Hereditary tyrosinaemia type I
Studies on the molecular genetics and DNA repair enzymes

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April, 2012

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1436

ISBN 978-82-8264-399-3

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Akademika publishing.
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Acknowledgements

This thesis has been written at The Institute of Clinical Biochemistry at Rikshospitalet, Oslo. I have worked part-time as researcher and part time as medical doctor at The Department of Medical Biochemistry at Rikshospitalet, except from the first seven months when I worked full time as a researcher at the Institute. This period was financed by the Medinnova Research Fund.

The first part of my thesis was produced between 2002 and 2004 with Professor Eli Anne Kvittingen as my supervisor, who sadly passed away in July 2005. Professor Kvittingen's work in the field of Tyrosinaemia type I was significant. Her scientific contribution was outstanding with pioneering work of a high international standard. I am deeply grateful to her for introducing me to the field of inborn errors of metabolism and for sharing her exciting ideas concerning tyrosinaemia type I with me. From this period I would also like to thank the head of our department Professor Oddvar Stokke for the opportunity to work both as a researcher and as a medical doctor in the field of inborn errors of metabolism. Not least I would like to thank Else Brodkorb for her extensive help with the analytical work in the lab. Without her effort and knowledge this would have been difficult to complete.

Eli Anne Kvittingen
† July 2005

The work in the second part of my thesis was done mainly from 2008 to 2011 with Professor Magnar Bjørås as supervisor. He is an excellent scientist in the field of DNA repair, and I am very grateful for the opportunity to work with and to learn from him, for his enthusiasm and his inspiring ideas. A special thank you to Amund Ellingsen who taught me many procedures and helped me significantly with the analyses in the lab.

I would like to thank Anne Marie Lund, Gro Guldal, Kari Høie and Mari Ytre-Arne for technical assistance, Paul Hoff Backe for advice about protein structures and Alexander Rowe for help with the English language.

I would also like to thank Lars Eikvar head of the Department of Medical Biochemistry for the oppertunity to carry out research. I am especially grateful to my close collagues Lars Mørkrid, Helge Rootwelt, Berit Woldseth and Katja Elgstøen. I thank Lars for his accurate and enthusiastic help with the statistical analyses, my co-supervisors Helge and Berit for our discussions, for their good advice and all of their help with the manuscripts. I thank Berit and Lars for our cooperation regarding patient work, and for giving me time, especially in the last year to complete my thesis. A special thank you to Katja for discussions, encouragement and friendship.

Finally I wish to thank from all my heart my dear Ingrid, my son Erlend, my parents and my siblings for all their support and encouragement over many years.

Oslo, April 2012

Yngve Thomas Bliksrud

Preface

Hereditary tyrosinamemia type I (HT1) is an inborn error of metabolism. It is caused by an enzyme deficiency in the tyrosine catabolism pathway and leads to accumulation of the metabolite fumarylacetoacetate (FAA). In humans FAA is naturally produced in very small amounts in the liver and kidneys throughout life, apparently with no negative consequences. However the accumulation of FAA in HT1 has striking pathological and life threatening effects including an extreme risk of cancer development (hepatocellular carcinoma). A mysterious mosaic pattern of hepatocytes, probably the result of a high local mutation frequency, was revealed in 1994 in a large majority of the HT1 patients. Evidence therefore points to HT1 being strongly associated with DNA instability.

The devastating effect of HT1 metabolites is the starting point for this thesis. We have described DNA alterations in Norwegian HT1 patients, and investigated the possible role of DNA repair systems in the pathogenesis of HT1.

List of publications

I Hereditary Tyrosinaemia type I in Norway: Incidence and 3 novel small deletions in the Fumarylacetoacetase gene

II Tyrosinaemia type I -
de novo mutation in liver tissue suppressing an inborn splicing defect

III Fumarylacetoacetate inhibits the initial step of the base excision repair pathway; implication for the pathogenesis of tyrosinaemia type I

Abbreviations and nomenclature

Aag	alkyladenine DNA glycosylase
Ala-DH	aminolevulinic acid dehydratase
AT	ataxia telangiectasia
BER	base excision repair
BS	Bloom syndrome
DR	direct repair
FAA	fumarylacetoacetate
Fah	fumarylacetoacetate hydrolase, fumarylacetoacetase
GC-MS	gas chromatography–mass spectrometry
GGR	global genome repair
HGMD	Human Gene Mutation Database
HR	homologous recombination
HT1	hereditary tyrosinaemia type I
4HPPD	4-hydroxy phenylpuruvate dioxygenase
MAA	maleylacetoacetate
Mbd4	methyl binding domain protein 4
MMR	mismatch repair
MS	mass spectrometry
Mth1	MutT homolog 1 (8-oxodGTPase)
Myh	MutY homolog DNA glycosylase
Neil1	endonuclease-eight-like DNA glycosylase 1
Neil2	endonuclease-eight-like DNA glycosylase 2
Neil3	endonuclease-eight-like DNA glycosylase 3
NHEJ	non homologous end joining
NIR	nucleotide incision repair
NTBC	2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione
Nth1	endonuclease three like DNA glycosylase 1
Ogg1	8-oxoguanine DNA glycosylase1
ROS	reactive oxygen species
SA	succinylacetone
Smug	single-strand selective monofunctional uracil-DNA glycosylase

TAT	tyrosine aminotransferase
Tdg	thymine-DNA glycosylase
TCR	transcription-coupled repair
Udg	uracil-DNA glycosylase
Ung	uracil-N glycosylase
XP	xeroderma pigmentosum

Introduction

Important years in the history of tyrosinaemia type I

1849 The amino acid tyrosine was purified for the first time (Bopp 1849)

1908 Inborn errors of metabolism were established as a specific group of diseases (Garrod) (Scriver 2008)

1955 The most important enzymes involved in tyrosine degradation were described for the first time. (Knox 1955)

1956 A probable tyrosinaemia type I patient was described for the first time. (BABER 1956)

1977 Succinylacetone was demonstrated in urine from patients with hypertyrosiaemia, and Fah deficiency was deduced as the primary enzyme defect (Lindblad, Lindstedt, and Steen 1977)

1979 Fah deficiency was demonstrated in liver tissue from patients with hypertyrosinaemia (Fallstrøm et al. 1979)

1992 The drug NTBC was introduced into the treatment. (Lindstedt et al. 1992)

1994 The phenomenon of self-induced correction was described. (Kvittingen et al. 1994)

Metabolism of tyrosine

Tyrosine

The amino acid tyrosine exists principally as either L-tyrosine or R-tyrosine. L-tyrosine is the only metabolically active form in mammals, thus the term “tyrosine” is used interchangeably with L-tyrosine. The molecular weight of tyrosine is 181.2 g/mol. The solubility is 2.5mM at 25°C in water which makes it one of the least soluble amino acids in cells and body fluids. In humans free tyrosine is classified as a semi essential amino acid with two sources: either hydrolysis of proteins from the diet and body tissues, or hydroxylation of the essential amino acid phenylalanine. Tyrosine is the starting point for the synthesis of several important substances like catecholamines, thyroid hormones and melanin pigments. Most of the free tyrosine is however, incorporated into proteins or broken down to fumarate and acetoacetate. Tyrosine is thus both a glucogenic and a ketogenic amino acid. (Mitchell et al. 2001) The reference values for tyrosine levels in body fluids from our laboratory (Department of Medical Biochemistry, Oslo University Hospital) are given in Table 1:

Table 1: Normal levels of tyrosine in humans

	Age	Normal Range
Plasma (µM)	< 1 month	30 – 120
	1 month – 6 year	30 – 110
	> 6 years	30 – 100
Cerebrospinal fluid (µM)	Adult	4 – 12
Urine (µmol/mmol creatinine)	< 1 month	6 – 70
	1 month – 1 year	15 – 90
	1 – 6 years	8 – 65
	> 6 years	3 – 40

The degradation of tyrosine

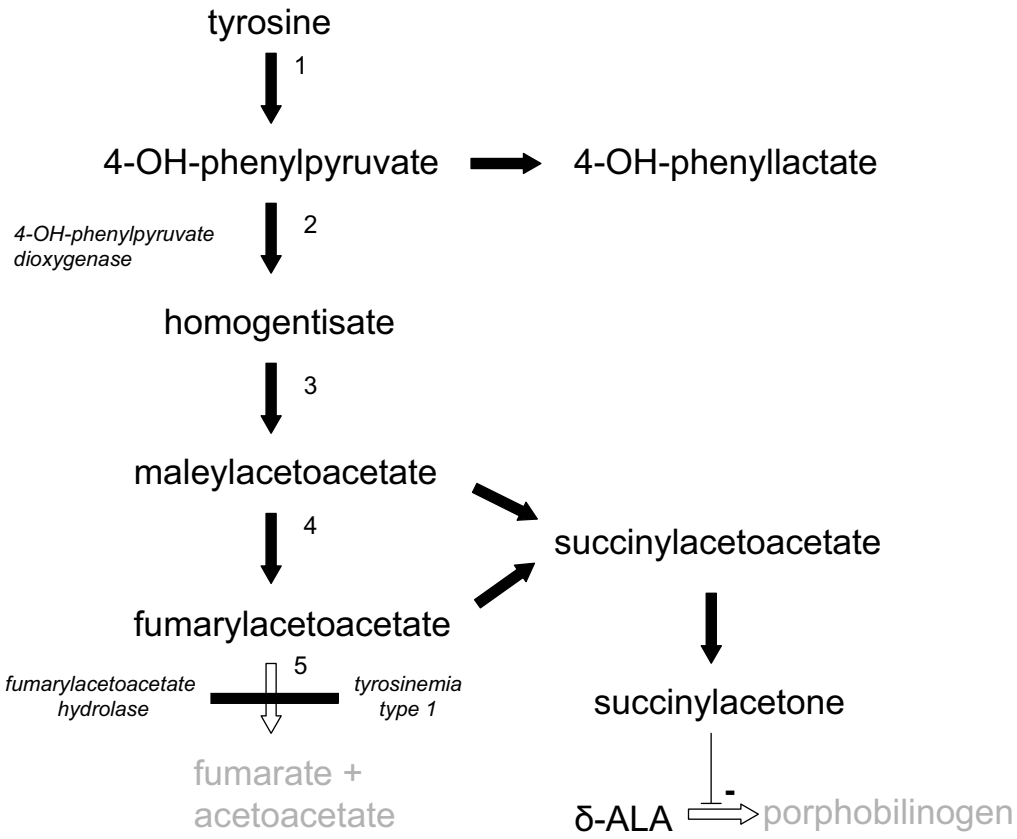

Figure 1 Tyrosine degradation and Fah deficiency. The steps are described in detail in the text below.

The complete degradation of tyrosine (Figure 1) takes place in the cytosol primarily of hepatocytes and to some extent in renal tubular cells *(Greenbeg D.M. 1969;LIN and Knox 1958)*.

Step 1: The formation of p-OH-phenylpyruvate from tyrosine by the enzyme tyrosine amino transferase (TAT) is the rate-limiting step of this catabolic pathway (Coufalik and Monder 1980;Dickson, Marston, and Pogson 1981;Ohisalo, Laskowska-Klita, and Andersson 1982).

Step 2: Homogentisic acid is formed from p-OH-phenylpyruvate through a complex series of reactions which involve decarboxylation, oxidation and migration of the side chain on the benzene ring. The reactions are catalysed by the enzyme 4HPPD. This enzyme is dependent on Cu and vitamin C, and is in humans exclusively expressed in the parenchymal cells of liver and kidney. (Fellman, Fujita, and Roth 1972) The enzyme is inhibited by the metabolite fumarylacetoacetate (FAA), which explains why tyrosine and p-OH-phenyllactate accumulate in the body fluids of HT1-patients. The enzyme is also the target for the enzyme inhibitor NTBC that is used in treatment of HT1.

Step 3: Homogentisic acid is oxidatively cleaved to maleylacetoacetate (MAA) by homogentisate dioxygenase. The enzyme contains reactive sulfhydryl groups and requires Fe++ (mercaptides).

Step 4: MAA is isomerized to FAA by maleylacetoacetate isomerase. This enzyme requires reduced glutathione probably in order to maintain its sulfhydryl groups in reduced form.

Step 5: FAA is cleaved by fumarylacetoacetate hydrolase (fumarylacetoacetase) (Fah) to yield fumarate and acetoacetate. In Fah deficiency FAA and probably MAA accumulate and are then reduced to succinylacetoacetate and further decarboxylated to succinylacetone (SA). SA is a potent inhibitor of δ-aminolevulinic acid–dehydratase in the hemesynthesis, and accumulation of SA leads to accumulation of δ-aminolevulinic acid and possible porphyria like neurological crises.

Fumarylacetoacetate hydrolase

Fumarylacetoacetate hydrolase (fumarylacetoacetase) (Fah) (E.C.3.7.1.2) is a hydrolase with no cofactors. It is a soluble, cytosolic homodimer with a molecular weight of 46,3 kDa per subunit. Fah has been purified from rat, bovine and human liver (Berger et al. 1987;Mahuran et al. 1977;Van, van, I, and Berger 1990), and polyclonal antibodies have been developed (Berger et al. 1987). The structure of murine Fah is experimentally determined (Bateman et al. 2001) There is no solved structure for the human protein, but there is a high degree of homology between the murine and the human protein. 372 of 419 amino acids (89 %) are identical (paper I). Human Fah is mainly expressed in liver and renal tubular cells, is active over a wide pH range and has a Km of 1,3 µM FAA. (Kvittingen, Jellum, and Stokke 1981) In other tissues the activity is 2 - 5 % of the level in liver (Kvittingen, Halvorsen, and Jellum 1983).

Hypertyrosinaemia in general

Non-genetic conditions

Hypertyrosinaemia is seen subsequently to severe hepatocellular dysfunction (Fujinami et al. 1990;Iber et al. 1957) probably due to secondary inhibition of the first steps of tyrosine degradation. Transient hypertyrosinaemia of newborns is a common condition with uncertain clinical consequences (Scriver and Rosenberg 1973). The cause is probably immaturity of 4HPPD. Increased dietary protein load in the newborn period and a relative ascorbate deficiency are also possible explanations for the temporarily increased tyrosine levels (Mitchell et al. 2001).

Genetic diseases

Hepatorenal tyrosinaemia (tyrosinaemia type I) is caused by deficiency of Fah, the last enzyme of tyrosine degradation, and is the subject of this thesis.

Oculocutaneous tyrosinaemia (tyrosinaemia type II) is caused by TAT deficiency, the first enzyme in tyrosine degradation, and is characterized by strongly elevated plasma tyrosine levels. Palmoplantar keratosis and painful corneal erosions are typical symptoms and respond to a protein restricted diet. Approximately 50 % of

patients develop mental retardation that cannot be reversed by dietary control. The mechanisms behind the neurological impairments are not fully understood.

Primary 4HPPD deficiency (tyrosinaemia type III): A few patients with neurological symptoms but no symptoms from liver and kidneys have been described with this deficiency.

Epidemiology

Tyrosinaemia type I is a rare disease that occurs worldwide with varying frequency. The highest incidence is found in the province of Quebec in Canada, especially in the Saguenay-Lac St. Jean region, due to a complex founder effect. The carrier frequency in this area is about 1:20, with one patient per 1846 living births (prior to prenatal diagnosis) (De Braekeleer and Larochelle 1990;Grompe et al. 1995;Laberge 1969). The incidence in the entire Quebec province is one patient per 16786 living births. In the rest of the world, including Scandinavia, the incidence before our studies had been estimated to be 1: 100,000 – 120,000 (Halvorsen 1980). Recently a higher incidence than average was proclaimed in one study from the Middle East but no number was given (Imtiaz et al. 2011).

