extracts (one of the most widely used traditional medicines in South Africa for the treatment for diarrhoea, stomach cramps, colic, afterbirth cramps, headaches and wounds (reviewed in Vermaak *et al.* (2014)). Growth medium was also collected from these bioreactors for cfDNA extraction, quantification and DNA fragment analysis. However, the use of Uzara extracts form part of the thesis of PhD student, Carlemi Calitz, and it was requested that the obtained results must not be published until the student has published her own related articles. Permission is, however, provided for the addition of a summary of the results obtained for the purpose of this thesis. It is important to mention that this experiment served as a pilot study for the utilisation of the 3D cell culture method of Wrzesinski and Fey (2013) in pharmacognostic toxicity studies. The method followed is, therefore, not yet efficiently refined for this purpose and future experiments will include more intervals of glucose consumption and apoptosis analysis and the addition of proteonomic analysis. Later collaborations can be arranged once the method is refined for more detailed investigations regarding both toxicity assays and cfDNA characteristics in response to treatments.

Though the obtained results may not yet be an accurate analysis of pharmacognostic toxicity, it does reveal that Uzara extract can induce significant changes in cfDNA release and characteristics. As mentioned in Table 5-1, blood donors are allowed to take homeopathic and natural plant products without any form of deferral. It is regrettable that deferrals are not considered for these medicinal products as very little is known regarding (i) their exact chemical contents, (ii) whether chemical interactions can occur within combinations of different extracts, (iii) the pharmacological effects of each component of the extract and (iv) whether there are any synergistic or antagonistic effects within extract mixtures that could amplify or repress certain pharmacological effects. To further complicate the matter, homeopathic and natural plant products are not under the same level of scrutiny as synthesised medications regarding clinical trials and registration as safe to use medicines.

In this paragraph we, therefore, wish to both (i) further introduce the utilisation of 3D cell cultures in the evaluation of cfDNA characteristics in response to medicinal treatment and (ii) illustrate the risk of not having more stringent guidelines or regulations regarding natural product usage and blood donation. Uzara, extract (100 mg/kg) was administered every 48 hours to bioreactors for 336 hours. The methods and analysis of paragraph 6.2's article was performed (Fig. 6-1) and the results illustrated in Figures 6-2 – 6-4 and Tables 6.1 – 6.3.

