Chapter 4: Methodology

All reagents were obtained from Sigma-Aldrich (Johannesburg, South Africa), unless otherwise stated.

4.1 Cell cultures

Basic cell culturing conditions were the similar for all cell lines. In principle, all cell lines were cultured in a CO_2 incubator at 37℃ and 5% CO_2 in complete growth medium (Dulbecco's Modified Eagles Medium (DMEM); Thermo Scientific) supplemented with 10% foetal bovine serum (FBS) (BioWhittaker), 1X Penicillin/Streptomycin (100 U Pen./ml; 100 µg Strep./ml; BioWhittaker) and 1X MEM non-essential amino acids (0.1 mM; BioWhittaker)). Cells were harvested as follows: the cells were washed with 1X phosphate buffered saline (PBS), and incubated at 37℃ with 1% trypsin (in PBS) (BioWhittaker) for 5 min. Subsequently, 2 ml complete growth medium was added to stop the trypsinisation process. The cells were counted using trypan blue by mixing 25 µl trypan blue, 15 µl PBS and 10 µl cell suspension. Of this mixture, 10 µl was added to each chamber of a haematocytometer. The number of cells was then counted under a light microscope. The cell concentration was tailored for each application.

4.2 DNA and RNA samples

EDTA treated whole blood samples were obtained from two sibling, female HT1 patients, aged 4 years 10 months, and 1 year 3 months respectively. Both patients were referred to the Potchefstroom Laboratory for Inborn Errors of Metabolism at North-West University (Potchefstroom Campus), South Africa by a paediatrician for biochemical diagnosis. Informed consent from the relevant parties concerned and ethical approval by the NWU Ethics Committee (NWU-00096-08-A1) were obtained for the use of these samples. In both patients, elevated urinary and serum tyrosine, methionine, and phenylalanine levels, as well as increased urinary *p*-hydroxyphenyllactic acid, *p*-hydroxyphenylacetic acid, *p*-hydroxyphenylpyruvic acid and succinylacetone levels, confirmed the diagnosis of hereditary tyrosinemia type 1. For control purposes, DNA was also isolated from healthy control subjects. DNA isolation was performed with the FlexiGene DNA kit from Qiagen, according to manufacturer's instructions.

For RNA extraction, peripheral blood lymphocytes were isolated from the blood samples. Briefly, 2 ml of the EDTA treated whole blood were layered on top of 2 ml cold Histopaque® in a 15 ml centrifuge tube. The samples were then centrifuged for 15 min at 550*g*. The plasma layer was discarded, and the buffy coat transferred to a 1.5 ml microcentrifuge tube. To this, 1 ml PBS was added. The samples were briefly vortexed and then centrifuged for 3 min at 1100*g*. The supernatant was discarded, and RNA isolated from the cell pellets with the Nucleospin RNAII kit (Macherey-Nagel) according to the manufacturer's specifications.

Markus Grompe (Oregon Health Sciences University, Portland, OR, USA), kindly provided us with FAH deficient mice, and Raymond Hickey (Oregon Health Sciences University, Portland, OR, USA), performed DNA extractions from the livers of the FAH deficient mice. DNA was extracted from livers of 3 month old control and *fah*$^{-/-}$ mice on continuous NTBC treatment or off NTBC treatment for 40 days. DNA extraction was performed in Oregon by Raymond Hickey, with the DNeasy® Tissue kit from Qiagen, according to manufacturer's instructions.

To track the development of the HT1 cell culture model, RNA was isolated from HepG2 cells that were untransfected, transiently transfected, single stable (transfected with ptTS-Neo vector), and double stable (transfected with ptTS-Neo and pSIREN-RetroQ-Tet-shRNA constructs).