Genetics

The Fah gene consists of approximately 3,5 kb and is located on chromosome 15 q23-25. The gene contains 14 exons. The open reading frame consists of 1257 nucleotides coding for 419 amino acids. HT1 is an autosomal recessive disease. Prior to this work at least 68 disease causing mutations had been described. 55 of them are registered in the HGMD database, (Stenson et al (2009) 2011) At least two previously described splicing mutations (Q64H G192>T (Rootwelt et al. 1994), and Q279R, A836>G (Kim et al. 2000) (Dreumont et al. 2001)) are not registered in the database. 11 disease causing mutations (9 missense/nonsense, 1 splicing, 1 small deletion) have recently been reported (Imtiaz et al. 2011). This gives the following distribution of mutation types: 44 missense/nonsense, 18 splicing, 4 small deletions, 2 gross deletions, 1 small indel. The most common mutation worldwide is the splicing mutation IVS12+5g>a. Other mutations are typically found in certain areas, like G337S in Scandinavia, W262X in Finland and D233V in Turkey. Prior to this work 6 disease causing mutations had been described in Norwegian patients.

Pathophysiology

Severe hepatitis

HT1 may be considered as a chemical hepatitis. The accumulation of toxic substances leads to cell destruction and compensatory accelerated regeneration of hepatocytes. The inflammation ultimately causes liver cirrhosis, and the reduction in functional liver parenchyma leads to liver failure. Development of hepatocellular carcinoma is common. Enzyme deficiencies upstream of Fah in the tyrosine degradation process do not affect the liver or the kidneys, thus apoptosis, necrosis, cirrhosis and cancer development are attributed to Fah deficiency and the specific HT1 metabolites FAA, MAA and SA (Grompe 2001;Mitchell et al. 2001).

Apoptosis

The flux through the tyrosine degradation pathway, and thus the toxic effect of the accumulating metabolites, can be regulated in Fah deficient mice with the drug NTBC. Endo and coworkers have shown that apoptosis occurs when NTBC treatment is terminated (Endo et al. 1997). The same mouse model was used by Kubo and coworkers to show that FAA triggers the caspase cascade in hepatocytes (leading to apoptotic proteolysis and cell death), and that caspase inhibitors reduce hepatocyte damage in Fah deficient mice (Kubo et al. 1998). In other studies renal tubular cells became apoptotic in Fah deficient mice when NTBC treatment was abolished. (Sun et al. 2000) (Endo and Sun 2002)

Regenerating nodules and carcinoma

Hepatocellular carcinomas occur very frequently in untreated HT1 patients. The mechanism behind the cancer development is not fully understood. FAA, but not SA and MAA, is mutagenic *in vitro* and increases oxidative damage by disrupting the sulfhydryl metabolism and by glutathione depletion (Jorquera and Tanguay 1997). FAA and MAA are alkylating agents and may alkylate functional groups of many cellular proteins like thiols and amines. The alkylating potential is due to the molecular structure (α,β-unsaturated carbonyl compound) of FAA and MAA. This means that these compounds may act as Michael acceptors in the Michael addition reaction. DNA damage caused by uncontrolled alkylation may be cytotoxic and/or mutagenic. Disrupted mitosis may lead to cell death, and disrupted base pairing

during replication may result in a permanently altered nucleotide sequence. Alkylation may furthermore trigger apoptosis, as recently reported (Lim et al. 2012) Inhibition of apoptotic signals may contribute to development of hepatocellular carcinoma in HT1 patients. Increased resistance to cell death has been demonstrated in hepatocytes of $fah^{-/-}$ mice, induced by chronic liver disease (Vogel et al. 2004) and by activation of specific cell survival pathways (Orejuela et al. 2008). SA react with free and protein bound amino acids to form adducts (Manabe, Sassa, and Kappas 1985) (Prieto-Alamo and Laval 1998).

Renal tumour development in HT1 has not been reported despite of catabolism of tyorisne renal tubuli cells (Fellman, Fujita, and Roth 1972;Greenbeg D.M. 1969;Lin and Knox 1958). This is a bit surprising because the risk of cancer development is so high in hepatocytes in HT1. The reason is not fully understood, but a lower rate of tyrosine catabolism and a lower replication rate in renal tubuli cells are probably parts of the explanation.

Hypertyrosinaemia

Blood tyrosine is elevated in HT1 patients because of a secondary inhibition of 4HPPD activity. Although the hepatorenal symptoms are not attributed to tyrosine elevation, neurological development might be affected. Neurological symptoms are associated with both hypertyrosinaemia type I, II and III, and studies on animal models suggest that affected energy metabolism (inhibition of creatine kinase activity) and increased oxidative stress (decreased glutathione concentration) may be two possible mechanisms (de Andrade et al. 2011) (Sgaravatti et al. 2009).

Other pathological changes

Since SA is a potent inhibitor of the porphyrin synthetic enzyme Ala-DH (Figure 1), porphyria-like neurological crises may occur in untreated HT1 patients. In addition, SA is shown to have an immunosuppressive effect that might also contribute to the liver damage seen in HT1 (Tschudy et al. 1982).

The cell autonomous model

The metabolites immediately upstream of Fah (FAA and MAA) are not detectable in plasma and urine. This is probably because they react very rapidly by forming adducts with other molecules, or because they are transformed into

succinylacetoacetate and SA. The direct damage caused by FAA and MAA is therefore likely to be limited to the cells in which they are formed. In contrast, SA is transported out of the cells and may affect other tissues as well. Consequently, the renal Fanconi syndrome in HT1 patients is assumed to be caused by circulating SA (Spencer et al. 1988;Spencer and Roth 1987).

Clinical presentation

HT1 is characterised by clinical heterogeneity. The disease may present from infancy to adulthood and can be categorized as acute, subacute or chronic (Chakrapani A, Gissen, and McKiernan 2011;van Spronsen et al. 1994). The acute form presents before 6 months of age with symptoms of acute liver failure. The subacute form presents between 6 and 12 months of age with hepatosplenomegaly, failure to thrive, coagulopathy, rickets and hypotonia. The chronic form presents after 12 months of age, typically with chronic liver disease, hypophosphatemic rickets and/or porphyria-like neurological crises.

Liver disease

The accumulating toxic metabolites in HT1 lead to liver failure, cirrhosis and/or hepatocellular carcinoma. The most severe form presents a few weeks after birth with dramatic symptoms of acute liver failure like oedema, ascites and bleeding diathesis due to hypoproteinemia. Additionally, non-specific symptoms like vomiting, diarrhoea and hypoglycaemia may be present. Sepsis is common. Severely reduced protein synthesis is a striking finding in HT1. The hepatic excretory functions are not however correspondingly affected. Jaundice is a late sign indicating that the liver may be so damaged that transplantation must be considered despite NTBC treatment.

The chronic form of HT1 leads to liver cirrhosis which in most cases is described as a mixed micromacronodular type with a variable degree of steatosis (Dehner et al. 1989). Hepatocyte dysplasia is common, and the risk of malignant development is considerable. When the patient presents the first symptoms of HT1, cirrhosis or hepatocellular carcinoma may already be present.

Renal dysfunction

Hypophosphatemic rickets is common as a first symptom of the chronic form of HT1 due to dysfunction of the proximal renal tubuli. Nephromegaly may be present at time

of diagnosis (Paradis et al. 1990), and some patients present with Fanconi syndrome: generalised aminoaciduria, glycosuria and renal tubular acidosis. Renal failure has also been reported (Santra et al. 2008).

Neurological crises

Acute porphyria-like neurological crises may occur at any age in patients who are not being treated with NTBC due to the inhibition of heme synthesis by SA (Figure 1). The crises may in severe cases lead to respiratory paralysis and death (Mitchell et al. 1990).

Cardiomyopathy

Hypertrophic cardiomyopathy has been described as common but usually benign in HT1 patients (Arora et al. 2006). However, in one infant myopathy was reported as the cause of death (Lindblad et al. 1987).

Pancreatic dysfunction

Hypertrophy of the endocrine cells of the pancreas has been found in infants with HT1. The symptoms are that of hyperinsulinism with episodes of hypoglycaemia (Baumann et al. 2005).

Diagnosis

HT1 is diagnosed by demonstrating presence of the pathognomonic metabolite SA and/or its precursors in urine (GC-MS). If only small amounts of SA are found, mutational analysis of blood samples should be performed. The demonstration of two known disease causing mutations confirms the diagnosis. Determination of Fah activity in lymphocytes, fibroblasts or liver tissue is not recommended as the only diagnostic measure when HT1 is suspected. The results of such assays must be interpreted with caution. Normal Fah activity in a liver biopsy may be due to sampling from a revertant nodule in a liver that otherwise lacks functionally active Fah. On the other hand, a false negative result may be caused by the pseudodeficiency gene (Rootwelt, Brodtkorb, and Kvittingen 1994) yielding low, but functionally sufficient, activity in all tissues.

Standard biochemical analyses reflect the affected hepatic protein synthesis with elevated INR and reduced albumin. Coagulopathy is an important sign and may

be present without other signs of liver disease. ALAT, GT and bilirubin may be normal or slightly elevated. α-fetoprotein is often grossly elevated at the time of diagnosis in acute HT1, but may be normal in chronic HT1. Urinary excretion of δ-ALA may be increased. Analysis of amino acids in plasma shows elevated tyrosine, phenylalanine and methionine, but to varying degrees (Kvittingen and Holme 2000;Mitchell et al. 2001).

Newborn screening for HT1 has been performed since 1969 in the province of Quebec initially by measuring tyrosine levels in dried blood spots. In 1974 measurement of blood α-fetoprotein was added. Since 1980 newborn screening for HT1 in the province of Ouebec is done by measurement of SA in urine (Scriver 2006). SA is well suited as a newborn screening marker, giving low rates of both false positive and false negative results. SA has virtually eliminated the problems with false positive diagnoses in Quebec (Scriver 2006). The most common screening method today is MS-MS blood spot analysis of SA (Allard et al. 2004)

Treatment

Prior to 1992, a tyrosine- and phenylalanine restricted diet and liver transplantation were the only available treatment options for HT1. Since the drug NTBC (2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione) (nitisinone, Orfadin^{TM}) became available in 1992, the prognosis of HT1 has dramatically improved (Holme and Lindstedt 2000). NTBC is a potent inhibitor of the enzyme 4HPPD, which is upstream of Fah in the tyrosine degradation pathway. Blocking this enzyme prevents production of the toxic metabolites FAA, MAA and SA (Holme and Lindstedt 1998;Lindstedt et al. 1992).

Tyrosine and phenylalanine restriction is necessary to prevent excessive levels of plasma tyrosine that might otherwise lead to acute corneal erosions and possibly cause neurological deffects over time. In addition to a low protein diet, patients are given a protein hydrolysate of amino acids free of tyrosine and phenylalanine to secure a sufficient supply of other amino acids (Mitchell et al. 2001).

Liver transplantation must be performed in cases where the patient does not respond to NTBC or develops hepatocellular carcinoma in spite of adequate NTBC treatment (Holme and Lindstedt 2000).

The Norwegian patients

During the last three decades 30 patients (11 girls 19 boys) have been diagnosed with HT1 in our laboratory at the Department of Medical Biochemistry, Oslo University Hospital (Figure 2), the only laboratory for diagnosis of inborn errors of metabolism in Norway.

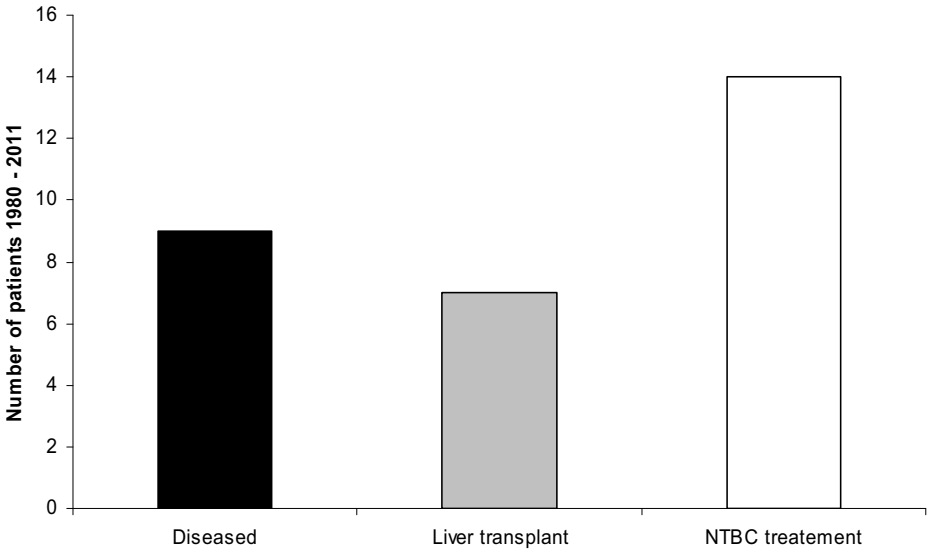

Figure 2 . The Norwegian HT1 patients diagnosed between 1980 and 2011.

29 of these patients are of Norwegian descent, and one patient is an immigrant from another European country. Altogether nine patients have died. Eight of them died before NTBC was available. The ninth died of acute HT1 in the newborn period after receiving only a few doses of NTBC. Of the remaining 21 patients, six were liver transplanted before NTBC was available. One patient, seven years old at the time of diagnosis and start of NTBC treatment, received a liver transplant because of hepatocellular carcinoma after two years on NTBC treatment. The remaining 14 HT1 patients are presently on NTBC treatment.

It has recently been suggested to classifiy HT1 clinically in three groups based on the age at onset of symptoms: before 6 months of age (acute form), between 6 and 12 months of age (subacute form), after 12 months of age (chronical form) (Chakrapani A, Gissen, and McKiernan 2011). This corresponds well, but not

perfectly with the clinical pictures. In a previous report we designated only phenotypes at the extremes of the clinical spectrum as “acute” or “chronic” to reveal an eventual genotype – phenotype correlation (Rootwelt et al. 1996). Thus many patients were categorized as intermediate in the midle category. Only one of our patients is categorized in the midle group based on the age at onset of symptoms.

Of a total of 29 patients of Norwegian descent 18 were diagnosed after 12 months of age. One was diagnosed between 6 and 12 monts of age, and four were diagnosed before 6 months of age. Four patients did not have an acute form of HT1, but further classification is difficult because accurate clinical information is missing. Two asymptomatic patients, one diagnosed at birth and one at seven months of age, cannot be fully clinically classified, because they were found as a result of an already diagnosed sibling and not because of their own symptoms (Figure 8). Altogether, the chronic form of HT1 dominates in Norway with hypophosphatemic rickets as the most common clinical symptom leading to diagnosis.

An expanded newborn screening program which includes HT1 was started in Norway March 1st, 2012. The relatively high proportion of chronic HT1 in the Norwegian population, possibly with milder biochemical findings at birth, may cause an increased risk of false negative screening results. The incidence of HT1 and the disease causing Fah mutations in the Norwegian population are the subjects of publication I.