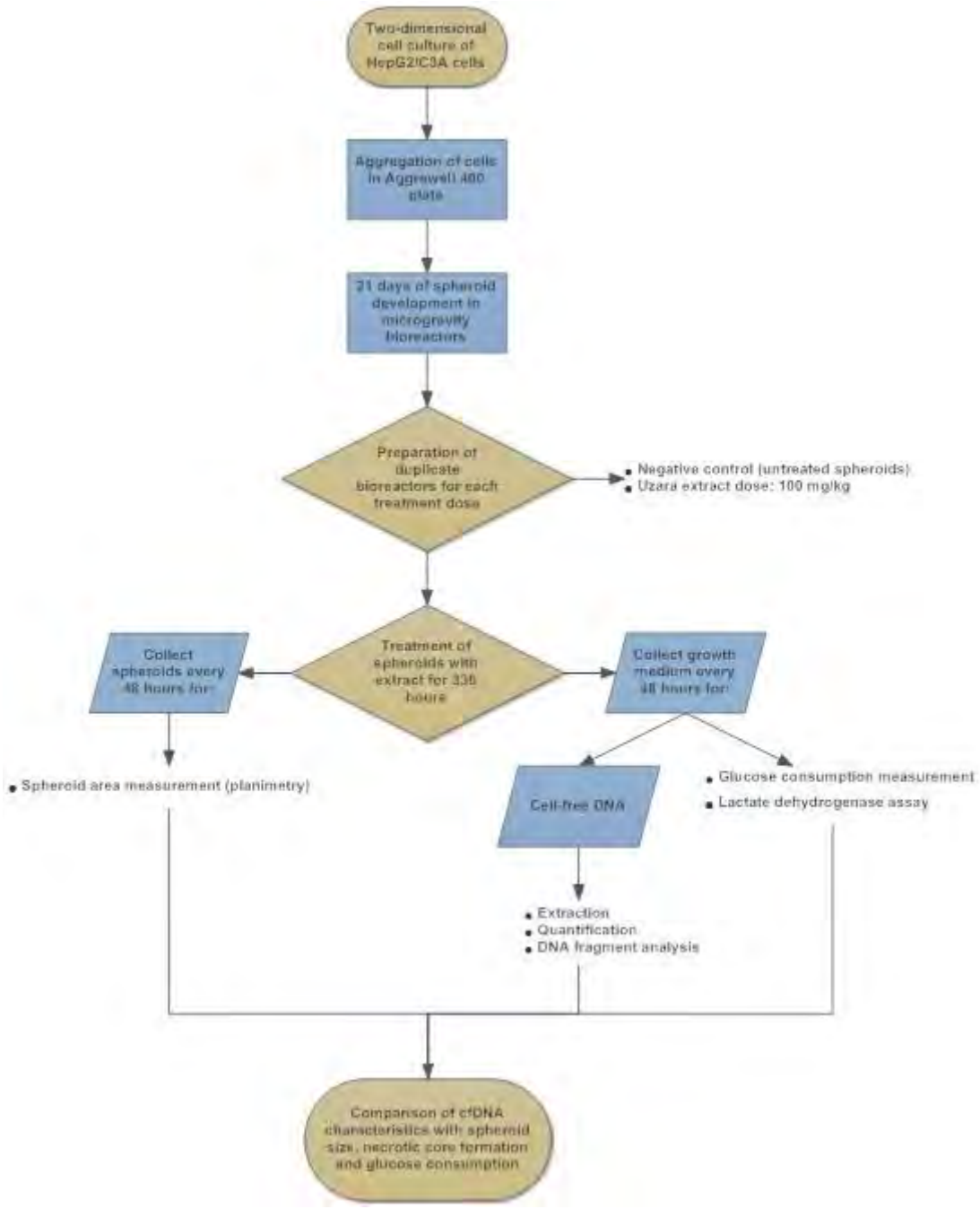


Figure 6-1: **Flow diagram of Uzara-treatment experiment.** Performed according to the methods of article in paragraph 6.2

The average size of the spheroids (Fig. 6-2A), LDH activity (Fig. 6-2B) and glucose consumption (Fig. 6-2C) were used to determine cell viability. The results obtained from the treatments were compared to that of the negative control results of paragraph 6.2. Treating spheroids with 100 mg/kg of Uzara extract resulted in reduced spheroid sizes compared to that of the negative control. The treatment also resulted in a gradual increase in LDH activity per spheroid area from 0 – 240 hours (though at levels no higher than the maximum level presented by the negative control at 96 hours) followed by a subsequent decrease. The results may indicate that the

spheroids weren't in the process of dying, but the growth rate was significantly reduced. The treatment resulted in an initial increase in glucose consumption at 48 hours with the levels being higher than that of the negative control spheroids, possibly indicating an inverse correlation between the spheroid size or growth and glucose consumption, as seen after 240 and 288 hours of paracetamol treatment (paragraph 6.2).