4.3 Comet assay modified for BER and NER

The comet assay or SCGE (Single Cell Gel Electrophoresis) is a rapid, sensitive and versatile technique for analysing and measuring DNA damage and repair *in vitro* and *in vivo* at individual cell level. This technique is a powerful tool to study factors modifying mutagenicity and carcinogenicity, and can also be used for measuring of DNA repair (Collins *et al.,* 2001:297, Dhawan, 2006). The comet assay protocol has previously been optimised for local conditions (van Dyk, 2005). However in the current study modifications were made for the use of cell cultures with the comet assay. Briefly, 1.5 x 10^5 HepG2 cells were seeded per well of a 6-well cell culture plate and covered with 2 ml complete growth medium. The cell culture plates were then incubated at 37℃ and 5% CO_2. After 24 h, the complete growth medium was aspirated and the cells harvested as described in section 4.1. The cell concentration was determined as described in section 4.1, and adjusted to 2.5 x 10^4 cells per 50 µl complete growth medium. To determine the optimum recuperation time of the HepG2 cells, the cell suspension was incubated for 1, 2, 3, and 4 h at 37℃ and 250 rpm to prevent cell adherence to the collection tube. In the meantime, frosted microscope slides were each coated with 300µl 1% high melting point agarose gel (HMPA, Appendix D). After incubation for the specific time interval, 50 µl of the cell suspension was mixed with 130 µl 0,5% low melting point agarose (LMPA, Appendix D) and evenly distributed on the

previously coated microscope slide. Standard comet assay conditions were then followed. Briefly, the coated microscope slides were submerged overnight in lyses buffer (5M NaCl, 0.4M EDTA, 1% Triton x-100, 10% DMSO) at 4℃. Afterwards, the mi croscope slides were placed in a horizontal electrophoresis tank and covered with electrophoresis buffer (0.6M NaOH, 50mM EDTA) for 30 min. Then an electrical field of 30 V was applied for 40 min. After electrophoresis, the slides were submerged in neutralising buffer (0.5M Tris-HCl, pH 7.5) for 15 min, followed by staining with 12.7 µM ethidium bromide for 30 min. After staining, the microscope slides were washed by submerging the slides in ddH_2O for 10 min. An Olympus 1x70 fluorescence microscope (200x magnification) was then used to study the slides. Comet IV® software was used to capture images and to determine the percentage of DNA in the comet head and tail of randomly selected comets.

The versatility of the comet assay allows it to be modified to achieve different outcomes. One such a modification was described by Collins *et al.,* where the comet assay was modified to determine the ability of the proteins involved in the initial steps of base excision repair (BER) to repair DNA damage (Collins *et al.,* 2001:297). This modification of the comet assay was later adapted to determine the capacity of proteins involved in the initial steps of nucleotide excision repair (NER) to repair DNA damage (Langie *et al.,* 2006:153).

We have adapted these modified comet assays to optimise for local conditions and to determine the effect of HT1 accumulating metabolites, SA and pHPPA, on the DNA repair capacities of BER and NER proteins. The optimized protocol is described in the paper: “Hereditary tyrosinemia type 1 metabolites impair DNA excision repair pathways” and is presented in chapter 6.

4.4 Gene expression

The effect of succinylacetone and *p*-hydroxyphenylpyruvic acid, as well as the sustained stress environment created by all of the accumulating metabolites on the expression of DNA repair genes, was assessed by determining the level of expression of *hOGG1* and *ERCC1*.

4.4.1 RT-PCR

cDNA was synthesized from extracted RNA using the MMLV high performance reverse transcriptase cDNA synthesis kit (Epicentre® Biotechnologies). For each reaction (reaction volume: 10 µl), 1.5 µg RNA, 1 µl oligo dT primer (10 µM), 1 µl *18S* rRNA reverse primer (10 µM)

and PCR grade water were mixed. The reactions were incubated for 2 min at 65℃, whereafter the reactions were placed on ice for 5 min. In the meantime, 2 µl MMLV reaction buffer (10X), 2 µl DTT (0.1 M), 3 µl HPLC grade water, 2 µl dNTP mix (10 mM of each dNTP; New England Biolabs®), 0.5 µl RNasin® (40 U/µl; Promega), and 0.5 µl MMLV (200 U/µl) were mixed. Of this mixture, 10 µl were added to each reaction. The reactions were then placed overnight at 37℃ in an Eppendorf® thermal cycler. The cDNA synthesis was terminated by increasing the incubation temperature to 85℃ for 5 min. The samples were st ored at 4℃.