DNA alterations and the phenomenon of self-induced correction

Different DNA alterations have been demonstrated in tissue from HT1 patients. In cultured skin fibroblasts from one patient with the chronic form of HT1, chromosomal instability with increased chromosomal breakage was demonstrated (Gilbert-Barness, Barness, and Meisner 1990). No chromosomal abnormalities were seen in fibroblasts from a patient with the acute form of HT1.(Wilson et al. 1994) In another study a murine HT1 model demonstrated a spectrum of different DNA alterations in hepatocytes. The DNA alterations were distributed as follows: 20 % point mutations, 30 % small deletions and insertions and, 50 % large DNA alterations (Manning et al. 1999). The high frequency of point mutations was emphasized by Kvittingen and coworkers in 1994 with the striking demonstration of self-induced correction of

disease causing mutations in liver tissue from the majority of HT1 patients (Kvittingen et al. 1994). In liver tissue from 16 of 19 HT1 patients investigated, immunohistochemical staining with an antibody against Fah surprisingly demonstrated a mosaic pattern of immunonegative and immunopositive cells indicating production of Fah protein in clones of cells. Molecular analyses demonstrated a correction of one of the causative point mutations in the immunopositive cell clones. Liver tissue from four of the patients was investigated. One patient was compound heterozygous for the splice site mutation IVS12+5g>a and an unknown mutation. One patient was compound heterozygous for IVS12+5g>a and the nonsense mutation G1069>T (E357X). One patient was homozygous for the combined splice site and missense mutation G1009>A (G337S). One patient was homozygous for the splice site mutation G192>T. IVS12g>a was corrected in the compound heterozygous patients. In one patient four different nodules were investigated, all of them showing the same reversion. Three of the patients showed the splice site reversion **g** (wildtype) >**a** (inborn mutation) >**g** (reversion), one patient showed the reversion CA**G**>CA**T**>CA**G**. Later studies have confirmed the reversion **g**>**a**>**g** and additionally demonstrated the reversion C**A**G>C**G**G>C**A**G (Demers et al. 2003;Dreumont et al. 2001;Poudrier et al. 1998). *In vivo* reversion of inherited mutations back to normal has also later been described in other diseases like adenosine deaminase deficiency (Ariga et al. 2001;Arredondo-Vega et al. 2002;Hirschhorn et al. 1994;Hirschhorn et al. 1996), Fanconi anemia (Gregory, Jr. et al. 2001;Gross et al. 2002;Lo, Jr. et al. 1997;Waisfisz et al. 1999) and epidermiolysis bullosa (Darling et al. 1999;Jonkman et al. 1997;Pasmooij et al. 2007;Schuilenga-Hut et al. 2002).

This phenomenon illustrates a principle of clonal expansion based on a competitive growth advantage. When a genetic alteration, random or otherwise, corrects one of the primary mutations, expression of functional Fah is restored. Consequently, the cell is able to eliminate the toxic metabolites FAA and MAA. The cell is “cured” with respect to HT1 and has gained a tremendous competitive growth advantage compared to the neighbouring dysfunctional cells. This also supports the idea of cell autonomy in HT1. FAA and MAA, reacting with other molecules before they are able to leak out of the cell, will have little effect on the “cured” immunopositive cell clone. And vice versa, the restored function in the reverted nodules cannot alleviate the dysfunction in the remaining immunonegative cells.

Investigations in a murine HT1 model receiving gene therapy have confirmed and elucidated this hypothesis. Transplanted wildtype hepatocytes showed a strong competitive growth advantage and were able to fully repopulate the livers of $fah^{-/-}$ mice (Overturf et al. 1996). The reversion phenomenon has been understood as a result of arbitrary events in the rapidly replicating cells. Thus, HT1 and the other conditions mentioned above, all affect tissues with sufficiently high cell turnover to allow for selection of reverted cells to occur. Other explanations of the reversion phenomenon seen in HT1 patients could be possible, but seem more unlikely. Homologous recombination is excluded at least in the homozygous patients where mutation correction has been demonstrated. An early embryonic mutation in a HT1 patient could give a mosaic pattern of hepatocytes, but the high incidence of self-induced corrections among the HT1 patients makes it unlikely. Repopulation by maternal cells after transplacental transport would be an interesting explanation, and could explain the high incidence and early occurrence of mutation correction in HT1 patients. But presence of the paternal mutation in the immunopositive nodules, as we have seen in 50 % of the cases in our laboratory, excludes this as a predominant explanation of the phenomenon. Furthermore, no evidence of maternal cell colonization was seen in four homozygous HT1 patients in one study (Bergeron et al. 2004).

Another explanation could be a new, second site mutation in the Fah gene stabilizing the transcript or protein sufficiently to provide immunoreactive material and enough enzyme activity to give the corrected cell a selective growth advantage. This is investigated in publication II.

The pattern of corrected nucleotides in the hepatocytes from HT1 patients could reflect mutation liability as a sequence dependent phenomenon (Seeberg and Fuchs 1990). This is investigated in publication III.

DNA damage

DNA in living cells is continuously exposed to damaging events like hydrolysis, oxidation and methylation caused by the normal metabolism. Figure 3 shows three examples of DNA damaging events.

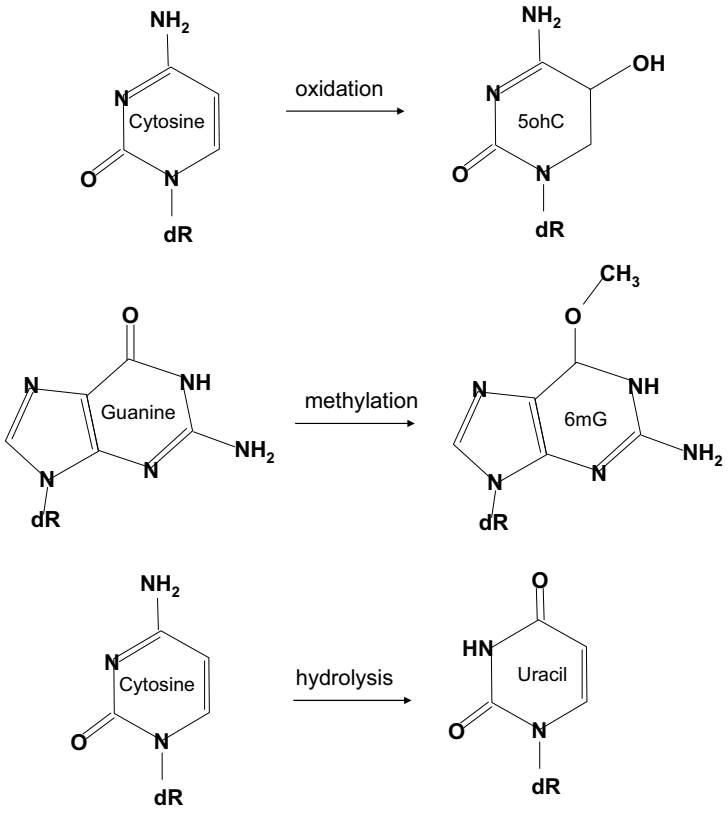

Figure 3 shows examples of spontaneous DNA damage through oxidation, methylation and hydrolysis producing 5-hydroxycytosine, 6-methylguanine and uracil, respectively.

In a single human cell under normal physiology 50.000 -100.000 DNA lesions occur each day (Friedberg EC 2006b). Table 2 gives an overview over the endogenous sources, frequencies and types of DNA lesions.

Table 2. Numbers of endogenous DNA lesions arising and repaired in mammalian cells in 24 hours (adapted from. (Friedberg EC 2006b)).

Endogenous source	Lesions pr day
Single strand breaks	50,000
Hydrolysis	
Depurination	18,000
Depyrimidination	600
Cytosine deamination	500
5-mC deamination	50
Oxidation	
8oxoG	1,000 -2,000
Ring saturated pyrimidines	2,000
Lipid peroxidation products	1,000
Nonenzymatic methylation by S-adenolsylmethionine	
7-mG	6,000
3-mA	1,200
1-mA, 3mC	200
Nonenzymatic methylation by nitrosated polyamines and peptides	
6-mG	20 - 100

The most quantitatively important damage forms are single strand breaks and depurination by hydrolysis. Hydrolysis can also result in depyrimidination and deamination. Other damaging metabolic events are oxidation, nonenzymatic methylation and replication errors. Reactive oxygen species (ROS) may damage the DNA molecule in different ways introducing strand breaks, abasic sites or modified DNA bases either in the DNA strand or those existing as free nucleotides. More than 80 different aberrant bases produced by ROS have been described (Bjelland and Seeberg 2003). Oxidation of guanine in the 8-position yields the mutagenic lesion 8oxoG, which is one of the most abundant oxidative DNA base alterations. Guanine is prone to oxidation due to its low oxidation potential. 8oxoG pairs with both adenine and cytosine and may therefore lead to the transversion GC > AT. The content of 8oxoG in purified samples of genomic DNA is detectable with HPLC with electrochemical detection and is frequently used as an oxidative stress marker. Thymine glycol and cytosine glycol are important pyrimidine lesions. The latter is in equilibrium with 5-hydroxycytosine (5ohC) which is the dehydrated form of cytosine glycol. Thymine glycol and 5ohC are cytotoxic and mutagenic, respectively. The ring

opening of purines is another important form of ROS induced damage and yields formamidopyrimidine (faPy). The faPy lesion may block the process of DNA polymerases. Recognition and initiation of catalysis of different oxidative DNA bases lesions is reviewed in (Dalhus et al. 2009).

DNA damage is also induced by exogenous sources such as UV-radiation, and alkylating- and oxidizing-agents.

DNA repair

To maintain DNA integrity all lesions in the DNA molecule have to be repaired. In Table 3 the sources of DNA base damages are listed with the most common corresponding lesions, major repair mechanisms and prototypical repair enzymes in human cells.

Table 3. DNA damaging agents, lesions, repair pathways and prototypical human repair enzymes (adapted from (Dalhus et al. 2009)) .

Damaging agent	Prototypical lesions	Major repair mechanism	Prototypical human repair enzymes
Alkylating agents	6-mG	DR	Tranferases: Agt
	1-mA	DR	Oxidoreductases: Abh2
	3-mA, 3mG, 7-mA, 7-mG	BER	Glycosylases: Aag
Hydrolysis	Abasic sites	BER	Endonucleases: Ape1
	Deamination forming uracil	BER	Glycosylases: Ung
	Deamination forming hypoxanthine	NIR	Endonucleases: EndoV
ROS	8-oxoG. faPyA/G, TG, 5-ohC, DHU, DHT	BER	Gylcosylases: Ogg1, Nth1
	DHU,DHT, 5-ohC	NIR	Endonucleases: Ape1
Replication errors	(a) Base mismatches (b) Insertion/deletion loops	MMR	Mismatch proteins: MutSα/β
UV radiation	Bulky adducts	NER	Xpa –Xpf + others
	CPDs, 6-4PDs	DR	Photolyases

Cells have several strategies to overcome the problems of DNA damage and its potentially fatal effect on transcription and replication. Different types of DNA damages require different repair pathways. Replication errors are repaired by the mismatch repair pathway (MMR) (Harfe and Jinks-Robertson 2000). The lethal double DNA strand breaks are repaired either by non homologous end joining

(NHEJ) or homologous recombination (O'Driscoll and Jeggo 2006). Five DNA polymerases in human cells by-pass different DNA lesions to ensure that the cell survives, but at the cost of a higher error frequency (Lehmann 2006) The complete repair of a DNA lesion is an alternative and vital strategy to maintain DNA integrity. Repair of DNA lesions was initially described in the bacterium *E.coli,* and many repair mechanisms are conserved from bacteria to man. Several mechanisms of direct repair and excision repair are well characterized in many species. Direct repair removes the lesion directly in one reaction without removing any part of the DNA molecule. As an example the aberrant methyl group in position six on O^6-methylguanine (6-mG) is removed by the specific enzyme 6-mG DNA-transferase (Figure 5). In base excision repair (BER) a part of the DNA molecule that surrounds the damage, from one to several bases, is removed, and the DNA strand is subsequently reconstructed. This process has several steps, requires different enzymes and can repair a broad spectrum of different DNA base lesions. The BER pathway removes one to ~10 bases (Liu et al. 2007). The nucleotide excision repair (NER) pathway is responsible for the repair of larger distortions in the helical structure of DNA, removing 20-30 bp at the lesion (Fleck and Nielsen 2004). NER is divided into global genome repair (GGR) and transcription-coupled repair (TCR). TCR maintains actively transcribed genes by excising DNA damage that blocks the RNA polymerase. GGR does not differentiate between transcribed and silenced genes.

Base excision repair

BER is considered to be the main pathway for repair of DNA base lesions in the cells. It consists of a complex series of reactions which takes place continuously. The base lesions require different DNA glycosylases to initiate the BER pathway. Several DNA glycosylases display overlapping substrate affinity ensuring less vulnerability to inherited enzyme deficiencies and emphasize the importance of the BER pathway.

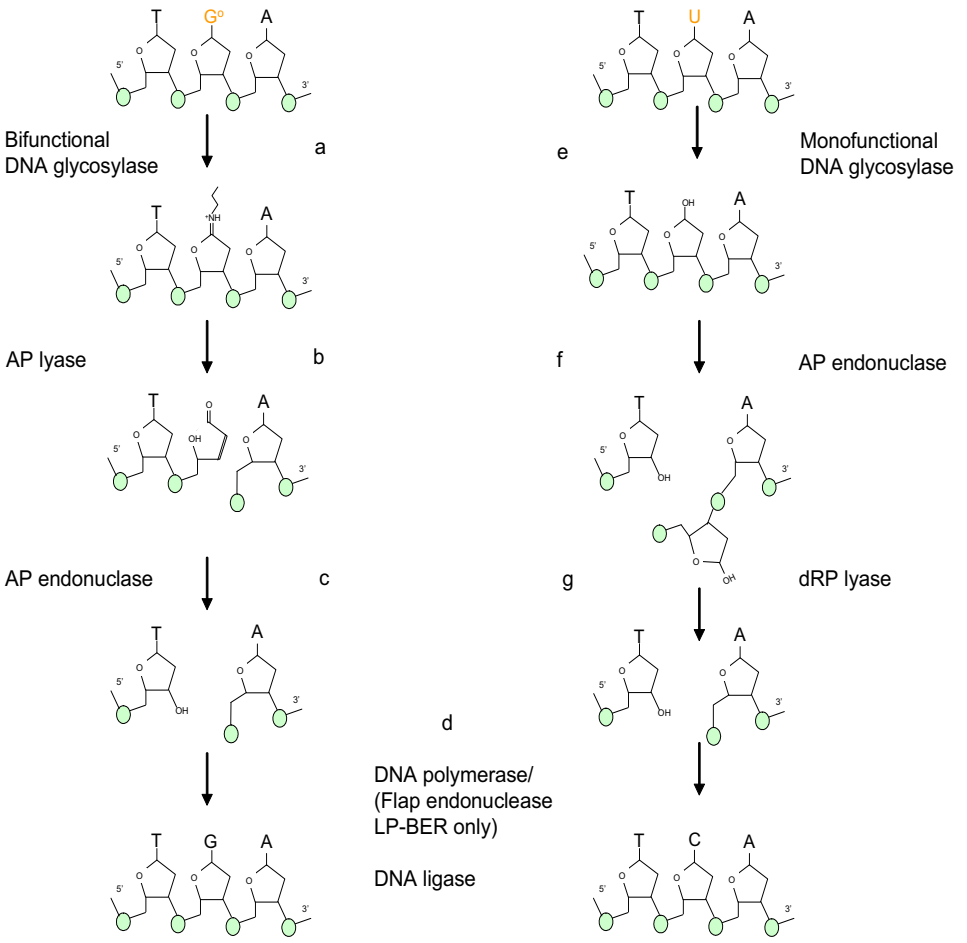

Figure 4 The BER pathway (adapted from (Dalhus et al. 2009)) (LP: long patch) The steps are described in detail in the text below.