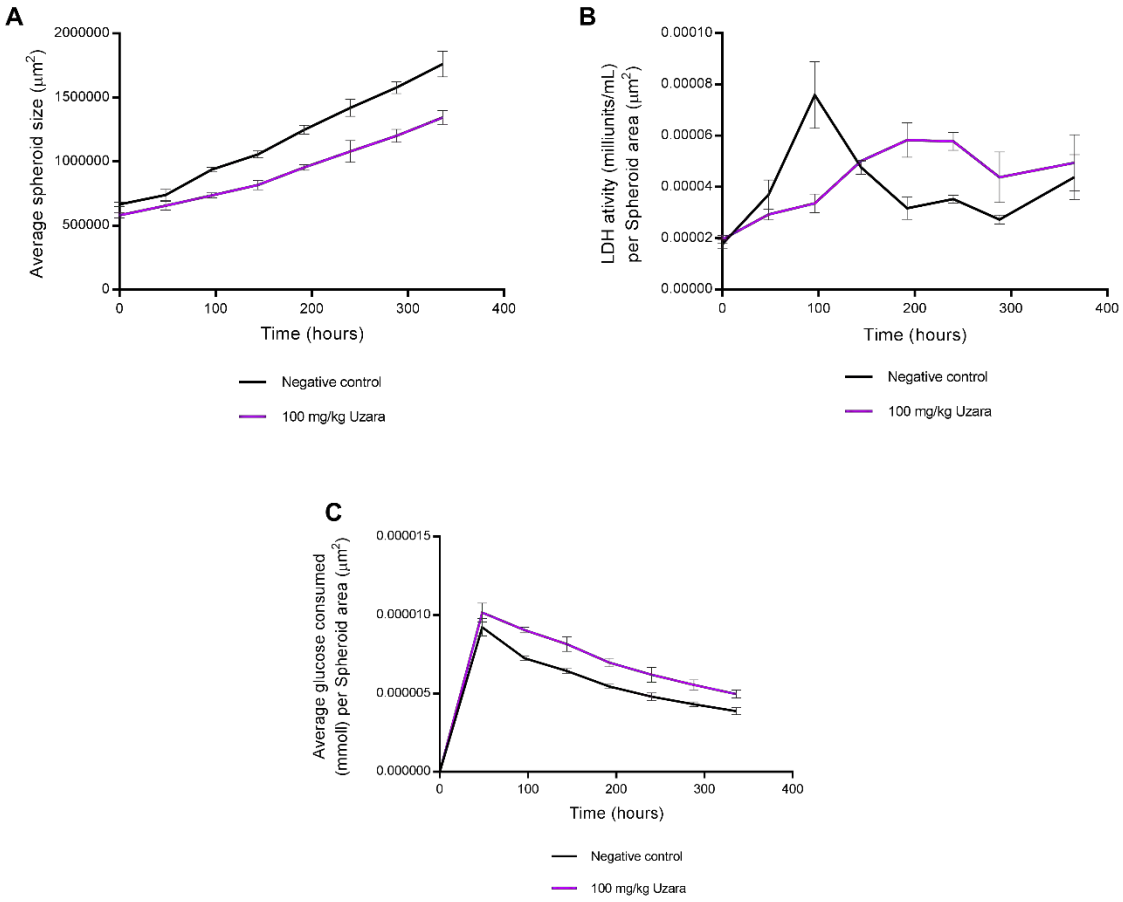


Figure 6-2: **Average growth rate and glucose consumption of 3D spheroids.** (A) Average spheroid size (± SD, n = 6), (B) LDH activity per spheroid area (± SD, n = 2) and (C) glucose consumption per spheroid area (± SD, n = 6) over time. Growth medium was exchanged and 6 spheroids removed from each reactor every 48 hours

Treatment of spheroids with 100 mg/kg Uzara extract resulted in a steady increase in cfDNA release per spheroid area from 0 – 96 hours, followed by a steady decline (Fig. 6-3). Compared to the cfDNA levels of the negative control, 100 mg/kg Uzara extract treatment resulted in significantly higher levels of cfDNA release. The total amount of negative control-derived cfDNA showed an increase in cfDNA levels from 2.79 to 3.89 µg/bioreactor during 0 – 144 hours and a small, gradual decrease to 3.17 µg/bioreactor by 336 hours (refer to paragraph 6.3). The total amount of Uzara treatment-derived cfDNA, on the other hand, showed an increase in cfDNA

levels from 1.77 to 7.08 µg/bioreactor after 288 hours and remained at 7 µg/bioreactor at 336 hours. Electropherogram data (Table 6-3) revealed initially high levels of nucleosomal fragments and a high nucleosomal fragment-to-actively released fragment ratio, as seen for the negative control (Table 2 of paragraph 6.3) likely due to the transfer of the spheroids to respective bioreactors. The negative control data showed a gradually decreasing nucleosomal fragment-to-actively released fragment ratio at 48 – 288 hours and a subsequent increase in both nucleosomal fragment and actively released fragment peak levels with slightly increased nucleosomal fragment-to-actively released fragment ratio at 336 hours. Uzara treatment, however, resulted in a gradual and continuous increase in nucleosomal fragment-to-actively released fragment ratio, with significantly higher levels of ~2 000 bp DNA fragments at 48 – 96 hours compared to the negative control.