4.4.2 Gene expression

Gene expression profiles of *fah*, *hOGG1*, and *ERCC1* were determined by real-time PCR with the 7500 Real-Time PCR instrument from Applied Biosystems™. TaqMan® primers and probes for each of the aforementioned genes of interest, as well as the *18S* rRNA reference gene, were also obtained from Applied Biosystems™. Sequence information about the primers and probes are not available but the assay reference numbers are supplied in table 4-1:

Table 4-1. Reference numbers for genes used in gene expression assays.

Gene	Applied Biosystems™ reference number
Fah	Hs00908443_m1
hOGG1	Hs00213454_m1
ERCC1	Hs01012158_m1
18S	Endogenous control: Part number 4319413E

All cDNA samples were diluted 250x with PCR grade water. Reaction mixtures for all gene expression assays contained: 10 µl TaqMan® mastermix (2X; Applied Biosystems™; part # 4364341), 1 µl *18S* primer/probe mix, 1 µl gene of interest primer/probe mix and 6 µl PCR grade water. To each reaction, 2 µl of the diluted cDNA were added. All samples were assayed in triplicate. The real-time PCR program was set to 95℃ for 10 min and then 40 cycles of 95℃ for 15 seconds and 60℃ for 1 min (fluorescence reading). All real-time PCR data were analysed with 7500 System SDS software version 2.0.5 from Applied Biosystems™.

4.5 Microsatellite analysis with Agilent 2100 Bioanalyzer

The effect on genetic stability was assessed by analysing microsatellite stability. A panel of five microsatellite markers as suggested by the National Cancer Institute (NCI) workshop in 1997 (Boland *et al.,* 1998:5248) was used to analyse human microsatellite DNA. The microsatellite markers included two mononucleotide repeats: BAT25, BAT26, and three dinucleotide repeats: D5S346, D2S123, and D17S250. DNA extracted from HepG2 cells exposed to SA and pHPPA and DNA from the two HT1 patients were used. A panel of four microsatellite markers (D3Mit21, D7Mit18, D7Mit10 and D11Mit7) as described by Zhang *et al.,* was used to examine microsatellite stability in DNA extracted from the *fah*$^{-/-}$ mouse livers (Zhang *et al.,* 2004:521).

Primer sequences for all markers are given in table 4-2.

Table 4-2. Primer sequences for microsatellite markers.

Microsatellite Marker	Primer sequence	Annealing temperature Tm (℃)
BAT25F	5'- TCGCCTCCAAGAATGTAAGT -3'	58
BAT25R	5'- TCTGCATTTTACTATGGCTC -3'	58
BAT26F	5'- TGACTACTTTTGACTTCAGCC -3'	58
BAT26R	5'- AACCATTCAACATTTTTAACCC -3'	58
D17S250F	5'- GGAAGAATCAAATAGACAAT -3'	52
D17S250R	5'- GCTGGCCATATATATATTTAAAC -3'	52
D5S346F	5'- ACTCACTCTAGTGATAAATCG -3'	55
D5S346R	5'- AGCAGATAAGACAGTATTACTAGTT -3'	55
D2S123F	5'- AAACAGGATGCCTGCCTTTA -3'	60
D2S123R	5'- GGACTTTCCACCTATGGGAC -3'	60
D3Mit21F	5'- AAAGCTCTACAGCGGAAGCAC -3'	
D3Mit21R	5'- CTGGGGAGTTTCAGGTTCCT -3'	
D7Mit10F	5'- GAAGATTGGGCTGTCTGCAC -3'	
D7Mit10R	5'- TGAGCTGATGGAGCTGATG -3'	
D7Mit18F	5'- GGGAGCCCAGCTTCTACTG -3'	
D7Mit18R	5'- TCCTAACACCCTTCCTGGTG -3'	
D11Mit7F	5'- AGGGTATTCTCTAGCCTCCACAC -3'	
D11Mit7R	5'- TTTGAGGCAAGATGTCATGATG -3'	