The BER pathway (Figure 4) is initiated by hydrolysis of the glycosylic bond between the base and the sugar (a and e). The bifunctional DNA glycosylase substitutes the damaged base with an activated amine moiety. The intrinsic AP lyase activity cleaves the DNA backbone 3′ of the lesion (b) and the remaining unsaturated fragment is a substrate for AP endonucleases (c). The monofunctional DNA glycosylase creates an abasic site with an activated water molecule (e), which is a substrate for AP endonucleases (f). 2′-deoxyribose-5′-phosphate (dRP), the remaining 5′-sugar

fragment, is removed by dRP lyase activities (g). The resulting gap is then filled (d) either with a single nucleotide (short patch repair) or by 2 or more nucleotides (long patch repair). The remaining nick is sealed by DNA ligase activity.

In mammalian cells there are 11 known glycosylases (Barnes and Lindahl 2004), which are classified according to enzyme activity as monofunctional or bifunctional. Alternatively, glycosylases can be divided into four distinct groups based on the three dimensional folds/motifs: (1) helix-hairpin-helix motif (HhH) (2) helix-2turn-helix motif (H2TH) (3) Udg fold and (4) Aag fold (Friedberg EC 2006a). Table 4 shows an overview of the human glycosylases and their major substrates.

Table 4, The 11 human Glycosyleses and their major substrates (from (Barnes and Lindahl 2004).

Protein	Major or significant substrates
Ung	Uracil
Smug	Uracil, 5-oh-meU
Tdg	Thymine, U:G, ethenocytosin:G
Mbd4	Thymine:G, U:G, Thymine:6-mG
Myh	8oxoG:A, 2-ohA:G
Ogg1	8oxoG:C
Nth1	Thymine glycol, Dihydrouracil, faPyG
Neil1	As Nth1, faPyA, 5S, 6R TG isomer, 8oxoG
Neil2	Overlap with Nth1/Neil1
Neil3	oxidation products of 8ohG
Aag	3mA, hypoxanthine, ethenoadenine

Ogg1: Ogg1 is the major DNA glycosylase for the removal of 8oxoG:C lesions in eukaryotic cells (Bjoras et al. 1997;van der Kemp et al. 1996). In humans there are two different forms of the protein located either in the cell nucleus or in the mitochondria (Nishioka et al. 1999). Ogg1 belongs to the HhH super family of glycosylases. The crystal structures of the apoenzyme and the enzyme in complex with DNA have been determined (Bjoras et al. 2002;Bruner, Norman, and Verdine 2000) Ogg1 is a bifunctional enzyme with the potential to remove the base and cleave off the sugar phosphate backbone at an AP site. However, it appears that Ogg1 primarily acts as a monofunctional DNA glycosylase (Morland et al. 2005). After cleavage of the N-glycosylic bond the 8oxoG residue remains for a short while in the recognition pocket and acts as a catalytic cofactor in the second reaction

(Fromme et al. 2003). *ogg1* deficient mice have no distinct phenotype. The level of 8oxoG is higher in hepatocytes from *ogg1*$^{-/-}$ than in cells from wildtype mice and increases continuously to become 3 -10 fold higher than in wildtype (Klungland et al. 1999). The spontaneous mutation rate, despite the 8oxoG accumulation is only increased three fold in *ogg1*$^{-/-}$ mice (Arai et al. 2003), and the 8oxoG lesions are slowly removed from *ogg1*$^{-/-}$ cells indicating overlapping enzyme activities (Osterod et al. 2001). *ogg1*$^{-/-}$ mice do not show increased tumour development, and the same is the case for *myh*$^{-/-}$ mice, but double knockout mice *ogg1*$^{-/-}$ *myh* $^{-/-}$ are significantly predisposed to tumours particularly of the lung, ovarian, lymphoma varieties. (Xie et al. 2004).

The “GO-system”: The cell has developed a complex defence against the mutagenic effect of 8oxoG (Figure 6). In addition to Ogg1, two other enzymes contribute to the detection and removal of 8oxoG/8oxodGTP. Together this defence cooperation is called “The GO-system” (The 8oxoG lesion is also called “GO”) (Fowler et al. 2003;Michaels and Miller 1992). If Ogg1 misses an 8oxoG:C lesion Myh gives Ogg1 a second chance. Myh recognises the mispaired 8oxoG:A (the mutagenic consequence of 8oxoG:C) and removes the adenine and incorporates a C opposite 8oxoG which is further processed in the Ogg1 repair pathway (Slupska et al. 1996;Takao et al. 1999). Mth1 contributes to DNA integrity indirectly by “cleaning” the pool of free nucleotides before they are incorporated into the DNA strand. Mth1 hydrolyses 8oxo-dGTP to 8oxo-dGMP which can not be incorporated into DNA (Nakabeppu et al. 2006).

Nth1, Neil1 and Neil2 are glycosylases in mammalian cells that remove oxidized and fragmented pyrimidine residues. Endonuclease III (*Nth*) was first described in *E.coli* as an endonuclease able to create nicks in DNA from cells exposed to DNA damaging treatment (Radman 1976;Strniste and Wallace 1975). Later Nth was described as a bifunctional protein with both glycosylase and AP lyase activities (Cunningham and Weiss 1985;Katcher and Wallace 1983). Spontaneous mutation rates increase only moderately in *Nth*$^{-/-}$ cells, which can be explained by the presence protein endonuclease eight (*Nei*) which acts as a backup DNA repair system (Saito et al. 1997).

E.coli cells lacking *Nei* do not demonstrate any phenotype. Double mutants lacking both *Nth* and *Nei* are hypersensitive to gamma-radiation (Jiang et al. 1997;Saito et al. 1997). There is a parallel situation in human cells. Nth1 in human

cells is the ortholog to *Nth* and acts on many substrates (Table 4), and Nth1 knockout mice have no clinical phenotype (Takao et al. 1999). Endonuclease VIII like proteins (Neil1, Neil2 and Neil3) act as DNA repair systems (Bandaru et al. 2002;Hazra et al. 2002a;Hazra et al. 2002b;Rosenquist et al. 2003;Takao et al. 2002). The substrate specificity of Neil1 and Neil2 are overlaps with Nth1 but also repairs some additional DNA lesions (Table 4). A *neil1* knockout mouse model has shown a metabolic phenotype with variable pentetrance (Sampath et al. 2011), a *neil3* knockout mouse model has shown profound neuropathology (Sejersted et al. 2011), neither of them have demonstrated tumour development. A *neil2* knockout mouse model has not been described so far. A double knockout $nth1^{-/-}neil1^{-/-}$ mouse model developed pulmonary and hepatocellular tumours with much higher incidence than either of the single knockout, $nth1^{-/-}$ and $neil1^{-/-}$ (Chan et al. 2009).

Udg: Uracil-DNA-glycosylases (Udg) removes uracil from DNA and was first described by Lindahl (1974) in *E.coli*. *Udgs* occur in most species and can be divided into eight different families based on the precise type of uracil damage (substrate) they remove (Friedberg EC 2006a). Of these eight Udg families four are found in mammalian cells: Ung, Smug1. Mbd4 and Tdg (Table 4). Structural studies show the same conserved core in the different Udg families reflecting common catalytic properties. The differences in the flanking parts of the proteins reflect the substrate specificity (uracil, 5-OH-methyluracil, uracil-mismatches etc.). Ung removes uracil from both single stranded and double stranded DNA. It occurs in two distinct forms from alternatively spliced mRNA from the same gene: Ung1 is sited in the mitochondria and Ung2 in the cell nucleus (Krokan et al. 2001). The major function of Ung in mammalian cells is to remove uracil when it is accidentally paired with adenine. The repair capacity is huge compared with other known glycosylases. In *E.coli Ung* catalyses the removal of 800 uracil residues from DNA per minute and there are ~300 *Ung* molecules present per cell (Lindahl et al. 1977). In contrast to many other glycosylases mouse models *ung* knockout mice demonstrate a specific pathological phenotype by developing B-cell lymphomas (Nilsen et al. 2003). Both the high repair turnover number and the fatal consequence of *ung* knockout emphasise the importance of uracil repair.

Direct DNA repair

Alkylating damage in DNA is both mutagenic and cytotoxic. It may be repaired by alkyltransferases. As previously mentioned the specific lesion is removed directly in one reaction without removing any part of the DNA molecule.

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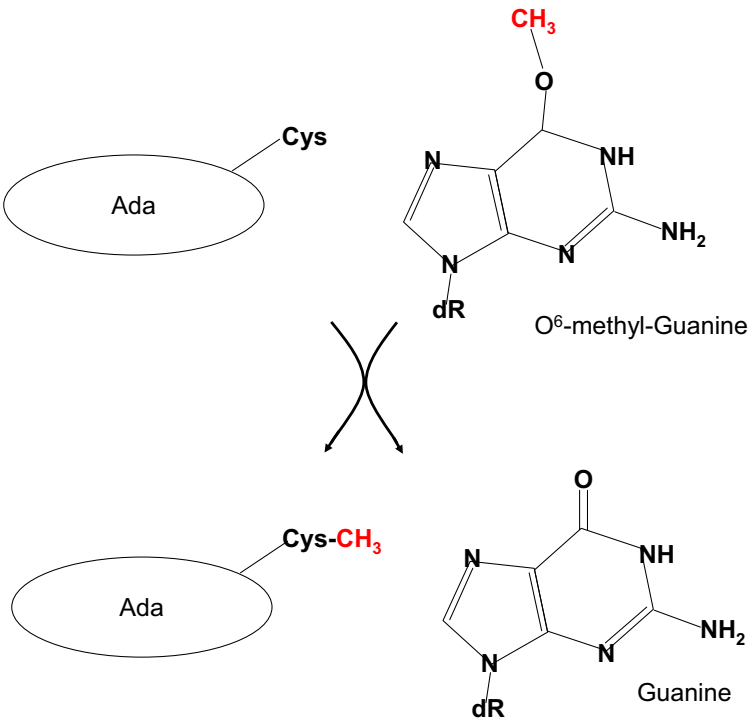

Fig 5. The direct repair of O6methylguanine by O^6-DNA-methyltransferase. (Ada: adaptive response protein)

The first 6-mG DNA methyltransferase was described in *E.coli* cells. Two different genes (*ada, ogt*) encode 6-mG DNA methyltransferases in *E.coli*. Similar enzymes are characterised in many species with a high degree of homology. 6-mG DNA methyltransferase can be characterised as a “suicide enzyme” because the protein is inactivated after the transfer of the methyl group. This direct reversal of DNA damage is a genetically efficient process; a single gene product is sufficient to remove the

damage. However the process is energetically very expensive because an entire protein is expended for each lesion.

The lesions 3-methylcytosine and 1-methyladenine are repaired by oxidative demethylation catalysed by AlkB proteins. The reaction is dependent on O_2, Fe^{++} and α-keto-glutarate and yields succinate and CO_2 (Begley and Samson 2003;Falnes 2004;Falnes, Johansen, and Seeberg 2002). In humans the ABH1 gene was identified and cloned in 1996 (Aas et al. 2003). Two other human homologues were identified in 2002 (Duncan et al. 2002).

Spontaneous mutations

Impaired DNA repair predisposes for introduction of permanent mutations (Friedberg EC 2006a). DNA lesions can lead to base mispairing and a permanently altered nucleotide sequence. This is of particulary interest in respect to HT1 because of the high frequency of true reversions (see defention in the dscussion). The highly mutagenic lesion 8oxoG is exemplified in Figure 6.

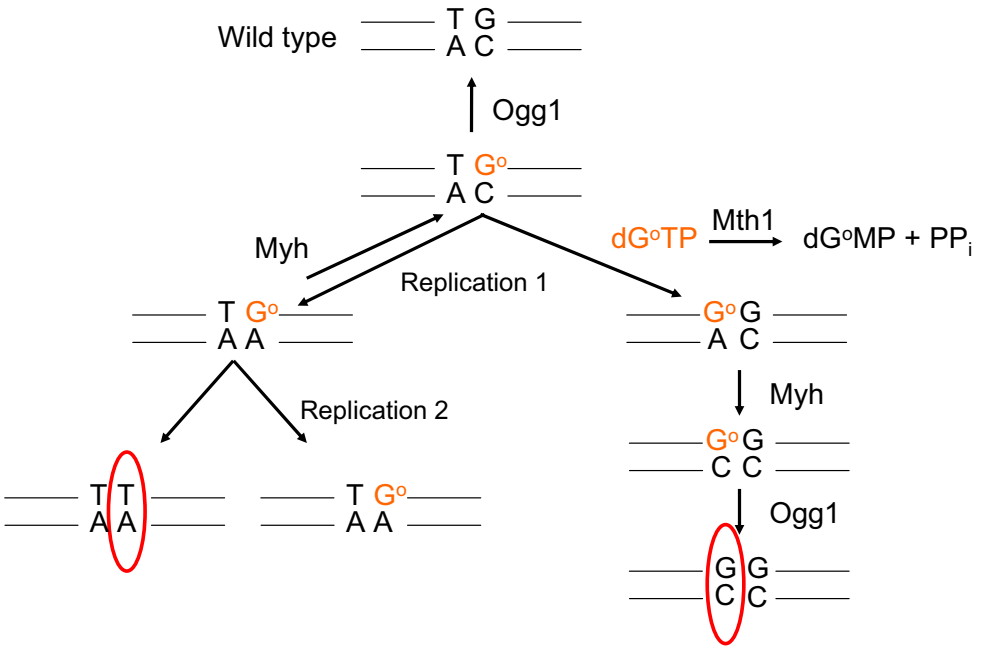

Figure 6. Repair of the 8oxoG lesion. The oxidative lesion 8oxoG can lead to base mispairing and a permanently altered nucleotide sequence in two different ways.

1. 8oxoG is a substrate for several DNA glycosylases, of which Ogg1 is the main enzyme. If such a lesion is not repaired 8oxoG can basepair with adenine instead of cytosine during replication. The 8oxoG:A pair may still be repaired in two steps by Myh which removes adenine and subsequently 8oxoG by Ogg1 or another glycosylase. If 8oxoG:A is not repaired before a second round of replication it will result in a permanently altered nucleotide sequence in one of the daughter cells. T:A is a normal base pair and "invisible" to the DNA repair systems, thus a permanent mutation (G:C > A:T) is established (red circle on the left).

2. Nucleotides can also be damaged prior to incorporation in the DNA strand. The repair enzyme Mth1 "cleans" the pool of free nucleotides by converting 8oxoGTP to 8oxoGMP. If free 8oxoG is incorporated by a DNA polymerase it will base pair with adenine instead of cytosine. The enzymes Myh and Ogg1 will subsequently "repair" the oxidative lesion and thereby produce the point mutation A:T > G:C (red circle on the right).

Human Disease and Deficient DNA repair

Several inborn syndromes are linked to impairments of different parts of the DNA repair system. Increased vulnerability to DNA damaging agents is a typical consequence. Tumour development and premature aging are typical of these diseases. Deficient DNA repair is suggested to be a central part of the aging process in general. Impaired DNA repair also plays a role in non-mitotic tissue. Alzheimers disease is an example of a neurodegenerative condition in which impaired BER may be a part of the pathophysiology.