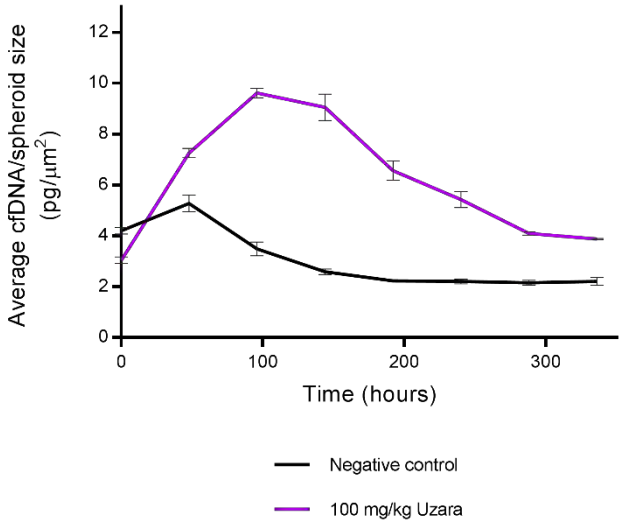
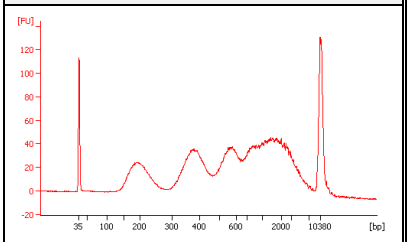
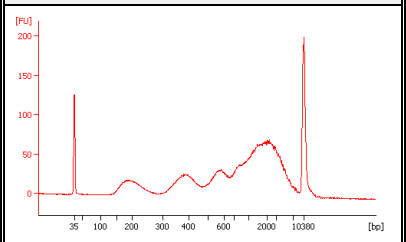
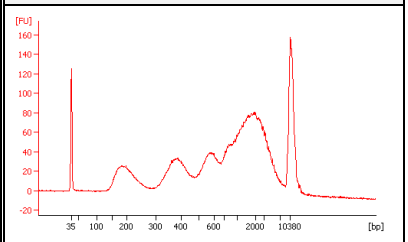
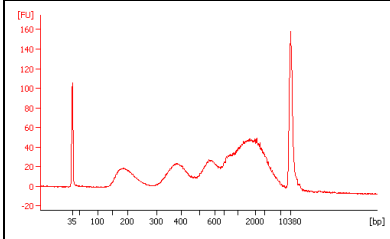
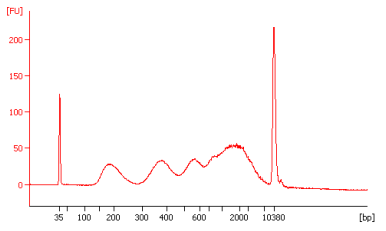
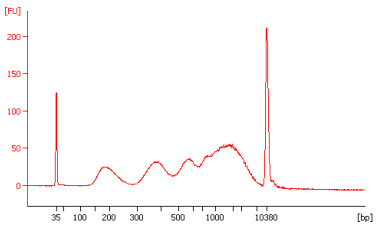
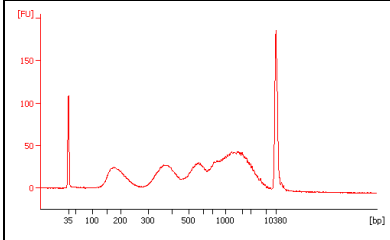
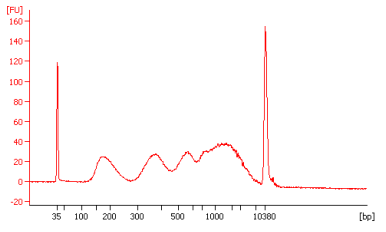


