Chapter 2: Literature review

The medical model of disease proposes that an abnormal process (pathogenesis), initiated by the cause of a disease, results in manifestations of the disease (Beaudet et al., 2010). In the case of hereditary tyrosinemia type 1 (HT1) the disease is caused by a defective fumarylacetoacetate hydrolase enzyme which results in manifestations such as the development of hepatocellular carcinoma (HCC) and somatic mosaicism. The molecular mechanisms responsible for the development of HCC and somatic mosaicism in HT1 are largely unknown (Mitchell et al., 2001:1777). In order to review what is currently known about the development of HCC and mosaicism in HT1, this chapter will explore the existing literature on hereditary tyrosinemia type 1, mosaicism, and DNA repair mechanisms.

2.1 Hereditary tyrosinemia type 1

2.1.1 Introduction to inherited metabolic disorders

Monogenic disorders present in approximately 10 of every 1000 live births, of which 7 have a dominant pattern of inheritance, 2.5 a recessive pattern of inheritance, and 0.4 are X-linked conditions (excluding colour blindness) (Beaudet et al., 2010).

These disorders are usually severe and can result in neurological impairment, mental retardation, and death. Fortunately, many of these effects can be reduced or avoided by early diagnosis and sustained dietary intervention (Fernandes et al., 2006:561).

Inherited metabolic diseases are identified by characteristic metabolite profiles, which have its origin in the block in a specific metabolic pathway due to a defective enzyme in that pathway. This deficiency prevents the normal metabolism of an intermediary compound and can result in the accumulation of this compound, its precursors and/or induced alternative metabolites to toxic levels. The accumulation of these metabolites gives rise to the specific metabolic profile of an inherited metabolic disease. Each of the inherited metabolic diseases has clinical features by which they are characterized, but in some cases there are no genotype-phenotype correlation (Arnold et al., 2010:263, Mitchell et al., 2001:1777, Poudrier et al., 1998:119, Valle et al., 2007:).
2.1.2 Inherited disorders of the tyrosine catabolism

Tyrosine is a non-essential or semi-essential amino acid, as it cannot be completely synthesised by mammals. It is the product of hydroxylation of phenylalanine in the para position of the benzene ring. Quantitatively the major natural fate of tyrosine is incorporation into proteins or degradation, when it is mainly converted into DOPA by the tyrosine hydroxylase enzyme. (Mitchell et al., 2001:1777). Since certain enzymes can phosphorylate tyrosine, it also plays an important role in signal transduction. Tyrosine is furthermore the precursor for the thyroid hormones: thyroxine and triiodothyronine, the pigment melanin, and the biologically-active catecholamines: dopamine, noradrenaline and adrenaline (Mitchell et al., 2001:1777).

Tyrosine degradation occurs primarily in the cytoplasm of hepatocytes and is both glucogenic and ketogenic. Under most circumstances the rate of tyrosine degradation is determined by the activity of tyrosine aminotransferase (E.C. 2.6.1.5) (Holme, 2003:141, Mitchell et al., 2001:1777).

Genetics defects are known for each of the enzymes involved in the degradation of tyrosine. A summary of the defective enzyme, gene involved, clinical symptoms, treatment, and alternative names for each of the inborn errors of metabolism found in the tyrosine degradation pathway is given in table 2-1.
Table 2-1. Summary of the inborn errors of metabolism of the tyrosine degradation pathway.

<table>
<thead>
<tr>
<th></th>
<th>Tyrosinemia 1</th>
<th>Tyrosinemia 2</th>
<th>Tyrosinemia 3</th>
<th>Alkaptonuria</th>
<th>Hawkinsinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme defect</strong></td>
<td>Fumarylacetoacetate hydrolase</td>
<td>Tyrosine aminotransferase</td>
<td>4-Hydroxyphenyl-pyruvic acid</td>
<td>Homogentisic acid oxygenase</td>
<td>4-Hydroxyphenyl-pyruvic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dioxigenase</td>
<td></td>
<td>dioxigenase</td>
</tr>
<tr>
<td><strong>Gene</strong></td>
<td>15q23-q25</td>
<td>16q22.1-q22.3</td>
<td>12q24-qter</td>
<td>3q21-q23</td>
<td>12q24-qter</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td>Acute liver failure, cirrhosis,</td>
<td>Palmar-plantar keratosis, painful</td>
<td>Intermittent ataxia, neurological</td>
<td>Darkening urine, cardiac disease,</td>
<td>Metabolic acidosis, failure to thrive</td>
</tr>
<tr>
<td></td>
<td>hepatocellular carcinoma, renal</td>
<td>corneal erosions, mental</td>
<td>abnormalities</td>
<td>urolithiasis, limitation of large joint</td>
<td>in infancy.</td>
</tr>
<tr>
<td></td>
<td>Fanconi syndrome,</td>
<td>retardation</td>
<td></td>
<td>movement, back pain</td>
<td></td>
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<tr>
<td></td>
<td>glomerulosclerosis, crises of</td>
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<tr>
<td></td>
<td>peripheral neuropathy</td>
<td></td>
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<tr>
<td><strong>Treatment</strong></td>
<td>NTBC administration, hepatic</td>
<td>Dietary restriction of tyrosine and</td>
<td>N.A.</td>
<td>Dietary restriction of tyrosine and</td>
<td>Dietary restriction of protein,</td>
</tr>
<tr>
<td></td>
<td>transplantation, dietary</td>
<td>phenylalanine</td>
<td></td>
<td>phenylalanine</td>
<td>administration of ascorbate</td>
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<tr>
<td></td>
<td>restriction of tyrosine and</td>
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<td></td>
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<tr>
<td></td>
<td>phenylalanine</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Alternative titles</strong></td>
<td>Hereditary tyrosinemia type 1,</td>
<td>TAT deficiency, Keratosis</td>
<td>4-Hydroxyphenyl-pyruvic acid</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>Hepatorenal tyrosinemia,</td>
<td>palmoplantaris with corneal</td>
<td>oxidase deficiency, 4-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fumarylacetoacetase deficiency,</td>
<td>dystrophy, Richner-Hanhart</td>
<td>Hydroxyphenylpyruvate oxigenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FAH deficiency</td>
<td>syndrome, Oregon type tyrosinemia, oculocutaneous type tyrosinosis</td>
<td>dioxigenase deficiency</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.1.3 Hereditary tyrosinemia type 1

