Chapter 8: Summary and conclusion

The inherited metabolic disorder, hereditary tyrosinemia type 1 (HT1), is the result of a defective fumarylacetoacetate hydrolase enzyme. The deficiency is caused by any of 51 mutations on *fah*. Under pathological conditions, tyrosine degradation metabolites such as fumarylacetoacetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA), and *p*-hydroxyphenylpyruvic acid (pHPPA) accumulate (Mitchell *et al.,* 2001:1777, Sniderman King *et al.,* 2008).

Several reports have shown that these metabolites are detrimental to cell homeostasis and may result in the following: mutagenic effects, glutathione depletion, induction of chromosomal instability, induction of cell cycle arrest and apoptosis, DNA ligase inhibition, and impairment of DNA repair (Bergeron *et al.,* 2006:5329, Jorquera and Tanguay*,* 1999:2284, Jorquera and Tanguay*,* 2001:1741, Jorquera and Tanguay*,* 1997:42, Prieto-Alamo and Laval*,* 1998:12614, van Dyk and Pretorius*,* 2005:815). These studies have, however, not shown which DNA repair mechanisms are affected and if the DNA repair affliction pertains to only protein functionality or if gene expression is also affected.

In order to study the mechanisms underlying pathogenesis in HT1, different models of the disease were developed. Most notably was the development of the *fah* deficient mouse by Grompe and colleagues (Grompe *et al.,* 1993:2298). This model was created by disruption of exon 5 of *fah*. The neonatally lethal phenotype is rescued by the administration of NTBC, which inhibits 4-hydroxyphenylpyruvate dioxygenase (Grompe *et al.,* 1995:453). Despite dietary and pharmacological treatment, these mice still show elevated levels of tyrosine and develop hepatocellular carcinoma (Al-Dhalimy *et al.,* 2002:38), making this the model that most closely resembles the disease in humans (Mitchell *et al.,* 2001:1777).

Although the fungal and animal models of HT1 are very useful, they are subject to limitations such as interspecies extrapolations (Mitchell *et al.,* 2001:1777). Consequently, in this study it was endeavoured to develop a HT1 hepatic cell model, which would be human genome based.

To develop the HT1 hepatic cell model, RNAi technology was employed. Given that tight control of *fah* expression is necessary, the Knockout Inducible RNAi system (Clontech Laboratories, Inc.), which allows tight regulation of expression of shRNAs, and thereby the shRNA target gene, was used. This is a tetracycline controlled system, i.e. a tetracycline-controlled transcriptional suppressor (tTS) regulates the expression of shRNAs (Gossen and Bujard*,*

1992:5547). To start, shRNA oligonucleotides for *fah* knock-down was designed, and acquired. The oligonucleotides were then inserted into the RNAi-Ready pSIREN-RetroQ-Tet vector, and subsequently sequenced to verify sequences. HepG2 cells were transfected with the ptTS-Neo vector, and cells stably expressing the necessary ptTS protein were selected. These cells were further propagated, and the cell lines were named HepG2tTS10 and HepG2tTS12. In parallel, the effectiveness of the *fah* knock-down shRNA constructs were determined, by transiently transfecting HepG2 cells with the *fah* knock-down constructs and an untargeted shRNA construct, respectively. From the results of the transient transfections, *fah* knock-down shRNA constructs 1 and 4 were chosen to establish the HT1 cell line. HepG2tTS10 and HepG2tTS12 cell lines were then separately transfected with either the *fah* knock-down constructs, or untargeted control or unrelated target control constructs. Cell colonies stably expressing the shRNAs were selected and propagated. To induce the knock-down of *fah*, doxycycline was added to the growth medium. RNA was isolated from the uninduced and induced cells and the expression of *fah* was determined by real-time PCR. Variable results were obtained. In the HepG2tTS10 cell line up to 84% knock-down of *fah* was achieved and in the HepG2tTS12 cell line up to 57% knock-down. The difference between the percentage of knock-down in the uninduced and induced cells were small, signifying that induction of *fah* knock-down with doxycycline did not have a noteworthy effect on *fah* knock-down. The possibility of 'leakage' of the inducible system, which would result in the continuous expression of the shRNAs was excluded. This was achieved by growing the double stable cells in complete maintenance medium supplemented with tet-system approved FBS, instead of regular FBS, and then inducing knock-down with doxycycline. The results showed no significant difference between the double stable cells grown in medium supplemented with either FBS.