Figure 6-3: **Average cfDNA release per spheroid area over time.** Growth medium was exchanged and 6 spheroids removed from each reactor every 48 hours (Error bars represent SD, n=3)

Table 6-1: **Capillary electropherograms of cfDNA from bioreactors treated with 100 mg/kg Uzara extract over time and the percentage of the ~2 000 bp actively released fragments and nucleosomal DNA fragments present in 800 pg of cfDNA sample**

0 HOURS		48 HOURS		96 HOURS	
					
42 – 883 bp (small + nucleosomal DNA fragments)	68 %	42 – 883 bp (small + nucleosomal DNA fragments)	52 %	42 – 883 bp (small + nucleosomal DNA fragments)	56 %
883 – 8 500 bp (actively released DNA)	32 %	883 – 8 500 bp (actively released DNA)	47 %	883 – 8 500 bp (actively released DNA)	42 %
144 HOURS		**192 HOURS**		**240 HOURS**	
					
42 – 900 bp (small + nucleosomal DNA fragments)	60 %	42 – 890 bp (small + nucleosomal DNA fragments)	63 %	42 – 890 bp (small + nucleosomal DNA fragments)	63 %
900 – 8 500 bp (actively released DNA)	39 %	890 – 8 500 bp (actively released DNA)	35 %	890 – 8 500 bp (actively released DNA)	36 %
288 HOURS		**336 HOURS**			
					
42 – 890 bp (small + nucleosomal DNA fragments)	66 %	42 – 890 bp (small + nucleosomal DNA fragments)	69 %		
890 – 8 500 bp (actively released DNA)	32 %	890 – 8 500 bp (actively released DNA)	30 %		

As the Uzara extract is of plant origin and the cfDNA levels of the spheroids treated with the extract were significantly increased compared to the negative control spheroids, it was suspected that the extract may contain DNA. Extraction of DNA was performed using the same method as for cfDNA extraction and quantification, revealing that Uzara extract does indeed contain small DNA fragments (Fig. 6-4). However, only mere nanogram amounts were administered to the bioreactors and the smaller fragments of DNA (37 – 140 bp, 140 – 280 bp and 280 – 9 000 bp, respectively) did not appear to be present in the growth medium electropherograms (Table 6-1 – 6.3).

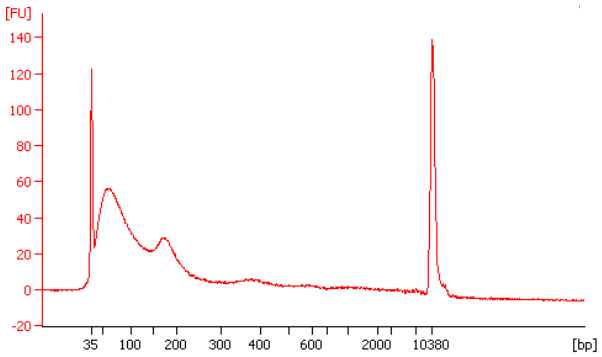


Figure 6-4: **Capillary electropherograms of the DNA extracted from Uzara extract.** Note the two prominent peaks at 37 – 140 bp (59 % of 800 pg cfDNA), 140 – 280 bp (24 % of 800 pg cfDNA) and 280 – 9 000 bp (17 % of 800 pg cfDNA), respectively

The results, therefore, support the observations described in paragraph 6.3 in showing that the cfDNA of spheroid cultures can effectively mirror changes in spheroid growth and glucose consumption. Furthermore, as seen during the VPA studies of paragraph 5.2, pharmaceutically-induced changes in cfDNA characteristics can have the potential of transferring or inducing biochemical and/or epigenetic changes in untreated recipient cells. The Uzara treatment results confirmed that homeopathic and natural plant products have the potential to significantly affect cfDNA release and characteristics. There is, therefore, a risk for the transfer of these changes to transfusion recipients and subsequent induction of biological effects, substantiating the fact that there are risks for not having more stringent guidelines or regulations regarding natural product usage and blood donation.