All microsatellite analyses proceeded along to the same basic protocol, i.e. DNA amplification by PCR on a Biometra thermocycler (Biometra, Germany), PCR product confirmation by gel electrophoresis on a 2% agarose gel (in 1X TAE buffer; Appendix D) and microsatellite analysis with an Agilent 2100 Bioanalyzer. All PCR reactions for microsatellite analyses were performed with the KAPA HiFi™ PCR kit from KAPABIOSYSTEMS. PCR reactions for the human microsatellite analyses, contained: 5 µl Fidelity buffer (5X), 0.5 µl dNTPs (10 mM of each dNTP),

0.5 µl of each primer (10 µM), 1.5 µl $MgCl_2$ (25 mM), 300 ng DNA, 0.5 µl KAPA HiFi polymerase (1 U/µl), and PCR grade water up to 25 µl. Thermocycling conditions were: 95℃ for 2 min, followed by 25 cycles of 98℃ for 20 sec, T_m for 15 sec, and 68℃ for 20 sec, and a final extension step of 68℃ for 1 min. PCR products were stored at 4℃ until further use.

The PCR reaction mixtures for studying the mouse microsatellite DNA contained: 3 µl GC buffer (5X), 0.3 µl dNTPs (10 mM of each dNTP), 300 ng DNA, 0.3 µl of each primer (10 µM), 0.6 µl $MgCl_2$ (25 mM), 0.3 µl KAPA HiFi polymerase (1 U/µl), and PCR grade water up to 15 µl. Thermocycling conditions were: 95℃ for 6 min followed by 15 cycles of 98℃ for 20 sec, 63℃ for 30 sec (decreased 0.5℃ of each cycle), 68℃ for 20 sec, and then 25 cycles of 98℃ for 20 sec, 58℃ for 30 sec, and 68℃ for 20 sec, with a final extension of 68℃ for 5 min. PCR products were stored at 4℃ until use.

Successful PCR was confirmed by means of electrophoresis on a 2% agarose gel. Subsequently, 1 µl of each PCR product was analysed on an Agilent DNA 1000 chip with the Agilent 2100 Bioanalyzer according to the manufacturer's instructions. Briefly, the gel-dye mix was prepared by equilibrating the DNA dye concentrate and DNA gel matrix to room temperate for 30 min. After equilibration, 25 µl of the DNA dye concentrate was added to the vial DNA gel matrix. The gel-dye mix was vortexed and briefly centrifuged. After the gel-dye mix was transferred to the spin filter, it was centrifuged for 15 min at 2240*g*. Between uses, the gel-dye mix was stored at 4℃.

For each analysis, the gel-dye mix was equilibrated to room temperature for at least 30 min. The DNA 1000 chip was primed by adding 9 µl DNA gel matrix to the assigned well and applying pressure for 1 min, with the plunger of the chip priming station at 1 ml. Another 9 µl gel-dye mix was added to each of the next two assigned wells. Then 5 µl marker was added to each of the 12 sample wells and the ladder well. Of each sample, 1 µl was added to a separate well, and 1 µl DNA ladder was added to the ladder well. The DNA 1000 chip was subsequently vortexed for 1 min at 2400 rpm. Within 5 min the DNA 1000 chip was run on the Agilent 2100 Bioanalyzer. Results were processed with Agilent 2100 expert software version B.02.03.SI307.

4.6 High resolution melting

High resolution melting (HRM) is a PCR-based method to detect variations in the DNA sequence by gauging changes in the melting temperatures (T_m) of a DNA duplex. HRM analysis was performed on HT1 patient PCR products of *fah* and *hprt1* and mouse *hprt1*.

All PCR and HRM analyses were performed by the Central Analytical facility (CAF) at Stellenbosch University (South Africa) on a Rotor-Gene™ 6000 real-time rotary analyser from Corbett Research. Genomic DNA, isolated from HT1 patient blood and *fah*$^{-/-}$ mice livers, and all PCR primers were supplied to CAF for HRM-PCR. PCR primer information is given in table 4-3.

Table 4-3. Sequence information of HRM-PCR primers.