Although a high level of knock-down was achieved in the HepG2tTS10 cell line, the level of *fah* expression in the cells transfected with the unrelated control shRNA construct and the untargeted control shRNA construct, were similar to the level of *fah* expression in the cells transfected with the *fah* knock-down shRNA constructs. The main issue arising from these results is: does the high level of knock-down of *fah*, seen in all these cells, pertain to only *fah*, or is gene expression in general affected by the developmental process. To elucidate this issue though, further investigation into this matter is needed. It was furthermore observed that the cell proliferation of the double-stable colonies was slow and after some time proliferation stopped. These cells were therefore not-viable. It was previously reported that genes involved in signal transduction, protein turnover and cell growth and proliferation are affected in HT1 cells, and that treatment with NTBC does not correct these changes (Luijerink *et al.,* 2003:901). Although a relevant a viable ideal hepatic cell model of hereditary tyrosinemia type 1 was not yet achieved, the establishment of such a model should not be discarded.

In an approach parallel to the development of the HT1 hepatic cell model, different molecular techniques were optimised to local conditions and applied to gain valuable insight into the pathological mechanisms of HT1. To this end, different HT1 related models and HT1 patient material were used, i.e. HepG2 cell cultures treated with the HT1 accumulating metabolites, SA and/or pHPPA; DNA samples from the *fah* deficient mouse model; and DNA and RNA samples from HT1 patients. In the past, this approach allowed us to obtain significant results pertaining to the elucidation of the molecular events characteristic of HT1 (van Dyk, 2005, van Dyk and Pretorius, 2005:815)

The comet assay was the first of these techniques that were applied. During the optimisation of this method, it was firstly determined that HepG2 cells need at least two hours recuperation time after cell harvesting, in order to be usable in the comet assay. Then the modified comet assay, which allows the user to assess the BER and NER capacity of cells, was used to determine the effect of SA and pHPPA on the ability of cells to perform base and nucleotide excision repair. Results indicated that exposure to either SA or pHPPA impairs base- and excision repair pathways. Exposure to these metabolites affected the repair mechanisms differently, since the metabolites had a larger detrimental effect on the initiating proteins of BER than on those of NER. SA, furthermore, did not have as big an impact on repair proteins as exposure to pHPPA. This observation also confirms our previous suggestion that exposure to pHPPA has an effect on DNA repair (van Dyk and Pretorius, 2005:815). These results therefore indicate that the metabolites accumulating in HT1 impairs the functionality of BER and NER DNA repair proteins. The results from the comet assay modified to measure BER and NER capacity, were published in *Biochemical and Biophysical Research Communication*, under the title “Hereditary tyrosinemia type 1 metabolites impair DNA excision repair pathways.” (van Dyk *et al.,* 2010:32).

To determine if the impaired BER and NER capacity was limited to protein functionality or if the level of gene expression of the repair proteins was also affected, the expression level of *hOGG1* and *ERCC1* was determined in HepG2 cells exposed to the SA and pHPPA, either separately or in combination, and in HT1 patient samples. Short term (96 h) exposure of the HepG2 cells to a combination of the two metabolites, showed an initial increase followed by a steady decrease in *hOGG1* expression. These changes are statistically insignificant, though. Similarly, the initial increase followed by a decrease in expression of *ERCC1*, was statistically insignificant. The expression levels of *hOGG1* and *ERCC1* by HepG2 cells after exposure to the HT1 metabolites separately, were also not statistically significantly altered. Short term exposure to these metabolites, therefore do not have a statistically significant effect expression of *hOGG1* or *ERCC1*. These results together with the results from the modified comet assay, suggest that short term exposure of cells to the accumulating metabolites, affect the capacity of cells for BER and NER DNA repair on a protein functionality rather than a gene expression level.

In the HT1 patient samples, the expression of both *hOGG1* and *ERCC1* were, however, markedly lower than in control samples. The expression of *hOGG1* and *ERCC1* were, furthermore, lowered to similar levels in each patient, confirming the strong correlation observations by Vogel and colleagues (Vogel *et al.,* 2002:1505).

Therefore, in addition to diminished DNA repair protein functionality the expression of the DNA repair genes are also affected in HT1. The low expression of the DNA repair proteins coupled with the decreased BER and NER DNA repair protein functionality, suggests that the capacity for DNA repair, is severely affected in HT1 cells.

Since impaired DNA repair negatively affects genome stability (Wilson *et al.,* 2007:138), microsatellite analysis was performed to assess genetic stability in HT1. Four different microsatellite markers, as described by Zhang *et al.,* (Zhang *et al.,* 2004:521), were used to determine microsatellite stability in the *fah*$^{-/-}$ mouse genome. The D7Mit18 microsatellite marker showed allelic imbalance on chromosome 7 of the mouse genome. Seeing that allelic imbalance is a molecular level trait of chromosomal instability, this observed allelic imbalance, confirmed the existing reports of chromosomal instability in HT1 (Gilbert-Barness *et al.,* 1990:243, Jorquera and Tanguay*,* 2001:1741, Zerbini *et al.,* 1992:1111). In addition, the instability of specifically the D7Mit18 microsatellite marker, suggests the possible involvement of the growth arrest specific 2 (*gas2*) and *ras* genes in HT1.