CHAPTER 7

CONCLUSIONS AND DISCUSSION

7.1 Discussion

Comprehensive literature reviews regarding the history and development of cirDNA research (chapter 2) and the putative sources of cirDNA and causes of cirDNA release (paragraph 3.2) have provided perspective as to the potential purpose or biological function of cirDNA as homeostatic intercellular messengers (paragraph 3.4). They have also raised the question of whether these intercellular messaging functions of cirDNA could have unforeseen clinical implications. Looking into (i) the guidelines or policies regarding blood donation by donors taking medication and (ii) the way that donated blood is processed, stored and administered to recipients (paragraph 5.1), it was concluded that there is little preventing the transfer of both freely circulating and cell-surface-bound cirDNA from donors to recipients. Is it then possible that the information contained in cirDNA of a donor can elicit genetic, epigenetic or biochemical effects in a recipient? The aim of this thesis was to evaluate the messaging ability of cirDNA, specifically focusing on the transfer of pharmaceutically-induced metabolic and epigenetic effects from donor to recipient cells, in order to determine the clinical implications or risks of the presence of cirDNA in blood.

However, as efficiently illustrated in the article presented in paragraph 3.2, the complexity of cirDNA contents in biofluids due to the vast number of putative sources of cirDNA and causes of cirDNA release serves as a severe obstacle in elucidating the biological and physiological significance of cirDNA. The utilisation of 2D and 3D cell cultures, on the other hand, can simplify the identification of the origins and biological functions of cfDNA by restricting the origins of the cfDNA to targeted cells. *In vitro* methods can, therefore, simplify the identification of the origins and biological functions of cfDNA by restricting the origins of the cfDNA to targeted cells. *In vitro* methods can also benefit biomarker research since one efficiently removes background noise from physiological factors other than that which are targeted. For this reason the utilisation of *in vitro* means in cirDNA research have been motivated in most, if not all, of the articles provided.

For the utilisation of cell cultures in the evaluation of the messaging ability of cirDNA (in order to determine the clinical implications or risks of the presence of cirDNA in blood), optimised methods for (i) growth medium collection and processing, (ii) cfDNA extraction and quantification, and (iii) cell culture seeding and incubation methods were identified (paragraph 4.1). Then, in the co-authored publication of Bronkhorst *et al.* (2016d), it was determined that capillary electrophoresis can be used to observe cfDNA fragment patterns, revealing the presence of both nucleosomal DNA fragment patterns and ~2 000 bp DNA fragments in cfDNA derived from 143B cells

(paragraph 4.2). With the aid of flow cytometry it was determined that the ~2 000 bp fragments can be associated with actively released DNA due to the absence of apoptosis and necrosis in samples where only this fraction is present. The nucleosomal DNA fragments were accompanied by the detection of apoptosis. The cfDNA release and characteristics of eight cell lines have subsequently been investigated and revealed that these fractions are present in all eight cell lines with different cfDNA release patterns (paragraphs 4.3.1 and 4.3.2). Capillary electrophoresis can, therefore, serve as a valuable method for the monitoring of changes in cfDNA release during cell growth and pharmacological treatment. Furthermore, several correlations between growth rate, cancer status and dependency on aerobic glycolysis have been observed (paragraphs 4.3.3 to 4.3.5). In particular, these results revealed statistically significant correlations between cfDNA release and glycolytic activity and no correlations with OXPHOS activity. Monitoring and/or targeting cellular metabolism during pharmacological treatments can, therefore, provide insight regarding cfDNA characteristics.

The next step was to determine whether the storage, processing and transfusion processes involved in blood donation can promote the transfer of DNA from donor to recipient (paragraph 5.1). Furthermore, guidelines or policies regarding medication usage in blood donors were also investigated to determine whether there is a potential for pharmaceutically-induced effects to be transferred via cirDNA. It was determined that the processing and storage of blood products after blood collection can preserve the cirDNA since the blood products, such as the plasma, are quickly frozen, thus inhibiting DNase activity. Cell-surface-bound cirDNA will also be protected from DNase activity, promoting the transfer of DNA from donor platelets and erythrocytes to recipients. CirDNA can also easily pass through blood transfusion filters used. To make matters worse, the guidelines for medication usage focuses primarily on blood thinning and teratogenic medication and not pharmacological activity. Medication with persistent adverse effects or epigenetic effects, for example, is not monitored. There is, therefore, a substantial risk that cirDNA can transfer pharmaceutically-induced effects from donors to patients should the medication used be able to alter cirDNA characteristics.