Organism	Gene	Exon / Intron	Amplification temperature	Primer name	Sequence
Human	*Fah*	12	63℃	FAH12FCPCR	5'-TCAACGGCTGCAACCTGCGG-3'
	Fah	12	63℃	FAH12RCPCR	5'-CAGGGATGCTCTGCGTGGGC-3'
	Hprt1	3	63℃	HPRT1/3FCPR	5'-ACTGAACGTCTTGCTCGAGATGTGA-3'
	Hprt1	3	63℃	HPRT1/3RCPR	5'-TGCTTTGATGTAATCCAGCAGGTCAGC-3 '
Mouse	*Hprt1*	6	63℃	HPRT1M6PP1F	5'-TGCAATCTAGTTCTGGGGACTGAC-3'
	Hprt1	6	63℃	HPRT1M6PP1R	5'-CCACAATTCACACAGAGATAGTCTGG-3'
	Hprt1	6	63℃	HPRT1M6PP4F	5'-AGGCCTTGGATCTAGAAACTACCTCAG-3 '
	Hprt1	6	63℃	HPRT1M6PP4R	5'-CCCACTGAGCCATCTCAGCA-3'
	Hprt1	6	63℃	HPRT1M6PP5F	5'-TGAGTTCAATCCTGGGACGC-3'
	Hprt1	6	63℃	HPRT1M6PP5R	5'-GCAAACACTCCTGCCATTAACA-3'

Unprocessed data were received from CAF and processed with the Rotor-Gene™ 6000 series software 1.7.

4.7 PCR and sequencing

To verify HRM results, the *fah* and *hprt1* PCR products were sequenced. Sequencing was performed by CAF with the ABI3730xl DNA analyser from Applied Biosystems™. Unprocessed sequencing data were received from CAF. Sequences were subsequently processed with Chromas version 1.42 from Conor McCarthy School of Health Science, Griffith University, Australia and DNAMAN version 4.1.2.1. from Lynnon Corporation.

Several clones of a mouse *hprt1* fragment, as well as the human *fah* fragment were amplified by co-amplification at lower denaturation temperature (COLD)-PCR. COLD-PCR proceeds on the same basic principle as HRM, i.e. a nucleotide mismatch on a double-stranded DNA sequence predictably changes the melting temperature (T_m) of the double-stranded DNA sequence (Liew *et al.,* 2004:1156). Each DNA sequence has a critical denaturation temperature (T_c) that is lower than the T_m of the sequence and below which PCR efficiency decreases (Li and Makrigiorgos, 2009:427). This fact is exploited in COLD-PCR to selectively enrich a minority allele

that differs by one or more nucleotide at any position in a sequence (Milbury *et al.,* 2009:632). Full-COLD-PCR proceeds as follows (Li and Makrigiorgos*,* 2009:427, Milbury *et al.,* 2009:632):

1. All DNA is denatured at a high temperature, e.g. 94℃,
2. temperature is decreased to an intermediate temperature, e.g. 70℃, to allow for cross-hybridisation of mutant and wild-type sequences,
3. temperature is increased to the T_c e.g. 86.5℃, to selectively denature the mismatched sequences,
4. temperature is decreased e.g. 55℃ to allow primer annealing,
5. temperature is increased e.g. 72℃ for extension of sequence, and
6. process is repeated from step 1.

To enrich for all possible mutations in the human *fah* gene fragment, 100 ng DNA (isolated from the control person and HT1 patients) was added to 22 µl mastermix containing: 5 µl Fidelity buffer (5X), 1 µl forward primer (10 µM) and reverse primer (10 µM), 1 µl dNTP mix (10 mM of each dNTP), 14.5 µl PCR grade water and 0.5 U KAPA HiFi DNA polymerase. Full-COLD-PCR was performed on a thermal cycler with the following program: 95℃ for 2 min, followed by 25 cycles of 98℃ for 20 sec, 64℃ for 15 sec and 68℃ for 30 sec, then 30 cycles of 98℃ for 15 sec, 70℃ for 8 min, T_c for 3 sec, 64℃ for 15 sec and 68℃ for 30 sec. A final step of 68℃ for 1 min was included to complete the PCR process, and the products were stored at 4℃ until use.