2.1.3.1 General considerations

Hereditary tyrosinemia type 1 (HT1, OMIM 276700) is a monogenic disorder with an autosomal recessive pattern of inheritance. The disease is caused by a deficiency in fumarylacetoacetate hydrolase (FAH, E.C.3.7.1.2), the last enzyme of the tyrosine degradation pathway (figure 2-1) (Lindblad et al., 1977:4641). In the absence of FAH, metabolites such as maleylacetoacetate (MAA), fumarylacetoacetate (FAA), succinylacetone (SA), \( p \)-hydroxyphenylpyruvic acid, \( p \)-hydroxyphenyllactic acid and \( p \)-hydroxyphenylacetic acid accumulate (Holme, 2003:141, Mitchell et al., 2001:1777, Sniderman King et al., 2008).

![Figure 2-1. Tyrosine catabolism. A defective fumarylacetic acid hydrolase enzyme results in the accumulation of upstream metabolites i.e. maleylacetoacetate (MAA) and fumarylacetoacetate (FAA) and its derivatives succinylacetoacetate (SAA) and succinylacetone (SA). FAA and MAA are not found in body fluids of patients but intracellularly, therefore the effects of FAA and MAA occurs mainly in the cells of organs where they are produced. SAA and SA can conversely be detected in plasma and urine of patients and consequently have widespread effects (Fernandes et al., 2006:561, Mitchell et al., 2001:1777).](image)

Most patients present with symptoms within the first few months of life, but some may only present 6 months after birth (Holme, 2003:141, Mitchell et al., 2001:1777, Scott, 2006:121). A failure to thrive precedes the appearance of more dramatic findings. HT1 patients have a ‘cabbage-like’ odour and characteristically present with liver dysfunction, renal tubular dysfunction and rickets (Holme, 2003:141, Roth, 2009, Sniderman King et al., 2008). Due to the effect of
succinylacetone on the heme-metabolism, porphyria-like neurologic crises are also observed (Scott, 2006:121, Sniderman King et al., 2008). However, treatment of patients with NTBC prevents the development of acute hepatic and neurologic crises (Mitchell et al., 2001:1777). HT1 is additionally characterised by a high incidence of hepatocellular carcinoma (Holme, 2003:141, Mitchell et al., 2001:1777, Sniderman King et al., 2008).

HT1 patients were historically classified as acute or chronic, with patients classified as ‘chronic’ when they survived more than two years on medical treatment. Classification of HT1 patients in this manner may be deceptive, as patients, classified as acute in the first year of life, may survive with a chronic course. Conversely, patients classified as chronic may still experience life-threatening neurologic and hepatic crises (Mitchell et al., 2001:1777). Van Spronsen et al., proposed the classification of HT1 as very early-, early- and late-presenting, based on the time of onset of symptoms, i.e. less than 2 months, 2-6 months and more than 6 months, respectively (Van Spronsen et al., 1994:1187). On the other hand, Demers et al., classified HT1 as acute, sub-acute and chronic, based on the time of liver-transplantation. Patients undergoing liver transplantation before age 2 are classified as acute, between age 2 and 6 as sub-acute and after age 6 as chronic (Demers et al., 2003:1313). The introduction of newborn screening programs and NTBC treatment have, however, altered the clinical course of HT1 by making early detection and treatment of HT1 possible. The different ‘forms’ of HT1 are therefore no longer distinguishable.