The exposure of 2D cell cultures to VPA (paragraph 5.2) and 3D cultures to paracetamol (paragraph 6.3) and Uzara extract (paragraph 6.4) has confirmed that commonly used and chronically taken pharmaceutical compounds, homeopathic remedies and natural plant products can significantly affect *in vitro* cfDNA characteristics. These treatments have resulted in significant changes in cfDNA levels, release patterns and nucleosomal fragment-to-actively released DNA ratios that is likely to also occur *in vivo*. Furthermore, the changes in cfNDA characteristics induced by VPA resulted in glycolytic effects and subsequent changes in cfDNA release when the extracted cfDNA was administered to untreated cells (paragraph 5.2.3). The *in vitro* induction of DNA methylation changes in tumour suppressor genes, RASSF1A and GSTP1,

and repetitive genetic elements, LINE-1 and ALU1, via cfDNA treatment has also been observed (paragraph 5.2.5). The transport of epigenetic effects from donor to recipient via the repetitive genetic elements in cirDNA, in particular, can result in significant gene transcription regulation effects (Ozer & Sezerman, 2017; Weber *et al.*, 2010) and requires urgent attention. CirDNA in the blood of donors taking medication can, therefore, consist of pharmaceutically-altered genetic and/or epigenetic information that has the potential to induce biochemical and/or epigenetic effects in recipient cells.

7.2 Research limitations and potential follow-up research

There are, however, certain research limitations that require further investigation. In particular, the observed transferred effects have been observed to be either of short duration (in the case of the glycolytic activity effects) or at statistically non-significant levels (in the case of the DNA methylation effects). One must keep in mind that:

1. The treatment regimens used represent acute treatment of both the medication used and the cfDNA administered. As mentioned in paragraph 5.2.4, studies have shown that environmentally-induced DNA methylation alterations undergo a form of a "kindling-like" phenomenon (Abdolmaleky *et al.*, 2015), where episodic exposure to chronic stimulation can induce stronger effects on the epigenome with each dose. Chronic medication use and the natural occurrence of constant cellular cirDNA exposure can, therefore, result in significantly stronger effects. As was discussed in chapter 6, spheroids developed in microgravity bioreactors can serve as the ideal *in vitro* model to investigate the chronic effects of medication and cfDNA exposure, as these cell cultures remain stable for up to three weeks (Wrzesinski & Fey, 2013).
2. The VPA concentrations used served as a therapeutic dose to the cancer cell line used. As mentioned in paragraph 5.2.5, the HDAC inhibiting activity and DNA methylation effects of VPA have anticancer properties (Blaheta *et al.*, 2005), implying that the therapeutic dosage used may not have been able to induce statistically significant effects and that increasing the dosage would result in cell death. As was reported in the article presented in paragraph 5.2.3, increasing the dose of VPA from 5 to 8 mM resulted in necrosis and a decrease in the actively released ~2 000 bp DNA fraction, the fraction of cirDNA that is particularly regarded to have intercellular messaging functions (Bronkhorst *et al.*, 2016d; Gahan, 2006). For the purpose of this study higher doses were, therefore, not appropriate, but the utilisation of non-cancer cell lines with less risk of cell death at higher doses is motivated in future studies.
3. Statistically non-significant does not necessarily imply biological insignificance. As mentioned in paragraph 5.2.4, DNA methylation changes as low as 5 % in specific genes can still affect cell phenotype through gene transcription regulation (Ozer & Sezerman, 2017). Whether such small changes will, inevitably, result in biological adverse effects will, therefore, depend on

whether the changes occurred in important genomic regions. Further investigation regarding pharmaceutically- and subsequent cfDNA-induced DNA methylation changes and the genomic positioning of these changes via next generation methylation sequencing may provide significant insight in the biological functions and characteristics of cfDNA and whether induced changes can elicit physiological effects that may negatively affect transfusion recipients.