Some diseases affect the **NER system**. Xeroderma pigmentosum (XP) is one of the most studied diseases. This is a rare autosomal recessive disease characterized by dermatological symptoms like photosensitivity, actinic keratoses and early cutaneous tumour development in sun-exposed areas of the body. Different ocular and neurological symptoms are also frequently seen. XP is genetically and phenotypically complex and involves at least seven genes which affect the NER system. The cells become vulnerable to DNA damaging agents resulting in increased mutagenicity and cell death. Onset of symptoms occurs in early childhood, and the therapy is protection against sunlight (Bootsma et al. 2001). Defective repair of **DNA strand breaks**: Ataxia telangiectasia (AT) is a rare autosomal recessive disease with an incidence of 1 in 40.000 live births. It is characterized by cerebellar ataxia, progressive mental retardation, small blood vessel dilation, immune dysfunction and neoplasms of the lymphoreticular system. The cells of AT patients are extremely sensitive to ionizing radiation, but not to UV radiation or DNA damaging agents that require NER. The patients have mutations in the ATM gene (ataxia telangiectasia mutated) which codes for a large 350 kDa protein consisting of 3056 amino acids. The protein is located mainly in the nucleus and is recruited to double-strand breaks and influences cell division (Chun and Gatti 2004). Defective **DNA helicase**: Bloom syndrome (BS) is a rare disease of autosomal recessive inheritance. It is characterized by a general tendency towards tumour development, thus the cancers may derive from many different tissues. The tumour types include leukaemia, lymphoma, Wilms tumour, medulloblastoma, osteogenic sarcoma, breast cancer, lung cancer etc (Willis and Lindahl 1987). **DNA replication**: Hereditary non polyposis colon cancer (Lynch syndrome) is typically characterised by impaired mismatch

repair and microsatelite instability, i.e. instability of simple repeated sequences. Slippage in the simple repeated sequences of the DNA-strand during replication leads to replicates of different lengths. In Lynch syndrome these replication errors are not effectively repaired. The patients have increased risk for many different cancer types, but especially colorectal cancer (Fishel et al. 1993). Four genes are involved, and the inheritance is autosomal dominant with varying penetrance.

Several studies show increased amount of oxidative DNA lesions in both neurons and lymphocytes of patients with **Alzheimers disease** (Reviewed in (Lovell and Markesbery 2007)). The increase is significant in both early and late stages of the disease suggesting that DNA oxidation is an early event in the disease progression. Other studies show a reduced capacity for DNA repair in a variety of lesions suggesting that impaired DNA repair contributes to the neurodegeneration observed in Alzeheimers disease.

Impaired DNA repair in HT1, a hypothesis

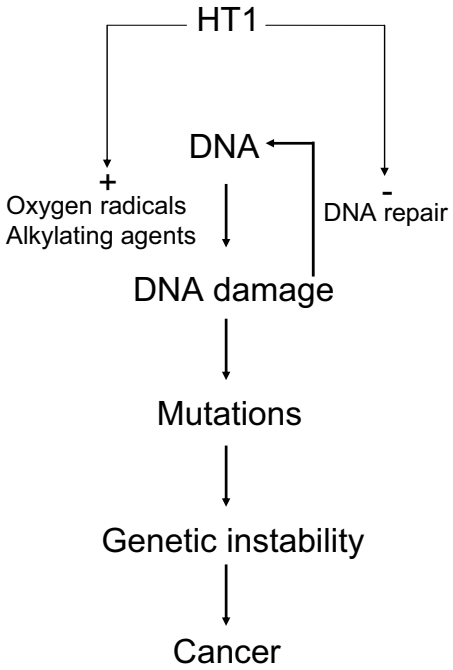

Figure 7. DNA instability in hepatocytes of HT1 patients. DNA is damaged by increased oxidative stress, the presence of alkylating agents and impaired DNA repair.

Figure 7 shows how accumulation of DNA damage can lead to increased mutation rates and cancer development in hepatocytes in HT1 patients. In general accumulation of DNA damage can result in cancer development through increased genetic instability and activation of oncogenes and inactivation of tumor suppressor genes. In HT1 patients the accumulation of oxidative DNA damage is possibly increased due to decreased levels of glutathione (Jorquera and Tanguay 1997). Reduced DNA repair capacity will accelerate this process. Our results in paper III suggest that inhibition of the BER pathway by FAA can lead to cancer development in HT1 hepatocytes.

Aims of the present study

The purpose of this thesis is to increase the understanding of genetical mosaicism and the frequent development of hepatocellular carcinoma in HT1 patients by:

- describing the molecular basis of HT1 in Norwegian patients.
- investigating DNA instability in HT1.
- describing the pattern of self-induced corrections in Norwegian HT1 patients
- describing of DNA damage in liver tissue from HT1 patients.
- searching for alternative explanations for the reversion phenomenon.
- measuring glycosylase activity under the influence of HT1 metabolites.
- measuring metyltransferase activity under the influence of HT1 metabolites.

Summary of published results

Paper I

28 Norwegians have been diagnosed with hereditary tyrosiaemia type I (HT1) over the last 30 years. In this study, 19 of these patients who had not previously been genetically characterized, are investigated. Three novel small deletions were found (NM_000137.1(FAH): c.615delT, p.Phe205LeufsX2, NM_000137.1(FAH): c.744delG, p.Pro249HisfsX55 and NM_000137.1(FAH):c835delC pGln279ArgfsX25), all of them leading to a change in the reading frame and a premature stop codon. 51 of the 56 disease causing alleles are hereby genetically characterized, identifying 9 different disease causing mutations in the Norwegian population. 65 % of the Norwegian HT1 patients are compound heterozygous for different mutations. Thus, the relatively high incidence of HT1 in Norway of 1 in 74,800 live births is not due to single founder effects or high incidence of parental consanguinity.

Paper II

Many patients with HT1 have a mosaic pattern of Fah immunopositive and negative nodules in liver tissue. This phenomenon has been explained by a spontaneous reversion of the mutation in one allele to a normal genotype, but only a few nodules have been examined. In this study we report a Norwegian patient, compound heterozygous for the mutations IVS12g^{+5}→a and G^{1009}→A, with liver mosaicism, but with an immunopositive nodule in which both primary mutations were intact. In the immunopositive hepatocytes of this nodule genetic analyses showed a new mutation C^{1061}→A 6 bp upstream of the primary mutation IVS12g^{+5}→a in the FAH gene. The splicing defect caused by the primary mutation is most likely suppressed by the new mutation due to improvement of the splicing site. In the same liver we demonstrate another nodule of regenerating immunopositive tissue due to reversion of one of the primary mutations to a normal genotype. Together with the original cells this results in a triple mosaicism of hepatocytes with 1, 2 or 3 point mutations in the FAH gene.

Paper III

Several reports suggest that intracellular accumulation of intermediates of tyrosine catabolism such as fumarylacetoacetate (FAA) and succinylacetone (SA) is an important cause of the liver and kidney pathology in HT1 patients. In this work we examined the effect of FAA and SA on DNA glycosylases initiating base excision repair, which is the most important pathway for removing mutagenic DNA base lesions. *In vitro* assays monitoring enzymatic activity of three different human DNA glycosylases, Ogg1, Neil1 and Neil2 demonstrated that FAA, but not SA, strongly inhibited base removal. These DNA glycosylases initiate excision of a broad range of mutagenic base lesions. Thus, it appears that inhibition of DNA glycosylases by FAA in HT1 patients increases mutagenesis and may contribute to hepatocellular carcinoma and somatic mosaicism.

Discussion

The incidence of HT1 in Norway

Norway is one of the areas in the world with an above average incidence of HT1. The incidence is by far the highest in the Quebec region of Canada with 1 patient in 16.786 living births. The incidence in Scandinavia is stated to be 1 patient in 100.000 living births in one of the most cited textbooks of the field of inborn errors of metabolism (The metabolic & Molecular Bases of Inherited Disease, online version 2009). The reference in this book for the Scandinavian incidence is however a chapter by S. Halvorsen in another textbook from as early as 1980 (Halvorsen 1980) The estimate is made only a few years after the first descriptions of the condition Fah deficiency, when the diagnostics were under development and before the treatment with NTBC was established. No recalculations of this number have been published in scientific papers, but Eli Anne Kvittingen suggested a higher number, 1 patient in 50.000 newborns, in her thesis from 1986. This estimate was also based on only a few years of HT1 diagnostics in Norway. After another 25 years it was possible to estimate the incidence better (paper I), and it seems that Halvorsen's and Kvittingen's numbers were too low and too high respectively. Our point estimate suggests 1 patient in 75.000 living births. The number of patients (14) diagnosed in our laboratory between 1991 and 2010 is however still low, and this makes our new estimate uncertain as well. If one extra or one less patient had been diagnosed in the period, the incidences would have been 1 in 70.000 and 1 in 80.000 live births, respectively. The incidence in the Quebec region of Canada is due to a complex founder effect, the possible elevated incidence in the Middle East is probably due to consanguinity (Imtiaz et al. 2011). The elevated incidence in Norway is caused by the coincidence of many different pathogenic mutations in the population. Figure 8 shows how the origins of the Norwegian HT1 patients (29) are distributed in the country.

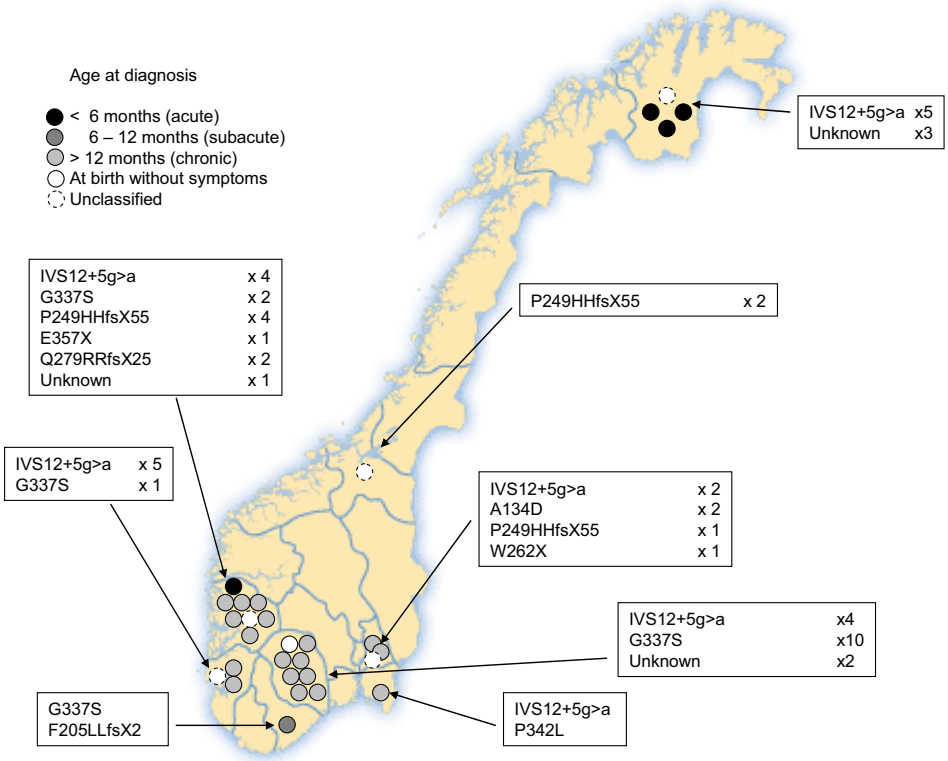

Figure 8. The distribution of HT1 patients in Norway. The numbers (x N) given identify the number of alleles.

There is only one patient diagnosed from the middle parts of Norway, and 67 % of the patients live in three counties: Hordaland, Telemark and Finnmark. The three mutations IVS12+5g>a, G337S and P249HfsX55 constitute 73 % of the disease alleles identified in Norway. The most common mutation worldwide IVS12+5g>a is also the most common in Norway (40,4 %). The other mutations: 26,9 % G337S, 13,5 % P249HfsX55, 3,8 % Q279RfsX25, E357X and A134D, 1,9 % F205LfsX2, W262X and P342L. Comments on methodology: Standard Sanger sequencing has been used to characterize the unknown FAH alleles. PCR and RFLP assays have been used to detect specific mutations. These methods are approved and routine in our laboratory. Therefore method employing realtime PCR were not developed for analyses of these rare mutations in the FAH gene.

Genetic mosaicism

The intragenic suppressor mutation (paper II) gives an alternative explanation to self-induced reversion and elucidates different points regarding the immunopositive nodules in livers of HT1 patients. It supports the assumption of high mutagenic activity in HT1 hepatocytes and the assumption that random point mutations explain the occurrence of immunopositive nodules. It indicates that the mosaic pattern of hepatocytes in HT1 patients may be more complex than previously assumed.

Kvittingen's report from 1994 of the self-induced reversion to normal genotype in hepatocytes from HT1 patients was surprising (Kvittingen et al. 1994). Genetic mosaicism per se had been well known for decades in advance. A spontaneous reversion of a disease causing mutation had also previously been descried (Yang et al. 1988), but only as a single event caused by gene rearrangements. "The shocking news" was the frequent occurrence of immunopositive cell clones in the liver samples (in 16 of 19 patients), "all" apparently genetically corrected through random mutations (samples from 4 patients were genetically characterized). Self-induced corrections of inborn errors have later been described in several other conditions like Fanconi anemia, Bloom syndrome and epiodermiolysis bullosae (Table 5). The reversions does not occur as frequently in these diseases as it does in HT1 patients, although a relatively high frequency (~20 %) has been found in epidermiolysis bullosae (EB) (German et al. 1977). The cellular conditions and the molecular mechanisms behind the corrections are however not necessarily the same for each condition. Table 5 shows that the described reversions are of different types, and that they take place in different tissues and in different diseases.

Table 5 (adapted from (Hirschhorn 2003))

Disease	Tissue	Type of reversion
HT1	Liver	Single nucleotide Second site mutation
ADA-SCID	B cells T cells	Single nucleotide Second site mutation
X linked SCID	T cells	Single nucleotide CpG hot spot
Wiskott-Aldrich (X-L)	T cells Lympocytes	Single nucleotide Second site mutation Second site del/ins
Bloom Syndrome	Fibroblasts Lymphocytes Lymphoid cell lines	Slippage Single nucleotide
Epidermiolysis Bullosae	Skin	Mitotic gene conversion Second site del/ins
FANC A	Blood cells Lymphoid cell lines	Second site insertions Ins/del repeat hot spot Single nucleotide Single nucleotide & repeat slippage
FANC C	Blood cells Lymphoid cell lines	Intragenic recombination Not intragenic recombination Second site CpG hot spot

The diseases for which intragenic recombination and gene conversion can be excluded are written in bold face.
ADA: adenosin deaminase, SCID: severe cmbined immunodeficiency,X-L: X-linked, Fanc: fanconi anemia, del: deletion, ins: insertion

Hirshorn suggests that there could be a possible common reversion mechanism in cases with the same type of reversion and when intragenic recombination and gene conversion can be excluded. The diseases for which such patients have been described are written in bold face in Table 5. The hypothesis of a sequencespecific common cause of reversion in these cases is interesting; however, many characteristics of HT1 differ significantly from the other diagnoses. Thus a common cause of DNA instability is not easy to conceive despite of common types of mutation reversion. HT1 phenotype characteristics including self-induced corrections and frequent cancer development in hepatocytes are connected to, and probably caused by, the specific accumulating metabolites. In X-linked SCID, Bloom syndrome and EB there are no known metabolite accumulation. DNA instability therefore must have

other causes. The competitive growth advantage of corrected/healed HT1 hepatocytes is “obvious” since the accumulation of metabolites of strong toxicity is prevented. Competitive growth advantage has been described after infusion of healthy cells (wildtype) in a X-linked SCID mouse model (Kume et al. 2002), but this pathological situation is also different from HT1. X-linked SCID is caused by mutations in the IL2RG gene and subsequent deficiency of the cytokine receptor common-gamma-chain. The deficiency leads to disturbed interleukin signalling including lack of cellular response to growth signals via IL-7 and other cytokines. Thus the growth advantage of infused wildtype cells in the X-L-SCID mouse is due to the regulation of a cellular signal system, not because of elimination of DNA damaging agents.