To assess the stability of the human genome after exposure to the accumulating metabolites, MSI analysis was performed on DNA isolated from HepG2 cells exposed to SA and pHPPA for 72 h. The Bethesda panel of microsatellite markers (Boland *et al.,* 1998:5248), was used to this end. No allelic imbalance or microsatellite instability was observed in the DNA from cells exposed to the metabolites.

The stability of microsatellite DNA was also assessed in DNA isolated from HT1 patient lymphocytes. This was done, since decreased gene expression DNA repair proteins was observed in the lymphocytes of HT1 patients (section 5.2), and the reduced DNA repair capacity in these cells might result in genetic instability in the lymphocytes. The Bethesda panel of microsatellite markers were also utilised here. In patient A, instability of the D2S123 and possibly D17S250 markers were observed. In patient B, instability of D5S346 and possibly D17S250 were observed. Since a diminished DNA repair capacity could lead to relaxed genomic stability, it is possible that the decreased DNA repair capacity, seen with the comet assay and gene expression assays, might be the effector in the genomic instability that is observed. Results from the gene expression levels of *hOGG1* and *ERCC1* in patient lymphocytes, and results from the assessment of microsatellite stability in human and mouse DNA samples, were compiled in the manuscript: “Impaired DNA

repair and genomic stability in hereditary tyrosinemia type 1." This manuscript was submitted to *GENE* (reference number GENE-D-11-00294).

As Loeb hypothesised that relaxed genomic stability could be the generator of the multiple mutations seen in cancer (Loeb, 1994:5059), HRM and sequencing was performed of selected parts of the *fah* and *hprt1* genes, to determine if an increase in mutations are seen in these genes. Since genomic instability were only seen in samples from the *fah* deficient mouse model and HT1 patients, only these samples were used for HRM and sequencing.

HRM and sequencing results of *hprt1* fragments analysed in both the human and mouse genome, were identical to control samples, indicating that no *de novo* mutations were present in these investigated locations. Similarly, HRM and sequencing results of *fah* fragments showed no *de novo* mutations in either of the human or mouse fragments. Although these results suggest the absence of a mutator phenotype as characterised by multiple mutations, the presence of a mutator phenotype can not be excluded, as only very small parts of the human and mouse genomes were analysed.

The use of these HT1 related models and HT1 patient material in the parallel approach, gave valuable new insights on the pathological mechanisms of HT1 pertaining to DNA repair (van Dyk *et al.,* 2010:32, sections 5.2 and 5.3). Also, the successful use of the different assays with HT1 related models and HT1 patient material, confirmed the suitability of these assays to assess the underlying pathological mechanisms of HT1, should an ideal HT1 hepatic cell model be developed.

The aim of this study was to contribute to the understanding of the molecular mechanisms underlying the development of HCC and mosaicism in HT1. In specific to:

- elucidate whether base- and nucleotide excision repair mechanisms are affected in HT1, and to what extent, i.e. protein functionality and/or gene expression levels,
- determine if microsatellite instability is present in HT1.

The results presented in this thesis show for the first time that:

- the HT1 metabolites, succinylacetone and *p*-hydroxyphenylpyruvic acid, affects base- and nucleotide excision repair mechanisms on a protein level;
- gene expression levels of DNA repair proteins, i.e. *hOGG1* and *ERCC1*, are low in HT1 patients;
- microsatellite instability occurs in HT1.

This study contributed towards elucidating the underlying mechanisms involved in the development of HCC and mosaicism, by showing that DNA repair mechanisms are severely affected in HT1 cells. The strong correlation that exists between a deficient DNA repair capacity and the development of cancer (Jackson and Loeb, 2001:7, Loeb and Loeb, 2000:379, Loeb, 1998:25), suggests that the observed severely affected capacity for DNA repair in HT1 could be a contributing factor to the development of the HT1-associated HCC.

Furthermore, characteristic of human cancers is the development of a mutator phenotype (Loeb, 1998:25). A mutator phenotype is typified by chromosomal aberrations, microsatellite instability and aneuploidy (Loeb and Loeb, 2000:379). The observation of microsatellite instability in the current study, therefore, contributes to the understanding of the developmental mechanisms underlying the HT1-associated HCC, by contributing evidence for the development of a mutator phenotype.

In addition, in chapter 9, a hypothesis is posited that suggests that the development of a point mutation instability (PIN) mutator phenotype may underlie both the development of HCC and the back mutations in HT1.