The gene for FAH has been cloned and mapped to chromosome 15q23-q25. It is a soluble cytosolic homodimer of 46.3 kDa subunits (Labelle et al., 1993:941, Mitchell et al., 2001:1777). The gene spans over 35 kb of DNA (Mitchell et al., 2001:1777) and has 2 protein coding transcripts (ENST00000407106; ENST00000261755). FAH is predominantly expressed in the liver but is also found in a wide range of tissue and cell types such as kidney, brain, adrenal glands, lungs, heart, bladder, intestine, stomach, pancreas, lymphocytes, skeletal muscle, placenta, chorionic villi and fibroblasts (Berger, 1996:107, Bergeron et al., 2001:15225). Fifty-one mutations responsible for HT1 have been identified in fah (figure 2-2), and include 33 nucleotide substitutions (missense/nonsense), 13 splicing mutations, two small deletions, two gross deletions and one small indel. Mutations are referenced from the Human Genome Mutation Database (HGMD®: http://www.hmgd.org) and (Cassiman et al., 2009:28, Mitchell et al., 2001:1777, Park et al., 2009:930, Vondrakova et al., 2010:411). Reversions to wild-type have been reported for four HT1 mutations: IVS12+5g→a, Q64H, G337S, and Q279R (Demers et al., 2003:1313, Dreumont et al., 2001:9, Kvittingen et al., 1993:1816, Kvittingen et al., 1994:1657, Poudrier et al., 1998:119). Although reversions were only reported for these mutations, reversion to wild-type of other HT1 mutations can not be excluded, as absence of evidence is not evidence of absence.
Figure 2-2. Schematic depiction of positions of mutations occurring in the *fah* gene. Exons are given as boxes and introns as lines. Note the clustering of mutations on exons 6, 7, 8, 9, 12 and 13 and introns 6 and 8.
2.1.3.2 Diagnosis and treatment

Hereditary tyrosinemia type 1 is usually diagnosed in patients with elevated plasma tyrosine, phenylalanine and methionine levels, although patients on a low-protein diet frequently have normal plasma tyrosine levels. The occurrence of elevated levels of SA in patient blood, plasma or urine is pathognomonic (Fernandes et al., 2006:561, Mitchell et al., 2001:1777, Sniderman King et al., 2008).

Diagnosis of HT1 has to be confirmed with enzyme or mutation assays of FAH. These assays can be performed on liver biopsies, lymphocytes, fibroblasts or dried blood spots (Fernandes et al., 2006:561).

The currently preferred treatment of HT1 is administration of NTBC, which is an inhibitor of the 4-hydroxyphenylpyruvic acid dioxygenase enzyme. In combination with NTBC treatment, a diet restricted of tyrosine and phenylalanine should be followed. In patients, who do not respond to NTBC treatment, a liver transplant may be necessary (Fernandes et al., 2006:561). Early treatment (<6 months) with NTBC reduces the risk of development of HCC (Fernandes et al., 2006:561). The true long-term efficacy of NTBC is, however, still unclear. Animal model trials, with fah⁻/⁻ mice, showed that HCC developed even under rigorous therapy or prenatal initiation of therapy (Al-Dhalimy et al., 2002:38). In humans, a case report showed that, despite long-term (~6 years) treatment with NTBC, HCC still developed (van Spronsen et al., 2005:90). Very recently, it was reported though that a HT1 patient, under NTBC and dietary treatment, was diagnosed with a liver neoplasm (histologically classified as HCC) at 15 months. After initial chemotherapy and partial hepatectomy, and a twelve-year disease free period, the liver neoplasm was re-evaluated and reclassified as hepatoblastoma. Hepatoblastoma in NTBC treated HT1 patients will no longer be a directive for liver transplantation (Nobili et al., 2010:e235).

2.1.3.3 Genetics of FAH

Hereditary tyrosinemia type 1 has a worldwide incidence of 1 in 100,000 persons but a much higher incidence is found in Quebec, Canada, where 1 in every 16,000 persons is affected. In the Saguaney-Lac St. Jean region of Quebec 1 in every 1,846 persons is affected (Roth, 2009, Sniderman King et al., 2008). Population genetics revealed that 94% of fah mutant alleles in the Saguaney-Lac St. Jean region are the IVS12+5g→a mutant allele, suggesting a founder effect (Mitchell et al., 2001:1777, St-Louis et al., 1997:291). This allele also accounts for a substantial fraction of mutant alleles in patients of European origin. Another frequently found mutant allele is the IVS6-1g→t allele. This allele together with the IVS12+5g→a allele accounts for 60% of mutant alleles in patients from America, Europe, Turkey and Morocco. Fah mutations that are frequently
found in other populations are the W262X mutation in Finns, the Q64H mutation in Pakistanis, the P261L mutation in the Ashkenazi-Jewish population and the D233V mutation in Turks (Mitchell et al., 2001:1777, Rootwelt et al., 1994:235, Sniderman King et al., 2008).

2.1.3.4 Mutation reversion

Molecular studies of HT1 patients revealed that identical genotypes and even family members could present different phenotypes, suggesting that genotypic variability on its own does not account for the different clinical forms observed in tyrosinemia (Mitchell et al., 2001:1777, Ploos van Amstel et al., 1996:51, Poudrier et al., 1998:119). A noteworthy observation was made by Kvittingen and colleagues when they described liver nodules in HT1 patients in which FAH activity was restored to normal (Kvittingen et al., 1993:1816, Kvittingen et al., 1994:1657). They proposed that nodule formation was due to a growth advantage of the reverted cells. It was later on suggested that the presence of varying numbers of reverted cells contributes towards the varying phenotypes observed for the same FAH mutations (Demers et al., 2003:1313).