True reversions and second site reversions

In the literature the reversion of a primary mutation is sometimes called a “back mutation” or a “true back mutation” (Ellis, Ciocci, and German 2001;Hirschhorn 2003;Pasmooij et al. 2005). Kvittingen’s report from 1994 of the self-induced reversion to normal genotype was an early example of a such “back mutation” in human cells. A suppressor mutation is by definition “a mutation that counteracts the effects of another mutation” (Anthony JF Griffiths 2000). The result is a “second site” revertant. The detection of a suppressor mutation (paper II) in the FAH gene was an early example of such second site reversions.

The triple mosacism in the liver of one HT1 patient discussed in paper II implies that a more complex mosaic pattern of hepatocytes is possible in other patients as well. Two cell clones with different back mutations in a single liver are possible in compound heterozygous patients. Different types of second site reversion are also imaginable. It is important to bear in mind that only a slight improvement of the Fah activity would be sufficient for the hepatocyte to obtain a selective growth advantage. The Fah pseudodeficiency illustrates this. An individual homozygous for the mutation R341W in the FAH gene showed very low enzyme activity but had no biochemical or clinical symptoms of HT (Rootwelt, Brodtkorb, and Kvittingen 1994). The need of only a slight improvement of the Fah activity increases the number of theoretically significant nucleotide alterations.

Possible types of suppressor mutations

We describe a second site suppressor mutation in one cell clone (paper II). The primary splice site mutation was suppressed by a new point mutation that improved the efficiency of the affected splicing site. Other alterations in the same splicing site could however also improve splice site efficiency. A theoretical example: Online scoring systems for 5' splice site efficiency (see details in the next section) predict that the theoretical mutation G1065>A in the FAH gene improves the splice site efficiency to about the same degree as the mutation (C1061>A) discussed in paper II.

The mutation G337S in the FAH gene introduces a cryptic splice site. An additional point mutations may weaken the cryptic splice site.

All but two of the proteinogenic amino acids have two or more triplets of nucleotides coding for them. In patients with the FAH mutation Arg381Gly, c.1141A>G a new point mutation could restore the amino acid sequence. (AGA (Arg) > AGT (Gly) > CGT (Arg)) Arg381Gly, is however, not among the mutations detected in Norwegian patients.

An additional missense mutation elsewhere in the FAH gene can introduce an amino acid substitution that might counter fatal effects on protein structure by the primary mutation.

A disrupted reading frame because of deletions/insertions of nucleotide(s) can be restored with other deletions/insertions.

In addition to intragenic nucleotide alterations, extragenic suppressor mutations are theoretically possible. An enzyme knockout upstream of Fah in the tyrosine degradation pathway would "cure" the cell just like the drug NTBC. The toxic metabolites would no longer be produced. The chance of such a knockout occurring is low because both alleles of the other gene must be disrupted; two independent events must take place instead of one. On the other hand there are many possible genetic alterations that could be effective. Knockout of one of three genes (TAT, 4HPPD and HAO) in the tyrosine degradation pathway would block the production of toxic metabolites. Furthermore many genetic alterations in each gene might disrupt the protein function. Such cell clones would however not be detected using antibody against Fah.

Extended investigation of hepatocyte nodules

We have investigated 18 Fah immunopositive hepatocyte nodules in addition to the nodules previously reported (paper II, (Kvittingen et al. 1994)). Five HT1 patients were investigated. No suppressor mutations were revealed; only reversions of primary mutations (true back mutations) were detected (unpublished data).

Table 6. Fah immunopositive hepatocyte clones in HT1 patients. Reverted alleles are indicated in bold face.

	Nodules investigated	Detected reversion	Not conclusive	Maternal mutation	Paternal mutation
Patient 1	1	1	0	G337S	**IVS12+5g>a**
Patient 2	6	4	2	**IVS12+5g>a**	P249HfsX55
Patient 3	4	4	0	**IVS12+5g>a**	E357X
Patient 4	1	0	1	P249HfsX55	W262X
Patient 5	6	5	1	F205LfsX2	**G337S**

Table 6 shows the number of investigated nodules from each patient. The reverted mutations are written in bold face. The reversion (g>a>g) of the mutations IVS12+5g>a and G337S was detected in 14 immunopositive nodules. No clear results were obtained in 4 nodules because of unsatisfactory technical quality. Only one type of back mutation was detected in each patient. This can be a coincidence because a low number of patients and nodules were investigated. It could also be due to the mutation types. Patients 2 and 5 are compound heterozygous for a point mutation and a small deletion. The frequencies of compensatory point mutations and deletions/insertions are not necessarily equal. It is also possible that the different immunopositive areas in the liver sections are derived from the same expanding immunopositive cell clone.

Complex mosaicisms have been reported in two patients with another autosomal recessive disorder; the dermatological disease epidermiolysis bullosa (Pasmooij et al. 2005). Patient 1 and 2 in were compound heterozygous for the nonsense mutation 3781C>T in the COL17A1 gene and the frame shift mutations 4424 – 5insC and 1706delA respectively. Both patients showed several discrete

patches of functionally restored skin, and genetic analyses of the different areas demonstrated different types of genetic reversions. Patient 1 showed a true back mutation of 3781C>T and a second site small deletion 4463delG (restores the reading frame). Patient 2 showed a second site suppressor mutation 3782G>C (prevents the nonsense mutation) from one skin area and two distinct gene conversions in two other areas.

The studies on HT1 and EB show that both the phenomenon of complex mosaicism and genetic reversion may not be so rare, but the characteristics of EB differ from HT1 in several ways. As previously pointed out there is no known accumulating metabolite connected to the disease. The mosaicism is suggested to be caused by random embryonic mutations, but the mutation rate is not increased. The restored skin areas remain unchanged throughout life, thus the mosaicism is not due to clonal selection and expansion.

Sequence requirements for spliceosome function

A new mutation ($C^{1061\rightarrow}A$) 6 bp upstream of the primary mutation IVS12g$^{+5}\rightarrow$a in the FAH gene is described in paper II. The splicing defect caused by the primary mutation is most likely suppressed by the new mutation due to improvement of the splicing site according to *in silico* analysis of the most common splice sites in the mammalian genome (Burset, Seledtsov, and Solovyev 2000). Several algorithms have been developed to calculate a splice site score to predict the effect of mutations in the splicing site and are available online. Table 7 shows the 5' splice site scores from four algorithms: maximum entropy (MAXENT) multiple dependence decomposition (MDD), Markov's model (MM) and a Weight Matrix Model (WMM) (Houdayer et al. 2008). The results are consistent with the analysis performed in paper II.

Table 7. Splicing efficiency estimates at the 5' donor splice site of intron12 in the FAH gene. Exonic and intronic nucleotides are shown in upper- and lowercase letters, respectively.

	Splice site sequence	MAXENT:	MDD	MM	WIMM
FAH wildtype (wt)	CCGgtgagt	10,90	15,68	10,63	9,35
immunonegative tissue	CCGgtgaat	4,89	6,48	6,16	5,90
immunopositive tissue	CAGgtgaat	6,60	10.78	7,40	8,24

(http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)

The algorithms produce absolute values for the 5' splice site, but the relative changes in splice site strengths are considered more important (Yeo and Burge 2004). All four algorithms predict that the new mutation in immunopositive tissue improves splicing efficiency. The average score for immunopositive tissue is 72 % of wildtype whereas the score for immunonegative tissue is 52 % of wildtype .

Comments on methodology: The effect of the suppressor mutation described in paper II was made probable by calculations concerning the splicosome. Site-directed *in vitro* mutagenesis would have been necessary to obtain a valid biological proof. However the findings were considered to be convincing at the time of publication without *in vitro* mutagenesis because of the *in silico* evaluation and the complete absence of other genetical differences in the FAH gene between the Fah positive and negative tissue. Consistent 5' splice site scores from the expanded *in silico* analysis (Table 7) supports this assumption.

Impaired Base Exission Repair (BER) in HT1

It is not obvious if or to what extent glutathione reduction and the alkylating activities of FAA and MAA explain the high frequency of point mutations, cancer, and the reversion phenomenon in HT1 hepatocytes. As shown in Figure 7 reduced DNA repair capacity will accelerate the accumulation of DNA damage and increase the instability of DNA. To elucidate the role of DNA repair in HT1 we have investigated the activity of four glycosylases (Ogg1, Neil1, Neil2 and Ung2) and one methyltransferase (6-mG methyltransferase) under the influence of FAA and SA.

The pattern of reversions

Deficiency of specific repair enzymes would increase the probability for specific sequence dependent changes. The pattern of reversions may therefore reflect the eventual type of impaired DNA repair activity. Six examples of true back mutations are previously published (Kvittingen et al. 1994) (Dreumont et al. 2001) Additionally we have detected back mutations in 14 immunopositive nodules from five patients (Table 6). Eighteen reversions are of the type: g>a>g, one is a>g>a and one is c>t>c. The second site reversion discussed in paper II is c>a. The number of patients and nodules are too small to conclude if this is a representative distribution of reversion types. A majority of the reversion g>a>g must be expected because the mutation IVS12g>a is the most common in the patient population. The pattern shows however that several types of point mutations (**a**>g, **g**>a, **t**>c and **c**>a) take place in HT1 hepatocytes. If this reflects reduced protection against specific DNA lesions, enzymes with substrates of more than one type must be impaired. We have investigated four glycosylases (Ogg1, Neil1, Neil2 and Ung2) that together cover a broad battery of substrates (Table 4).

In addition we have investigated 6-mG DNA methyltransferase (*Mycobacterium tuberculosis*) for two reasons: Firstly 6-mG is a highly mutagenic damage (Lawley 1989), and secondly the enzyme has an important sulfhydryl group in the active site (Daniels et al. 2004) which could be attacked by FAA.

FAA inhibits the first step of the BER.

BER is the most important pathway for removing mutagenic DNA base lesions. In paper III we demonstrate that FAA inhibits the three glycosylases Ogg1, Neil1 and Neil2 strongly. Ogg1 is the major DNA glycosylase for removal of 8-oxoG opposite C and Neil1 function as a backup activity (Bjoras et al. 1997;Morland et al. 2002). 8-oxoG is as previously stated one of the most common mutagenic adducts that result from oxidative DNA damage (Shibutani, Takeshita, and Grollman 1991). Neil1 and Neil2 remove oxidized pyrimidines which are mutagenic lesions as well (Feig, Sowers, and Loeb 1994). The inhibition by FAA was particularly strong on Neil1 and Neil2. Significant inhibition was detected with only a small excess of FAA. The approximate molar ratios at 50 % inhibition were; FAA/Neil1 and ~3 FAA/Neil2 ~1. This reflects a particularly efficient inhibition mechanism. The approximate molar ratio at 50 % inhibition of Ogg1 was FAA/Ogg1 ~6000 which also suggests efficient inhbition. The inhibition of three glycosylases means a probable significant inhibition of the first step of the BER pathway in HT1 hepatocytes because of the enzyme's overlapping substrate specificity and their function as a back up for each other. These results suggest that inhibition of Ogg1, Neil1 and Neil2 by FAA in liver cells of HT1 patients increase mutagenesis. Knockout mouse models for different DNA glycosylases support this assumption. The single knockouts (*neil1-/-, ogg1-/-, nth1-/-* or *myh-/-)* show no or modest increase in cancer development. Several double knockouts show however increased mutagenesis and carcinogenesis (Chan et al. 2009;Xie et al. 2004). The double knockout *ogg1* $^{-/-}$*myh*$^{-/-}$ mouse model started to develop tumors at the age of two months (Xie et al. 2004). *nth1*$^{-/-}$*neil1*$^{-/-}$ mice even developed hepatocellular tumours (Chan et al. 2009) which are of particularly interest in HT1. We have not investigated Nth1 in our study, but the structural similarity with Ogg1 suggests that Nth1 may be inhibited by FAA as well. Nth1 has overlapping substrate specificity with Neil1 and Neil2. See "Future perspectives" for further possible investigations.

The inhibition of the glycosylases may be due to structural changes of the proteins caused by adduct formation or competition between FAA and oxidative DNA base lesions for the active site in these enzymes. See "Future perspectives" for possible further investigations.

FAA did not inhibit Ung2, and SA did not inhibit any of the investigated enzymes.

BER and HT1 pathophysiology

Our study elucidates the role of FAA in the HT1 pathophysiology. FAA induced BER impairment fits well into the theories of accumulating ROS induced DNA damage in HT1 hepatocytes. The increased accumulation of oxidative base lesions, due to reduced glycosylase activity, has both mutagenic and cytotoxic consequences, thus the impaired BER pathway contributes to the understanding of the mutagenicity, the cancer development and the hepatocyte decay in HT1 patients. The fact that only FAA and not SA acts as an inhibitor fits with the cell autonomous model. FAA affects the cells of the organ in which it is produced; no HT1 patients are described with primary cancer in any other tissue. The inhibition of the purified glycosylases Ogg1, Neil1 and Neil2 is demonstrated *in vitro*. We have attempted to measure Ogg1, Neil1 and Neil2 DNA glycosylase activity in protein extracts from transplanted HT1 livers, but the level of glycosylases in the protein extracts were below the detection limit.

Content of 8oxoG in HT1 hepatocytes

Detection of DNA damage in HT1 hepatocytes which corresponds to the specific base lesions removed by Ogg1, Neil1 and Neil2 would have confirmed the impact of our findings and strongly supported our hypothesis. We performed a pilot study that showed a significant increase in 8oxoG accumulation in genomic DNA from one HT1 patient compared with one control sample, but the subsequent investigations could not confirm the results from the pilot study. We have measured the 8oxoG content in 20 DNA samples from 6 liver transplant HT1 patients and compared them with 31 liver samples from 8 control liver transplant patients (cancer coli).

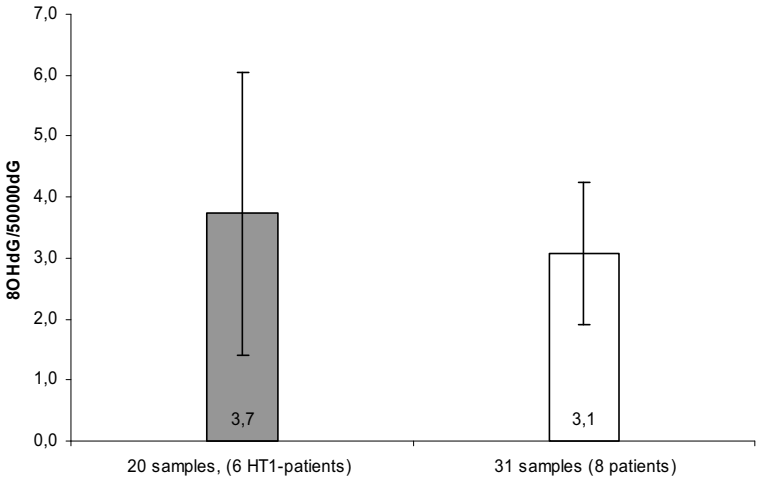

Figure 9. Analyses of 8oxoG content in hepatocytes of HT1 patients and controls. The columns show the mean value with 95 %- confidence interval.

The results were difficult to interpret. The average 8oxoG:C-content was 19 % higher in the samples from HT1 patients compared to the controls, but the confidence interval was broad and the difference between HT1 samples an the control samples was not statistically significant (p = 0.5 Mann-Whitney-U test). Varying amounts of DNA damage in hepatocytes from HT1 patients could perhaps reflect the heterogeneity of HT1 hepatocytes. But it could also reflect poor age matching between our patient samples and control samples. The control samples used in this study were collected over a period of two years prior to analysis. Our HT1 patient samples are 15 – 20 years old with one exception (5 years old).