7.3 Conclusions

The success of this study lies in its contribution to the literary component of cirDNA research and the introduction of two important concepts to the field, namely (i) the utilisation of *in vitro* methods in cirDNA research and (ii) the clinical risks of cirDNA during transfusion.

A well received, comprehensive review of the history and development of cirDNA research was provided in order to familiarise researchers with previous works in order to better understand the nature of cirDNA, to show that there are still many unanswered questions relating to the nature and biological functions of cirDNA that have been neglected due to the pursuit of clinical applicability and to emphasise that it is this lack of answers that is hindering clinical successes (paragraph 2.2). A comprehensive compilation of putative sources of cirDNA or causes of cirDNA release then followed to illustrate that cirDNA is a complex collection of information from vast numbers of interacting biological features, which overly complicates the discovery of both the biological function of cirDNA and novel biomarkers in biological fluids. For this purpose, this study illustrated that the utilisation of both standard 2D (chapters 4 and 5) and more physiologically relevant 3D (chapter 6) cell cultures can significantly simplify cirDNA research by reducing the complexity of DNA samples to only that of targeted cells. The use of cfDNA derived from *in vitro* methods to identify tissue- or disease-specific markers or elucidate the biological significance of cirDNA prior to the attempt of such discoveries in biological fluids is, therefore, encouraged.

Together, the two reviews painted the picture of cirDNA as homeostatic genetic entities or messengers capable of transferring informational updates throughout the body to maintain a form of genetic information equilibrium (paragraph 3.4). Changes in equilibrium could, for example, be caused by epigenetic changes in the nuclear DNA of cells reacting to environmental changes or xenobiotic exposure, including medication, resulting in subsequent cirDNA release in order to maintain homeostasis of information. It is this homeostatic function of cirDNA that causes the concern of whether cirDNA inadvertently transferred to patients during transfusions can become clinical risk factors that can elicit adverse physiological effects. This study has illustrated that (i) pharmaceutical compounds, homeopathic remedies and natural plant products can significantly affect *in vitro* cfDNA characteristics, (ii) blood donation guidelines regarding medication use and deferrals do not defer all medication use or take persistent adverse effects into account and,

therefore, cannot prevent the transfer of pharmaceutically-altered cirDNA, (iii) the processing, storage and administration processes of donated blood to recipients cannot efficiently remove freely circulating and cell-surface-bound cirDNA from the blood products, making the transfer of cirDNA to recipients a given fact and (iv) pharmaceutically-altered cirDNA has the potential of inducing biochemical and/or epigenetic effects. The presence of cirDNA in blood, therefore, has clinical risks or implications, especially regarding chronic medication use and blood donation, that require critical attention.

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ANNEXURE A

Reference list of co-authored publications

Bronkhorst, A.J., Aucamp, J. & Pretorius, P.J. 2015. Cell-free DNA: Preanalytical variables. *Clinica chimica acta*, 450(2015):243-253.

Bronkhorst, A.J., Aucamp, J. & Pretorius, P.J. 2016. Adjustments to the preanalytical phase of quantitative cell-free DNA analysis. *Data in brief*, 6(2016):326-329.

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Bronkhorst, A.J., Wentzel, J.F., Aucamp, J., Van Dyk, E., Du Plessis, L. & Pretorius, P.J. 2016. Enquiry concerning the characteristics of cell-free DNA released by cultured cancer cells. (In Gahan, P.B., Fleischhacker M. & Schmidt B., *eds*. Circulating nucleic acids in serum and plasma - CNAPS IX. Advances in experimental medicine and biology. Switzerland: Springer International Publishing. p. 19-24).

Bronkhorst, A.J., Wentzel, J.F, Aucamp, J., Peters, D.L., De Villiers, E.P. & Pretorius, P.J. 2017. Satellite DNA and transposable elements are actively released by cultured cancer cells. In preparation.

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