In 88% of HT1 patients’ livers FAH-immunopositive nodules are found, which contain cells that appear to function normally (Demers et al., 2003:1313, Kvittingen et al., 1994:1657, Youssoufian and Pyeritz, 2002:748), thereby creating a liver mosaic. Molecular studies showed that the mosaic pattern was because in some cells reversion to the wild-type of one allele of the original point mutation in fah (RefSeq NM_000137.2) has occurred. This reversion of one allele to the wild-type is sufficient to re-establish a normal metabolic milieu giving such cells a growth advantage over cells with non-corrected mutations (Eyre-Walker et al., 2007:610, Kvittingen et al., 1994:1657, Marusyk et al., 2008:1). The extent of the observed surface of reversion can vary between 0.1% and 85% (Demers et al., 2003:1313).

Usually only one type of reversion is reported in each individual, but in one Norwegian patient a triple mosaic was observed. A reversion to wild-type of the HT1 causing mutation as well as a second-site repressor mutation was seen (Bliksrud et al., 2005:406), suggesting that more than one type of reversion might be present in one individual. A correlation appears to exist between the extents of the reversion, the age of the patient and the severity of the disease, i.e. the higher the extent of surface reversion, the less severe the phenotype (Demers et al., 2003:1313).

Table 2-2 gives a summary of the currently reported HT1 mutations that are observed to revert to the wild-type, the original mutagenic nucleotide change, and the effect of the original mutation.
Table 2-2. Summary of the observed mutation reversions and their effects as seen in HT1.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nucleotide change</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q64H (Missense)</td>
<td>c.192G&gt;T</td>
<td>Partial intron 2 retention and premature termination</td>
</tr>
<tr>
<td>Q279R (Missense / Nonsense)</td>
<td>c.836A&gt;G</td>
<td>No significant effect on enzyme activity</td>
</tr>
<tr>
<td>G337S (Abnormal splice / Missense)</td>
<td>c.1009G&gt;A</td>
<td>Cause aberrant splicing by introducing an acceptor splice site within exon 12, thereby deleting the first 50 nucleotides of this exon. The following exon-intron boundary is frequently missed, and a cryptic donor splice site within intron 12 causes a partial intron 12 retention of 105 bp.</td>
</tr>
<tr>
<td>IVS12+5g→a (Splice)</td>
<td>G&gt;A</td>
<td>Alternative splicing with retention of the first 105 nucleotides of intron 12, exon 12 skipping, and a combined deletion of exons 12 and 13</td>
</tr>
</tbody>
</table>


The mechanism often invoked for ‘reversions’, such as gene conversion and mitotic recombination, cannot explain the site specific reversion in cases of homozygotic inheritance of recessive monogenic diseases (Hirschhorn, 2003:721). It seems therefore that the reversion to wild-type, in the case of HT1, is the result of a true back mutation.

2.1.3.5 Accumulating metabolites

As described, the block of tyrosine degradation due to a defective FAH, results in the accumulation of upstream metabolites. The accumulation of the intermediates may contribute the establishment of a sustained stress environment in the cell, by disrupting cell homeostasis.

The metabolites immediately upstream of the block, i.e. FAA and MAA (figure 2-1) have not been found as excreted or circulating metabolites, suggesting compartmentalisation (Mitchell et al., 2001:1777). Their effects are therefore within the cells where they are formed. Reduction and decarboxylation reactions rapidly transform FAA and MAA to SA, which is then excreted (Langlois et al., 2006:1648, Prieto-Alamo and Laval, 1998:12614).

The chemical structure of FAA and MAA, in that both molecules have α,β-unsaturated carbonyl compound structures, suggests that these molecules may alkylate macromolecules, such as DNA, or disrupt important sulfhydryl reactions by complexation with proteins or glutathione (Lindblad et al., 1977:4641). Jorquera and Tanguay have confirmed that FAA, but not MAA or SA,
is highly mutagenic. They furthermore showed that its mutagenicity is potentiated by depletion of glutathione (Jorquera and Tanguay, 1997:42). Singularly, FAA furthermore:

- activates the ERK pathway, causing chromosomal instability, aneuploidy and transformation (Jorquera and Tanguay, 2001:1741),
- induces cell cycle arrest (in G2/M) and apoptosis (Jorquera and Tanguay, 1999:2284, Kubo et al., 1998:9552), and
- disrupts endoplasmic reticulum function, by early activation of GRP17/BiP and phosphorylation of eIF2α, which leads to the induction of CHOP (Bergeron et al., 2006:5329).

At physiological pH and temperature, the HT1 pathognomonic metabolite succinylacetone (SA) forms stable adducts via Schiff base formation with amino acids, either free or in proteins (lysine being the most reactive) (Manabe et al., 1985:1060). Prieto-Alamo and Laval showed that SA inhibits T4 DNA ligase in this manner by reacting with the lysine residue in the ligase. This inhibition results in the slow rejoining of Okazaki fragments (Prieto-Alamo and Laval, 1998:12614). We observed that DNA repair in liver cells is impaired by p-hydroxyphenylpyruvic acid (pHPPA) (van Dyk and Pretorius, 2005:815).

Still further contributing to the establishment of the sustained stress environment is the glutathione depleting properties of FAA, which in turn potentiates its mutagenicity (Jorquera and Tanguay, 1997:42). In addition, FAA also induces tumour hypoxia (Edwards et al., 1991:419). Hypoxia causes genomic instability by down-regulating at least five DNA repair genes (Galhardo et al., 2007:399).