Investigation of Ogt

Purified 6-mG methyltransferase from *Mycobacterium tuberculosis* was investigated under the influence of increasing amounts of FAA and SA. There is a high degree of homology between the human enzyme and the enzyme from *M. tuberculosis*. No significant decrease in enzyme activity was detected for either of the HT1 metabolites. The active site of 6-mG methyltransferase contains a cysteine unit (Daniels et al. 2004), thus an inhibiting effect of FAA would not have been surprising. Lack of inhibition suggests that the SH-group of the cysteine is protected within the protein structure.

The methyltransferase assay

N-[H3]-*N*-methyl-*N'*--nitro-*N*-nitrosourea (MNU; 1.5 Ci $mmol^{-1}$) was used to prepare methylated calf thymus DNA (6000 d.p.m. μg^{-1} DNA). The methyltransferase activity was measured in a reaction buffer (90 mM Tris, 90 mM Borate, 2 mM EDTA (TBE buffer)) with 500 fmol Ogt and 1.5 µg MNU treated calf thymus DNA in a total volume of 3 ml at 37°C for 60 min. Increasing concentrations of FAA or SA were added to the reaction mixtures. The protein was precipitated by incubating with 100 µl of 10 mg/ml BSA (in TBE buffer) and 1 ml of 4 M perchloric acid ($HClO_4$) for 30 min at 70°C. The precipitate was spun down at 4000 rpm for 20 min. The pellet was washed with 4 ml of 1M $HClO_4$ and resolved in 0.4 ml of 0.1 M HCl. The samples were transferred into tubes containing ULTIMA GOLD^{TM} MV scintillation liquid (Packard BioScience B. V., Netherlands), and the radioactivity was measured in a scintillation cou nter (Liquid Scintillation Analyzer, TRI-CARB 2900TR).

Comments on previous studies on DNA instability in HT1

Cytogenetic studies on skin fibroblasts from one HT1 patient were reported in 1990 by Gilbert-Barnes et al. (Gilbert-Barness, Barness, and Meisner 1990). The patient was a girl with chronic HT1 who died from hepatocellular carcinoma at four years of age. Increased chromosomal breakage affecting 71 % of the cells was demonstrated and related to the alkylating agents FAA and MAA. On this basis genetic instability was suggested to be a part of the pathophysiology and carcinogenesis in HT1. The importance of the chromosomal breakage in fibroblasts in this study seems however to be unclear for several reasons. Fibroblasts are most likely not exposed to FAA and MAA because tyrosine is exclusively degraded in the liver. SA is distributed throughout the body, but the relation to cancer development is less clear because primary tumours have only been detected in the liver of HT1 patients. The study was based on only 14 cells from one patient and has not been confirmed in later work (Wilson et al. 1994).

Prieto-Alamo et al (1998) described reduced DNA ligase activity in protein extracts from HT1 fibroblasts (Prieto-Alamo and Laval 1998). In a separate assay SA was shown to inhibit T4 DNA ligase *in vitro* at high concentrations (100μM). Thus inhibition by SA was suggested as an explanation for the reduced DNA ligase activity in the protein extracts. However the SA concentration was not measured in the protein extracts. The results from this study have not been verified in other studies, thus both the role of DNA ligase in HT1 and the influence of SA on DNA ligase seem unclear. FAA was not investigated in this study.

More recently van Dyke *et al* have used a comet assay to examine whether BER was inhibited in cells treated with SA or p-hydroxyphenylpyruvate (van Dyk et al. 2010). However it is unclear from this work whether DNA glycosylase activities are affected by SA or p-hydroxyphenylpyruvate. A carcinogenic effect of p-hydroxyphenylpyruvate is not likely. This metabolite is not specific for HT1 and it increases to a much higher level in tyrosinaemia type II (Mitchell et al. 2001), a condition that is not associated with cancer development. FAA is not tested in this study. In a recent paper, van Dyk showed reduced gene expression of Ogg1 in lymphocytes from two HT1 patients, indicating that down-regulation of DNA

glycosylases at the transcriptional level could impair BER of oxidative damage (van Dyk E. and Pretorius 2011).

Future perspectives

To further investigate the DNA repair capacity in HT1 more DNA enzymes should be tested under the influence of HT1 metabolites. The activities of Nth1, Myh and Mth1 should be investigated to elucidate the total effect of HT1 metabolites on the defence against oxidative DNA damage. Additionally it would be of interest to investigate the defence against methylating DNA damage provided by the Aag DNA glycosylase for example. Both FAA and SA should be investigated as potential inhibitors.

Detection of oxidative DNA damage in HT1 hepatocytes would support our findings in paper III. Investigation of aberrant methylation and hydrolytic damage to the DNA molecules would complete the picture. MS-MS technology makes it possible to detect all sorts of damage modifications to the DNA molecule. With appropriate age matched control samples it would be of interest to investigate methylation, oxidative and hydrolytic damage in DNA extracts of hepatocytes from HT1 patients.

In paper III we suggest a strong inhibiting effect of FAA on glycosylase activity. Our studies do not reveal the mechanism of this inhibition. Changes in protein structure may be a part of the explanation. Thus it would be of interest to investigate structural changes to Ogg1, Neil1, and Neil2 and eventually other DNA repair enzymes. Electrophoretic mobility shift assays can be used to detect protein-DNA interactions. MS analyses may also reveal structural changes of the proteins after FAA exposure.

To develop a combined fah$^{-/-}$ glycosylase$^{-/-}$ knockout mouse would perhaps be the ideal way to explore the effect of FAA on BER capacity. Two *fah* deficient mouse models have been engineered by others (Grompe et al. 1993;Russell, Russell, and Kelly 1979). In our laboratory, lines of different double and triple knockout mice are developed. A cooperation to develop a combined fah$^{-/-}$ and glycosylase $^{-/-}$ knockout mouse line would be of interest. Three possibilities are the triple knockouts: *fah*$^{-/-}$ *ogg1*$^{-/-}$ *mutY*$^{-/-}$, *fah*$^{-/-}$ *neil1*$^{-/-}$ *neil2*$^{-/-}$ and *fah*$^{-/-}$ *neil1*$^{-/-}$ *nth1*$^{-/-}$.

There is still one unknown FAH mutation in the Norwegian HT1 population. One of our patients has only had the paternal mutation identified after sequencing (paper I). The other mutation may affect the promoter region of the FAH gene. No

FAH promoter mutations have yet been identified in HT1 patients. A promoter mutation can be hard to exclude. Most promoter mutations are located within the first 500 nucleotides upstream of the start codon, although regulatory regions may also be found 10kb or more upstream of the gene (de Vooght, van, and van Solinge 2009). Furthermore, mutations deep into introns or far downstream of the coding region may also impair gene function.

Conclusion

Our studies give a new estimate of the incidence of hereditary tyrosinaemia type I (HT1) in Norway, and we have described the molecular basis of HT1 in the Norwegian population in more detail than previous reports with three novel small deletions and all together nine different disease causing mutations (paper I). HT1 is characterized by a high hepatocellular mutation frequency, hepatocellular mosaicism and frequent development of hepatocellular carcinoma. In paper II we explore the phenomenon of self-induced genetic correction in HT1 hepatocytes and describe a new mechanism behind the reversion phenomenon. A triple mosaicism was detected in one patient. In one cell clone the primary splice site mutation was suppressed by a new point mutation due to improvement of the splicing site. The triple mosaicism of hepatocytes supports the assumption of an increased frequency of point mutations in HT1. The studies on DNA glycosylases suggest a common pathophysiology behind the high frequency of point mutations and cancer development (paper III). Three glycosylases with overlapping substrate specificity were strongly inhibited by FAA *in vitro*. Thus the first step of the base excision repair (BER) pathway is probably suppressed in hepatocytes in HT1. Such inhibition of BER is associated with both increased mutation frequency and increased cancer development. Impaired BER may thus be an important factor in the pathophysiology of HT1.

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Errata

Page 13, step 2:
“This enzyme is dependent on Cu,” should be: “This enzyme is dependent on ferrous iron, “

“The enzyme is inhibited by the metabolite fumarylacetoacetate (FAA),” should be: “The enzyme is deficient in tyrosinaemia patiens as a secondary phenomenon to the primary enzyme defect,”

Page 13, step 4:
“This enzyme requires reduced glutathione probably in order to maintain its sulfhydryl groups in reduced form.” should be: “This enzyme is a glutathione-S-transferase (glutathione-S-transferase, Zeta 1) and requires reduced glutathione.”

Publication I

Scand J Clin Lab Invest., Accepted 11 March 2012
Hereditary Tyrosinaemia type I in Norway: Incidence and 3 novel small deletions in the Fumarylacetoacetase gene

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Original Article

Received: 30-Dec-2011

Accepted: 11-Mar-2012

Hereditary Tyrosinaemia type I in Norway: Incidence and 3 novel small deletions in the Fumarylacetoacetase gene

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Acknowledgements: We would like to thank Lars Mørkrid for help with statistical analyses and Anne Marie Lund, Gro Guldal and Kari Høie for technical assistance.

Abstract

28 Norwegians have been diagnosed with hereditary tyrosinaemia type I (HT1) over the last 30 years. In this study, 19 of these patients were investigated. Three novel small deletions were found (NM_000137.1(FAH): c.615delT, p.Phe205LeufsX2, NM_000137.1(FAH): c.744delG, p.Pro249HisfsX55 and NM_000137.1(FAH):c835delC) pGln279ArgfsX25, all of them leading to a change in the reading frame and a premature stop codon. 51 of the 56 disease causing alleles are hereby genetically characterized, identifying 9 different disease causing mutations in the Norwegian population. 65 % of the Norwegian HT1 patients are compound heterozygous for different mutations. Thus, the relatively high incidence of HT1 in Norway of 1 in 74,800 live births is not due to single founder effects or high incidence of parental consanguinity.

Key words:

Inborn Errors of Metabolism, Metabolic Disease, Single Gene Disorders, Amino Acids, Epidemiology, Genotype, Frameshift Mutation, Liver Disease, Hepatocellular carcinoma

Background

Hereditary tyrosinaemia type I [(HT1) MIM 276700] is an autosomal recessive disorder caused by deficiency of fumarylacetoacetase [(FAH) E.C. 3.7.1.2], the last enzyme in the tyrosine degradation pathway, which leads to accumulation of the pathogenic metabolites fumarylacetoacetate (FAA) and maleylacetoacetate (MAA) and their derivatives succinylacetoacetate and succinylacetone. FAA and MAA are alkylating agents and form glutathione adducts that are assumed to cause the organ damage symptomatic of the disorder. HT1 is a disease with highly variable clinical symptoms with onset occurring between infancy and adolescence. It is characterized by progressive liver disease and secondary renal tubular dysfunction. In the most acute form the patients present with severe liver failure within weeks of birth, whereas hypophosphatemic rickets may be the first symptom in chronic HT1. Untreated HT1 patients die from liver cirrhosis or hepatocellular carcinoma at a young age. The treatment consists of a protein restricted diet, and the drug NTBC (Orfadine®) which inhibits the breakdown of tyrosine and thus the production of the toxic metabolites. The drug has dramatically improved the prognosis. [1].The human FAH gene (*FAH*) is located on chromosome 15q23-q25, spans 30-35 kb and consists of 14 exons. The cDNA has an open reading frame of 1257 bp encoding 419 amino acids [2;3]. 55 disease causing mutations are registered in the HGMD Professional database (Human Gene Mutation Database; release date December 9th, 2011) [4]. In addition, 11 new mutations of Middle Eastern origin were recently reported [5]. The missense mutation Q279R reported previously [6] is not in the HGMD database. Three of the total of 67 mutations are small deletions. HT1 is a rare disease, but the incidence is extraordinarily high (1 in 16,700 live births) in the province of Quebec in Canada due to a founder mutation [7]. The incidence in the rest of world, including Scandinavia, has previously been estimated to be 1 in 100-120,000[1]. Before this study 6 different disease causing mutations had been found in Norwegian patients [8].

Materials and methods

Patients

During the last three decades 28 Norwegian HT1 patients (17 males and 11 females) have been diagnosed with HT1 in our laboratory (The Norwegian National Laboratory of Inborn Errors of Metabolism, Department of Medical Biochemistry, Oslo University Hospital.) No DNA material is available for 2 patients. 7 patients have previously been investigated and fully characterized. [8] Three additional patients in the same study were not fully characterized, since only one of the mutated alleles was identified in each of them. In this study the latter three patients and 16 new patients are investigated. DNA from leukocytes or fibroblasts was used from all patients. From one of the three partly characterized patients, DNA material was unavailable. Since the patient's paternal mutation was known, maternal DNA was used for further analyses. DNA from 100 control individuals (200 alleles) was used to exclude polymorphisms.

Sequencing and Restriction enzyme analyses:

PCR products from three of the HT1-patients with one or two unknown mutations and from the mother of one patient were generated. The products from all 14 exons of *FAH*, including 28-101 intronic bp of the exon-intron boundaries, were subjected to sequencing. The primers used are listed in table I. Sequence analysis was performed after treatment with ExoSAP-IT® (Affymetrix Inc. Santa Clara, California 95051, USA) using Big Dye® Terminator v3.1 Cycle Sequencing Kit and an automated ABI 3100 or 3730 auto sequencer (Applied Biosystems, CA, USA). The novel mutations (c.615delT, c.744delG and c.835delC) were confirmed by PCR amplification and restriction enzyme digestion. The enzymes and primers used are listed in table II (mismatch bases are underlined). Other mutations (IVS12+5G>A , G337S, E357X) in *FAH* were detected by restriction enzyme analyses as reported previously[8]. The rest of the patients were investigated by restriction enzyme analyses only.

Protein structure

The consequences of the novel mutations were analyzed with PYMOL (http://www.pymol.org), using the experimentally determined structure of mouse FAH [9].

Statistics for calculating the incidence of HT1 in Norway.

The treatment with NTBC is likely to have increased the awareness of HT1. Thus the year of the first trial of NTBC, 1991 [10], was chosen as the starting point for calculating the incidence of HT1 in Norway. December 31st, 2010 was chosen as end point of the interval. 14 patients born in this period were diagnosed in our laboratory, which is the only one in Norway offering a selective metabolic screening. A point estimate of the undiagnosed patients was made based on our patients' ages at the time of diagnosis. Additionally we have diagnosed one patient after December 31st, 2010. Of this total of 15 patients, 5 were found as a result of an already diagnosed sibling and not because of symptoms, leaving 10 as index patients with ages at time of diagnosis equal to 0.055, 0.214, 0.386, 0.430, 1.158, 1.478, 1.739, 2.209, 4.652, and 7.337 years. This age distribution was made normal by means of the Box-Cox transformation with parameter λ = 0.1775, which yielded μ = 0.0807, σ = 1.4293, and skewness virtually zero. The number (n_j) and average age (a_j) of individuals in each monthly cohort were calculated from annual data of live births in Norway from January 1991 to December 2010. Incidence, f, was assumed to be unchanged over this period. The same Box-Cox transformation was applied to all a_j. Transformed ages were denoted α_i. The number of diagnosed individuals in the period should be equal to $f \cdot \sum_{j=1}^{240} n_j \cdot P_j = 14$. P_j is the area under the standard normal distribution up to the z-score point = $(\alpha_j - \mu)/\sigma$. In this expression all values for n_j are known from the birth registry, and each P_j can be calculated from the standard normal distribution, given α_i, μ, and σ. Summing up this

gives f· 1047079.3 = 14. Hence f = 1.33705E-05, and the number of expected undiagnosed individuals is $f \cdot \sum_{j=1}^{240} n_j \cdot (1 - P_j) = f \cdot 134556.7 = 1.80$.