Similarly, to enrich for all possible mutations in the *hprt1* mouse gene fragment, approximately 100 ng DNA from the control and *fah* deficient mice were separately amplified in the presence of 5 µl Fidelity buffer (5X), 1 µl HPRT1M6PP4F (10 µM) and 1 µl HPRT1M6PP4R (10 µM) primers, 1 µl dNTP mix (10 mM each dNTP), 10.5 µl PCR grade water and 0.5 U KAPA HiFi DNA polymerase. Full-COLD-PCR was performed on a thermal cycler with the following program: 95℃ for 2 min, then 10 cycles of 98℃ for 15 sec, 63℃ for 20 sec and 72℃ for 30 sec, followed by 30 cycles of 98℃ for 15 sec, 69℃ for 8 min, T_c for 3 sec, 63℃ for 20 sec and 72℃ for 30 sec, and final extension for 1 min at 72℃. After PCR, all samples were kept at 4℃.

The HPRT1M6PP4 amplicons were then phosphorylated by mixing 7.5 µl reaction buffer A (10X; Fermentas), 2 µl ATP (65 µM), 10 µl amplicon, 53.5 µl PCR grade water, and 20 U T4 polynucleotide kinase (10 µl/U; Fermentas). The mixtures were vortexed and incubated at 37℃ for 30 min. The enzyme reactions were terminated by incubating the mixtures at 70℃ for 10 min. A clean-up step followed. Briefly: 1 volume (75 µl) chloroform was added to a phosphorylation mixture and vortexed. This mixture was then centrifuged for 5 min at 3400*g*. The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube and left open for 5 min. The phosphorylated samples were stored at 4℃ till further use. The cleaned, phosphorylated HPRT1M6PP4 amplicons were then separately ligated into pEZSeq™ HC Amp vectors (for vector

map see Appendix A) by mixing 2.5 µl pEZSeq vector premix (4X), 6 µl PCR grade water and 1 µl Clonesmart DNA ligase, and then adding 0.5 µl of the amplicon (~500ng DNA). This was then mixed and incubated for 2 h at room temperature. The ligation reactions were stopped by incubating the mixtures at 70℃ for 15 min. The ligation products were then transformed into eCloni bacterial cells, briefly: the cells were thawed on ice for 10 min. After gentle resuspension of the cells, 2 µl of a ligation mixture was added to 50 µl of the bacterial cell suspension. The transformation mixtures were kept on ice for 30 min, followed by heating in a water bath at precisely 42℃ for exactly 90 sec. The mixtures were immediately placed on ice for 2 min. Subsequently, 950 µl of SOC broth (Appendix D) were added to each transformation mixture and the tubes incubated at 37℃ for 60 min while shaking at 250 rpm. Of each transformation mixture, 50 µl and 100 µl were separately plated on yeast-tryptone (YT) agar plates containing 100 µg/ml ampicillin (Appendix D). All plates were incubated overnight at 37℃. The following day colony PCR was performed for several of the colonies. In short, a mastermix was made by adding together: 5 µl GoTaq® flexi buffer (5X), 0.5 µl dNTP mix (10 mM of each dNTP), 6.7 µl $MgCl_2$ (25mM), 1 µl M13 forward primer (10 µM), 1 µl M13 reverse primer (10 µM), and 0.25 GoTaq® DNA polymerase (5 U/µl). Several colonies were then separately resuspended in 10.55 µl PCR grade water. To each suspended colony, 14.45 µl of the mastermix was added. PCR was then performed on an Eppendorf® thermal cycler with the following program: 94℃ for 3 min, then 30 cycles of 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 1 min, followed by a final extension step of 1 min at 72℃. After PCR, all samples were kept at 4℃. The amplicons were run on a 2% agarose gel to confirm the presence of the correct size product. The amplicons were subsequently sent for dideoxy sequencing by CAF. Unprocessed sequencing results were received from CAF. The sequencing results were processed with Chromas and DNAMAN.