The reduction products of FAA and MAA, maleylacetone (MA) and fumarylacetone (FA), alkylate and inhibit human glutathione transferase zeta (GSTZ1-1). GSTZ1-1 is identical to maleylacetoacetate isomerase, and catalyses the cis-trans isomerisation of MAA to FAA (Lantum et al., 2002:707).

The discontinuation of NTBC treatment in fah deficient mice showed that accumulation of the intermediates collectively, resulted in progressive liver pathophysiology. The ensuing hepatic stress causes the activation of the AKT survival pathway and inhibition of apoptosis, conferring cell death resistance (Orejuela et al., 2008:308).
2.1.3.6 HT1 models

Model organisms are commonly used in research to study disease. In this regard, genetically modified organisms, which include a fungal model, a nematode model, and three different mouse models, have been created to study HT1.

Fernandez-Canon and Penalva (Fernandez-Canon and Penalva, 1995:9132) created a fungal model by disrupting the *fahA* gene in an *A. nidulans biA1 methG1 argB2* strain. This disruption resulted in the secretion of succinylacetone and prevented growth on phenylalanine or phenylacetate. They also determined that the loss of homogentisate dioxygenase activity prevented the effects of Fah deficiency in *A. nidulans*.

A nematode model of HT1 was created by Fisher et al., (Fisher et al., 2008:9127) in *Caenorhabditis elegans* (*C. elegans*). In *C. elegans* the K10C2.4 gene encodes for a homolog of FAH. When Fisher and colleagues disrupted the K10C2.4 gene through RNAi, a lethal phenotype was observed, which included intestinal damage, reduced fertility and activation of oxidative stress and endoplasmic reticulum stress response pathways and resulted in death in young adulthood. They found that the phenotype is sensitive to tyrosine levels, as increased dietary tyrosine enhanced the phenotype and disruption of enzymes upstream of K10C2.4 in tyrosine degradation, decreased the phenotype.

In 1979, Gluecksohn-Waelsch created mice with a homozygous c\(^{14}\)CoS deletion, which is an X-ray induced deletion of chromosome 7. These albino mice have a neonatal lethal phenotype as a result of liver dysfunction by way of abnormal expression of hepatic mRNAs (Grompe et al., 1993:2298, Grompe et al., 1998:518). In 1993, Grompe and colleagues determined that *fah* maps to this deletion interval, making the c\(^{14}\)CoS mice the first of the mouse models for HT1. In order to determine whether the c\(^{14}\)CoS deletion is attributable to a loss of *fah*, Grompe et al (Grompe et al., 1993:2298) created the second of the HT1 mouse models by disruption of exon 5 of the *fah* gene. Both the c\(^{14}\)CoS mice and the *fah*\(^{Δ exon5}\) mice had the same phenotype. The third HT1 mouse model was developed by Endo et al (Endo et al., 1997:24426). This model is a double mutant, which is defective in both fumarylacetoacetate hydrolase and 4-hydroxyphenylpyruvate dioxygenase (HPD) (Endo et al., 1997:24426). In both the *fah*\(^{Δ exon5}\) (fah\(^{−/−}\)) and *fah*\(^{−/−}\) *hpd*\(^{−/−}\) mice models the early lethality is avoided by a defective 4-hydroxyphenylpyruvate dioxygenase enzyme. In the first of these models this is achieved through the inhibition of 4-hydroxyphenylpyruvate dioxygenase by NTBC and in the second model through knock-out of the *Hpd* gene (Endo et al., 1997:24426, Grompe et al., 1995:453). However, *fah*\(^{−/−}\) mice treated with NTBC still had elevated levels of tyrosine and developed hepatocellular carcinoma (Ali-Dhalimy et al., 2002:38). The fourth mouse model was created by Manning et al (Manning et al., 1999:11928) through cross-breeding of *fah*\(^{−/−}\) mice with *aku* (alkaptonuria) mice. The offspring of these mice are deficient in fumarylacetoacetate hydrolase and homogentisate-1,2-dioxygenase. Similar to the fungal model, the defective
homogentisate-1,2-dioxygenase (HGD) rescues the lethal phenotype. Unlike the fah\(^{-}\) hpd\(^{-}\) mice, the fah\(^{-}\) hgd\(^{ak/uak}\) mice do not have elevated tyrosine levels. Similarly, however, in neither of the two double mutant mouse models hepatocellular carcinoma developed (Manning et al., 1999:11928, Nakamura et al., 2007:1556S). Aponte and colleagues also created HT1 mouse models, through the induction of point mutations by N-ethyl-N-nitrosourea (ENU) (Aponte et al., 2001:641). The fah\(^{6287SB}\) mice have a missense mutation in exon 6 and the fah\(^{5961SB}\) mice have a splice mutation that causes the loss of exon 7. The fah\(^{6287SB}\) mice have a milder phenotype and the fah\(^{5961SB}\) mice have a more severe phenotype. In both of these mice, the level of succinylacetone is elevated (Aponte et al., 2001:641).

### 2.2 Mosaicism

#### 2.2.1 Introduction to mosaicism

The NCBI defines mosaicism in genetics as the presence of two populations of cells with different genotypes in one patient, (http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0002294/). Mosaicism may result from DNA mutation, epigenetic DNA alterations, chromosomal abnormalities or spontaneous reversion of inherited mutations during development which is propagated to only a subset of the adult cells (Youssoufian and Pyeritz, 2002:748). Mosaicism is different from chimerism. In mosaicism the two genotypically different cell populations are the result of DNA changes in post-zygotic cells and in chimerism the genetically different cell populations originate from different zygotes (Youssoufian et al., 2002:748). Differences in tissues and cell types due to normal development are not considered a true mosaicism, as the nuclear DNA sequence is the same in all cells (Hirschhorn, 2003:721).

Mosaicism is typified by unexpected differences in phenotype. For instance, individuals who are genotypically identical may have different disease phenotypes, or when improvement of a disease is seen rather than an expected worsening (Hirschhorn, 2003:721).

Genetic mosaicism can be sub-divided into germ-line and somatic mosaicism. In germ-line mosaicism the two genotypically different cell populations are limited to the egg and sperm precursor cells (http://ghr.nlm.nih.gov/glossary=germlinemosaicism). Alternatively, in somatic mosaicism, the genotypically different cell populations are in any of the cells of the body, which may or may not include germ-line cells (http://ghr.nlm.nih.gov/ glossary=somaticmosaicism). These two sub-types of mosaicism may occur in the same individual (Youssoufian and Pyeritz, 2002:748). Somatic mosaicism may be the result of a de novo mutation or the reversion to wild-type of an inherited mutation.
• **Somatic mosaicism due to *de novo* mutation**

This type of mosaicism usually originates during embryogenesis. The stage of embryogenesis and the site of the mutation determine the phenotype (Hirschhorn, 2003:721). Processes like ageing and generation of immune diversity, as well as the pathogenesis of neoplasia and mitochondrial disorders may be the result of somatic mosaicism (Youssoufian and Pyeritz, 2002:748).

• **Somatic mosaicism due to reversion to wild-type**

In Mendelian disorders, the mechanisms for the spontaneous reversion to wild-type of an inherited mutation, include: back mutations, homologous recombination, second-site repressor mutations, substitutions in the mutant codon and transposition or changes in activity of mobile elements in the genome (Youssoufian and Pyeritz, 2002:748). Patients may be abnormal, possibly milder than expected or occasionally normal (Hirschhorn, 2003:721).

The specific disorder, the tissue of origin and selective pressure may all have bearing on the frequency of the mosaicism (Davis and Candotti, 2010:46, Youssoufian and Pyeritz, 2002:748).

Some genetic disorders where reversion of point mutations occurs:

1. Adenosine deaminase deficient severe combined immuno deficiency
2. Bloom syndrome
3. Epidermolysis bullosa
4. Fanconi anaemia
5. Wiskott-Aldrich syndrome
6. X-linked severe combined immunodeficiency
7. Hereditary tyrosinemia type 1

### 2.2.2 Mosaicism in HT1

Mosaicism is a frequent observation in HT1 patients (Demers *et al.*, 2003:1313). Reports on mosaicism in HT1, show that the mosaicism seen in the livers of HT1 patients are the result of reversion to wild-type of the inherited HT1 causing mutations (Kvittingen *et al.*, 1993:1816, Kvittingen *et al.*, 1994:1657).

For a more detailed explanation of mosaicism in HT1, as well as the possible cause thereof, please see the prepared manuscript in chapter 9. The manuscript is entitled: “Point
mutation instability (PIN) mutator phenotype as model for true back mutations seen in hereditary tyrosinemia type 1."

2.3 DNA repair

The human genome is under constant insult from endogenous and exogenous sources, which may cause genetic instability. Genetic instability is commonly linked to the development of cancer (Friedberg et al., 2006:1118, Loeb et al., 1974:2311, Loeb et al., 2008:3551). Maintenance of genomic integrity is for this reason of particular importance to organism survival (Christmann et al., 2003:3, Mitra et al., 2002:15). Different DNA repair mechanisms exist to safeguard genomic integrity (Christmann et al., 2003:3, Mohrenweiser et al., 2003:93). In HT1 the accumulating metabolites affect these repair mechanisms (Prieto-Alamo and Laval, 1998:12614, van Dyk et al., 2010:32, van Dyk and Pretorius, 2005:815).

2.3.1 Introduction to DNA repair mechanisms

There are many different biological responses to DNA damage. These mechanisms include direct reversal of the DNA damage, excision repair, strand break repair, tolerance of base damage, cell cycle checkpoint activation and apoptosis (Friedberg et al., 2006:1118). According to Friedberg, the definition of DNA repair only considers biological responses that restore the normal nucleotide sequence and structure to be true DNA repair. DNA lesions in the form of incorrect, altered or damaged DNA bases are repaired through DNA excision repair pathways (Devlin, 2002, Friedberg et al., 2006:1118, Lodish et al., 2008, Mohrenweiser et al., 2003:93).

2.3.2 DNA excision repair mechanisms

DNA excision repair pathways are those repair mechanisms that repair DNA lesions through the removal of damaged bases as free bases or as nucleotides. These mechanisms include base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). (Christmann et al., 2003:3, Devlin, 2002, Friedberg et al., 2006:1118, Lodish et al., 2008).
2.3.2.1 *Base excision repair*

BER is the most frequently used repair mechanism in nature. During BER, DNA glycosylases recognize and remove damaged DNA bases. These lesions include oxidized and alkylated DNA bases. Oxidized DNA bases, e.g. 8-oxoG, arise spontaneously within the cell during inflammatory responses or from exposure to exogenous agents and alkylated DNA bases e.g. 7-meG and 3-meA are induced by endogenous alkylating species and exogenous carcinogens (Almeida *et al.*, 2007:695, Christmann *et al.*, 2003:3, Friedberg *et al.*, 2006:1118).

The mechanism of repair for both of these types of modified bases can be summarised as follow:

- Recognition, base removal and incision, through mono- or bifunctional glycosylases and AP endonucleases.
- Nucleotide insertion, by DNA polymerase $\beta$.
- Repair synthesis and DNA ligation by DNA ligase I or a DNA ligase III$\alpha$ and XRCC1 complex.

2.3.2.2 *Nucleotide excision repair*

NER repair bulky DNA adducts, such as UV-light-induced photo-lesions, intra-strand cross-links, large chemical adducts generated from aflatoxine, benzo[a]pyrene and other genotoxic agents (Christmann *et al.*, 2003:3, Friedberg *et al.*, 2006:1118, Mohrenweiser *et al.*, 2003:93). NER can be subdivided into two pathways: global genomic repair (GGR) and transcription-coupled repair (TCR).

The process of GGR can be summarised as follow:

- Recognition of DNA lesion by XPC-HR23B, RPA-XPA or DDB1-DDB2.
- Unwinding by TFIIH.
- Excision of the DNA lesion by XPG and XPF-ERCC1.
- Repair synthesis by polymerase $\delta$ or polymerase $\epsilon$.
- Ligation by DNA ligase I.

Transcription-coupled repair is initiated when a lesion blocks RNAPII. This blockage leads to the compilation of CSA, CSB and/or TFIIIS at the lesion site. RNAPII is thereby removed or displaced from the DNA or lesion. Similar to GGR, the exonucleases XPF-ERCC1 and XPG then cleave the lesion containing DNA strand, polymerase $\delta$ or polymerase $\epsilon$ resynthesises the DNA.
and DNA ligase I performs the final ligation (Christmann et al., 2003:3, Friedberg et al., 2006:1118).

### 2.3.2.3 Mismatch repair (MMR)

Contrary to BER, MMR occurs after DNA replication (Lodish et al., 2008). Spontaneous and induced base deamination, oxidation, methylation and DNA mismatches incurred during replication are removed by MMR (Christmann et al., 2003:3, Fukui, 2010:260512).

The steps by which MMR proceeds are as follow (Fukui, 2010:260512, Lodish et al., 2008):
- Recognition and incision of DNA lesions by MutSα and MutLα respectively.
- Excision of the lesion by EXO1.
- Repair synthesis by DNA polymerase δ and ligation by DNA ligase I.

### 2.3.3 Consequences of defective DNA repair

The frequency of spontaneous mutations in the human genome is very low, i.e. less than $1 \times 10^{-8}$ mutations per base pair (Bielas et al., 2006:18238). To maintain this low frequency of spontaneous mutations, the necessity of well functioning DNA repair mechanisms is underlined. In addition to the approximately $2 \times 10^4$ spontaneous, potentially mutagenic, lesions that occur per diploid mammalian cell per day, replicative DNA polymerases make a mistake every $10^4$ to $10^5$ nucleotides polymerised (Preston et al., 2010:281, Albertson et al., 2009:17101). As described, most of the spontaneous DNA lesions are repaired by the base excision repair pathway (Friedberg et al., 2006:1118, Preston et al., 2010:281). The errors made by the replicative DNA polymerases are mostly corrected by the intrinsic exonuclease activity of the polymerases, but the errors that escape the proofreading of the polymerase are repaired by the mismatch repair pathway (Friedberg et al., 2006:1118, Albertson et al., 2009:17101).

Recently Bielas and colleagues showed that the frequency of mutations in cancer cells is up to $1 \times 10^{12}$ mutations per tumour consisting of $1 \times 10^9$ cells (Bielas et al., 2006:18238). Unrepaired spontaneous mutations may activate oncogenes or repress tumour suppressor genes (Friedberg, 2003:436). Defective mismatch repair mechanisms, through loss of function of MSH2 or MLH1, result in changes in microsatellite DNA (microsatellite instability, MSI) and tumour suppressor genes. A well-known example hereof is hereditary nonpolyposis colon cancer (Friedberg et al., 2006:1118). Defects of the nucleotide excision repair pathway predispose an individual to the development of skin cancer, as can be seen in the hereditary disease: Xeroderma pigmentosum (Friedberg et al., 2006:1118). The predisposition for skin cancer in these patients
comes from the increased mutational burden in the skin cells that are exposed to sunlight (Friedberg, 2003:436).

Loeb et al., hypothesised that a mutator phenotype underlies tumourigenesis (Loeb et al., 1974:2311). A diminished capacity for DNA repair may result in relaxed genomic stability, (chromosomal, microsatellite and point mutation instability), which may result in a mutator phenotype. This may cause random mutations in the genome, some of which may occur in genes that influence the ability of cancer cells to proliferate, invade and metastasise (Loeb, 1994:5059).

2.4 Summary

A defect of the tyrosine degradation enzyme, fumarylacetoacetate hydrolase, results in the inborn error of metabolism, hereditary tyrosinemia type 1 (Mitchell et al., 2001:1777, Roth, 2009). Consequently, upstream metabolites such as fumarylacetoacetate (FAA), succinylacetone (SA) and p-hydroxyphenylpyruvic acid (pHPPA) accumulate (Holme, 2003:141, Sniderman King et al., 2008).

Several studies have shown that these metabolites are detrimental to cell homeostasis in a variety of ways. Both in vitro and in vivo studies have contributed to the elucidation of these detrimental effects. For in vivo studies, several models of HT1 was developed, however, none of these models are human genome based (Aponte et al., 2001:641, Endo et al., 1997:24426, Fernandez-Canon and Penalva, 1995:9132, Grompe et al., 1993:2298, Manning et al., 1999:11928).

Two of the in vitro studies have reported that the detrimental effects of SA and pHPPA, pertains to impairment of DNA repair capacity. In one report it was shown that SA inhibits T4 DNA ligase, causing a slow rejoining of Okazaki fragments (Prieto-Alamo and Laval, 1998:12614), and in the other it was only suggested that pHPPA may affect DNA repair mechanisms (van Dyk and Pretorius, 2005:815). In an in vivo study with the fah<sup>−/−</sup>hpdt<sup>−/−</sup> mice, Kubo et al speculated that FAA affect mitochondria directly, causing apoptosis in the short term and DNA damage in the long term (Kubo et al., 1998:9552). Until now, it is unclear which of the multitude of DNA repair mechanisms are affected and if the impairment of the DNA repair mechanisms is limited to protein functionality or if expression of repair proteins are affected. A reduced capacity for DNA repair results in instability of the genome i.e. chromosomal, microsatellite and point mutation instability (Loeb, 1994:5059). Yet, reports of genome instability in HT1 are limited to the observation of chromosomal instability (Gilbert-Barness et al., 1990:243, Jorquera and Tanguay, 2001:1741, Zerbini et al., 1992:1111).
Characteristic of HT1 is the high prevalence of hepatocellular carcinoma and the development of a liver mosaicism (Demers et al., 2003:1313, Kvittingen et al., 1994:1657, Mitchell et al., 2001:1777, Youssoufian and Pyeritz, 2002:748). Kvittingen and colleagues observed that the liver mosaicism is the result of reversion to wild-type of the HT1 causing mutations (Kvittingen et al., 1994:1657). Although, general consensus suggests that these reversions are true back mutations (Demers et al., 2003:1313, Hirschhorn, 2003:721, Kvittingen et al., 1994:1657, Youssoufian and Pyeritz, 2002:748), the mechanisms underlying the back mutations are unknown. The high frequency of HCC and mosaicism, as well as the observation that both HCC and reversion can co-exist in one patient (Bliksrud et al., 2005:406), suggest that the mechanism underlying the development of HCC and reversion may be causally linked.

It is commonly suggested that cancer develop by way of a mutator phenotype. A mutator phenotype is the result of deficient DNA repair mechanisms (Bielas et al., 2006:18238, Loeb, 1994:5059, Venkatesan et al., 2006:294). Characteristic of a mutator phenotype is relaxed genomic stability, as can be observed by chromosomal instability, microsatellite instability and an increase in random point mutations (point mutation instability) (Charames and Bapat, 2003:589, Klein, 2006:18033, Loeb, 1994:5059). The observation of chromosomal instability in HT1 suggests that a mutator phenotype might be present, however, this has not been investigated before.

The aim of this study was therefore to contribute to the understanding of the molecular mechanisms underlying the development of the HT1-associated HCC and mosaicism. Specifically, the aims of this study were to:

- elucidate whether BER and NER DNA repair mechanisms are affected in HT1, and to what extent, i.e. protein functionality and/or expression of repair genes.
- determine if microsatellite instability is present in HT1.

Achievement of these aims would contribute to the understanding of the mechanisms underlying the development of HCC in HT1, as well as possibly shedding light on the mechanism underlying the reversion to wild-type of HT1 causing mutations.

A two-pronged approach was followed in parallel to achieve the aims of the study. Seeing as no human genome based model of HT1 is available, the first of the approaches was to develop a hepatic cellular model of HT1. This model can then be used to assess the effects on DNA repair mechanisms and genome stability in HT1. The second leg of the parallel approach was to use HT1 models and HT1 patient material, to firstly optimise the techniques, and secondly to gain important information on the effects on DNA repair mechanisms and genome stability in HT1. The parallel approach of the study is given in the diagram below (figure 2-3).
Figure 2-3. Diagram of the two-pronged approach of the study.

1. **HT1 hepatic cell culture model**
   - Design and acquire shRNA oligonucleotides
   - Stably transfect HepG2 cells with pTS-Neo vector
   - Transfect HepG2(TS) cells with pSIREN-RetroQ-Tet-shRNA constructs
   - HT1 hepatic cell culture model
   - Apply methods

2. **HT1 related models and HT1 patient material**
   - HT1 patient DNA and RNA samples
   - fah−/− Mouse DNA
   - HepG2 cells exposed to SA and/or pHPPA
   - Optimise methods
   - BER and NER Comet Assay
   - Gene expression profiles
   - Microsatellite analyses
   - High resolution melt analysis and sequencing

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**CHAPTER 2 – LITERATURE REVIEW**