Ethics

For all living individuals contributing biological material, written, informed consent has been obtained. Dispensation was given by "The Norwegian Directorate of Health" for the deceased HT1-patients. The study was approved by the regional committee for medical research ethics.

Results

Sequencing *FAH* from the HT1 patients identified 3 novel, small deletions; F205LfsX2 (NM_000137.1(FAH):c.615delT, p.Phe205LeufsX2), P249HfsX55 (NM_000137.1(FAH):c.744delG, p. Pro249HisfsX55) and Q279RfsX25 (NM_000137.1(FAH):c835delC, pGln279ArgfsX25). All three mutations were confirmed by restriction enzyme analyses (figure 1). The novel mutations lead to frame shift and premature termination codons. F205LfsX2 changes the next triplet to a premature stop codon (TAG). Both P249HfsX55 and Q279RfsX25 lead to a premature stop codon (TGA) towards the end of exon 10. None of the three novel deletions were found in the 200 control alleles. F205LfsX2 was found in one patient who was compound heterozygous with the missense mutation G337S. P249HfsX55 was found in 5 patients (7 alleles), 2 were homozygous, 2 were compound heterozygous with the intron 12 splice site mutation IVS12+5G>A (NM_000137.1(FAH):c.1062+5G>A), and one patient was compound heterozygous with the nonsense mutation W262X. Q279RfsX25 was found in 2 patients. Both were compound heterozygous with the missense mutation G337S.

1,181,636 children have been born in Norway between January 1st, 1991 and December 31st, 2010 [11]. 15.8 patients (14 diagnosed and 1.8 undiagnosed) suggest an incidence of 1 in 74,800 live births.

Discussion

The three novel mutations in *FAH* result in premature stop codons with probable deleterious effects on enzyme function. Figure 2 shows the structure of the murine FAH protein and the predicted consequences of the mutations P249HfsX55 (A), F205LfsX2 (B) and Q279RfsX25 (C). In all cases large parts of the FAH protein are affected and normal folding, function and stability of the protein can not be expected. Although this is a murine protein structure, the same can be expected for the human protein, for which no structure is available, since there is a high degree of homology between the murine and the human protein. 372 of 419 amino acids (89 %) are identical (fig. 2D) [12]. A mismatch mutation generating Q279R has previously been reported as a disease causing mutation[6], further supporting the Q279RfsX25 deletion mutation as a causal mutation. In addition to a detrimental effect on the enzyme function, premature termination codons frequently result in mRNA degradation through nonsense-mediated mRNA decay when they occur more than 50 nucleotides 5′ to the terminal exon-exon junction, as they do with these three mutations [13]. This drastically reduces the amount of expressed protein in vivo.

51 of 52 alleles from the Norwegian HT1 patients with available DNA material are now characterized in this and previous studies[8], identifying 9 different *FAH* mutations in the Norwegian HT1 population (figure 3). IVS12+5G>A is the most common, found in 40.4 % of the alleles. P249HfsX55, Q279RfsX25 and F205LfsX2 are found in 13.5 %, 3.8 % and 1.9 % of the alleles, respectively. A single mutated allele was found in one patient despite a classical clinical and biochemical phenotype. The second mutation may be located outside the regions analyzed, affecting *FAH* transcription, splicing or mRNA stability. It could also be principally due to a germ cell mutation in the mother or an early de novo mutation in the patient that would not appear in the *FAH* of the mother's fibroblasts, which we have investigated in this particular case. 9 patients are homozygous; 4 for IVS12+5G>A, 3 for G337S and 2 for P249HfsX55. The remaining 17 (65 %) of the 26 Norwegian patients with available DNA-material are compound heterozygous for different mutations, demonstrating the lack of major founder effects and parental consanguinity.

This work gives the best possible estimate of the incidence of HT1 in the Norwegian population. Our laboratory is the only laboratory in Norway performing selective metabolic screening, and to our knowledge there are no other patients in Norway with a HT1 diagnosis made elsewhere. Newborn screening has not included HT1 in Norway. Furthermore, some cases of HT1 may also have been missed due to death by liver failure or hepatocellular carcinoma prior to or without the appropriate analyses being performed. This work shows that the incidence of HT1 in Norway is among the highest reported. The incidence in the Middle East is also proclaimed to be higher than average, but no number is given [5]. The high incidences in the Quebec region of Canada and the Middle East are due to a founder mutation and consanguineous mating, respectively. In Norway, however, the high incidence is caused by a high number (at least 9) of different disease causing *FAH* mutations in a relatively small population.

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Table I, PCR and Sequencing primers

Exon (*FAH*)	PCR primer, sense	PCR primer, antisense	Sequencing primer
1	GGGTGTTCACGGTGAGACCAA	CATTCCACTCCATTCCACTCCA	CTCCGCACGCCACCTTAG
2	GGGGGACCTGTGGACTCTTC	TCGGAAATGGAACGCAAGGA	CTTTCTGAGTAAATGAGCCA
3	AAGAGCAGAGGTCCTGAGAGTTT	AGAATGGCACTGATAACCACA	TCCATTGGAAGGAGGGATAC
4	AGGTGCCCACTGGAGATTGT	GCCTGGATGCTGGGAAAGGTC	GGGCTGAGCCCGTGGGTGGG
5	GGCTGAGCTCTGGATGTGTA	GACATTTGGCCCAGTTCATA	GTGCTTGGAACAGTCCATTC
6 + 7	TAACAGCTCTGATGCCCTGCATTC	GCTGAAGAGGAGGGGAGTG	GACAACATATAACAGCTCTG
8	TCTGCTTTCCTGAGGCGTCCAT	TGAGTGGCCCGAGTTCTTAGAG	TCTCTAGGTGACAAGTGACC
9	TGGAGATGGCAGTGCTAGGT	GGCTGCCTGGAAGTAATCGT	GTTGGGAGATGCCCTGATCA
10	GGTTTGGCTCCTGTGACCTT	AGCTCCTGGCCATGTCAGTC	GCTCAGCCCACCTGCCAGTG
11	CCTCCCTGATGCATGGAATT	TCTCTCTTTCCCTCAATTCT	TTTTCCTCCCTGATGCATGG
12	AGCAGAGGAGCTGAGGTTGGA	TGCACCCAGAGAGAAAGGCTA	GCCTCCGGGATGCTAGGCTA
13	TCGGGACTCCCAGGTCTTGCT	CCGCAGCTAGAACAGTGCAT	CATGGTCCATGCCAGAGCCCAAAGG
14	CGCTGCCTAGGTGTTGGTT	CACTGAATAGCGGACCAG	GTGTTGGTTCCGGTGAGCCC

Table II Restriction enzyme analyses

Mutation	Restriction enzyme	Sense primer	Antisense primer
P249HfsX55	ApaI	GTTGGGAGATGCCCTGATCA	TCCCAAAACTCTTCCCAAGGAAGGG
F205LfsX2	Mwo I	GTGACCTGGGCGGCAGAT	CTCTCCCAATCTGTTTCCAGGGCCTGCA
Q279RfsX25	Mbo II	TGGAGATGGCAGTGCTAGGT	GGCTGCCTGGAAGTAATCGT

Figure 1

A: Sequencing of exon 9 of *FAH*. The yellow frame shows the small deletion c.744delG.

B: Verification of the mutation Pro249HisfsX55 by restriction enzyme digestion of a 119 bp PCR product. The mutated allele remains undigested, whereas the normal allele is digested into fragments of 96 and 23 bp. Lane 1: DNA Molecular Weight Marker V (Roche Applied Science) Lane 2: Undigested PCR product. Lane 3: Homozygous sample. Lane 4: Heterozygous sample. Lane 5: Negative control.

C: Sequencing of exon 8 of *FAH*. The yellow frame shows the small deletion c.615delT.

D: Analyses of the mutation Phe205LeufsX2 by restriction enzyme digestion of a 90 bp PCR product. The mutated allele remains undigested, and the normal allele is digested into fragments of 61 and 29 bp. Lane 1: DNA Molecular Weight Marker V Lane 2: Undigested PCR product. Lane 3: Heterozygous sample. Lane 4: Negative control.

E: Sequencing of exon 9 of *FAH*. The yellow frame shows the small deletion c.835delC.

F: Analyses of the mutation pGln279ArgfsX25 by restriction enzyme digestion of a 415 bp PCR product. The normal allele contains 2 restriction sites. The digested normal allele consists of fragments of 243, 92 and 80 bp. The deletion c.835delc introduces a third restriction site, thus the digested mutated allele consists of fragments of 167, 92, 80 and 76 bp. Lane 1: DNA Molecular Weight Marker V Lane 2: Undigested PCR product. Lane 3: Negative control. Lane 4: Heterozygous sample.

* 123 and 124 bp. ** 267 bp. *** 434 and 458 bp.

Figure 2

A, B and C: The three-dimensional structure of the murine FAH protein (PDB id: 1HYO) and the predicted consequences of the mutations P249HfsX55 (A), F205LfsX2 (B) and Q279RfsX25 (C). The arrows point to the mutated site. The resulting changes in the amino acid sequence are coloured orange. The parts of the protein downstream of the termination codon are coloured grey. D: Amino acid sequence alignment of the human and the murine FAH protein showing that 372 of 419 amino acids (89 %) are identical (blue colour).

Figure 3

FAH with the location of the 9 disease causing mutations found among Norwegian patients. The rectangles represent the 14 exons separated by 13 introns. F205LfsX2, P249HfsX55 and Q279RfsX25 are novel mutations. The frequency of each mutation in the Norwegian HT1 population is indicated.

Figure 1

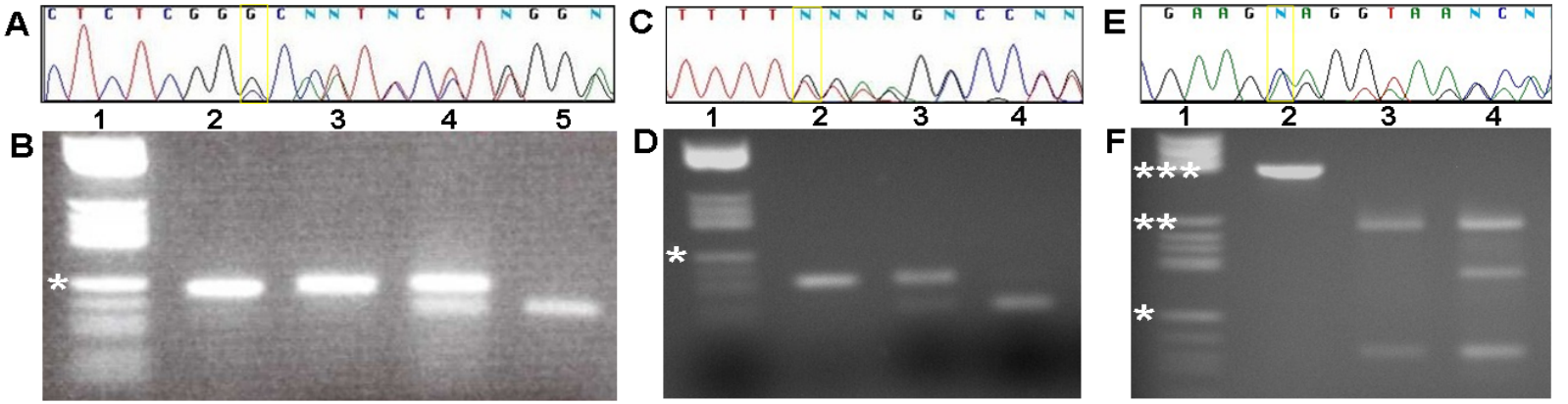

A
C T C T C G G G C N N T N C T T N G G N
1 2 3 4 5
B
*
C
T T T T N N N N G N C C N N
1 2 3 4
D
*
E
G A A G N A G G T A A N C N
1 2 3 4
F
***
**
*

Figure 2

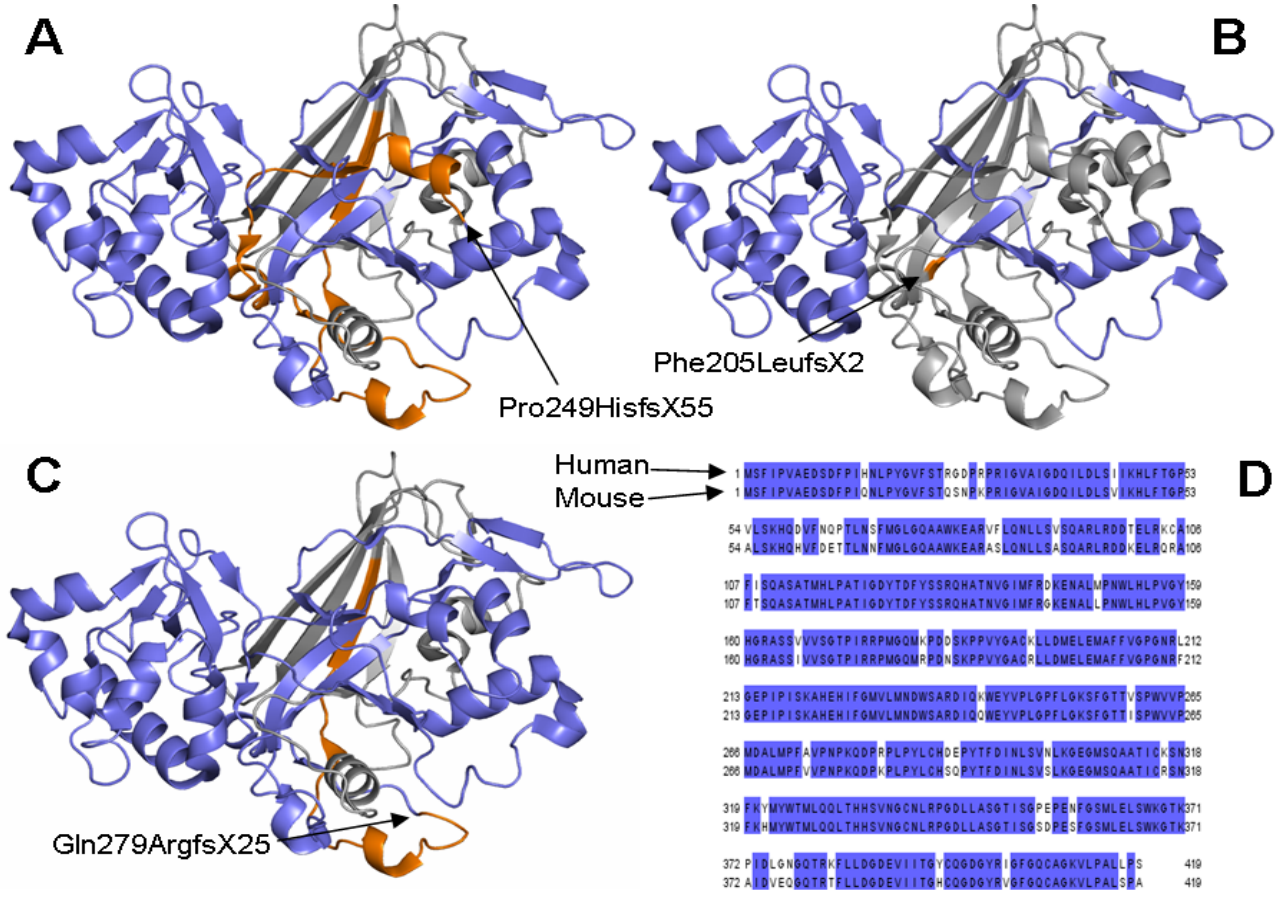
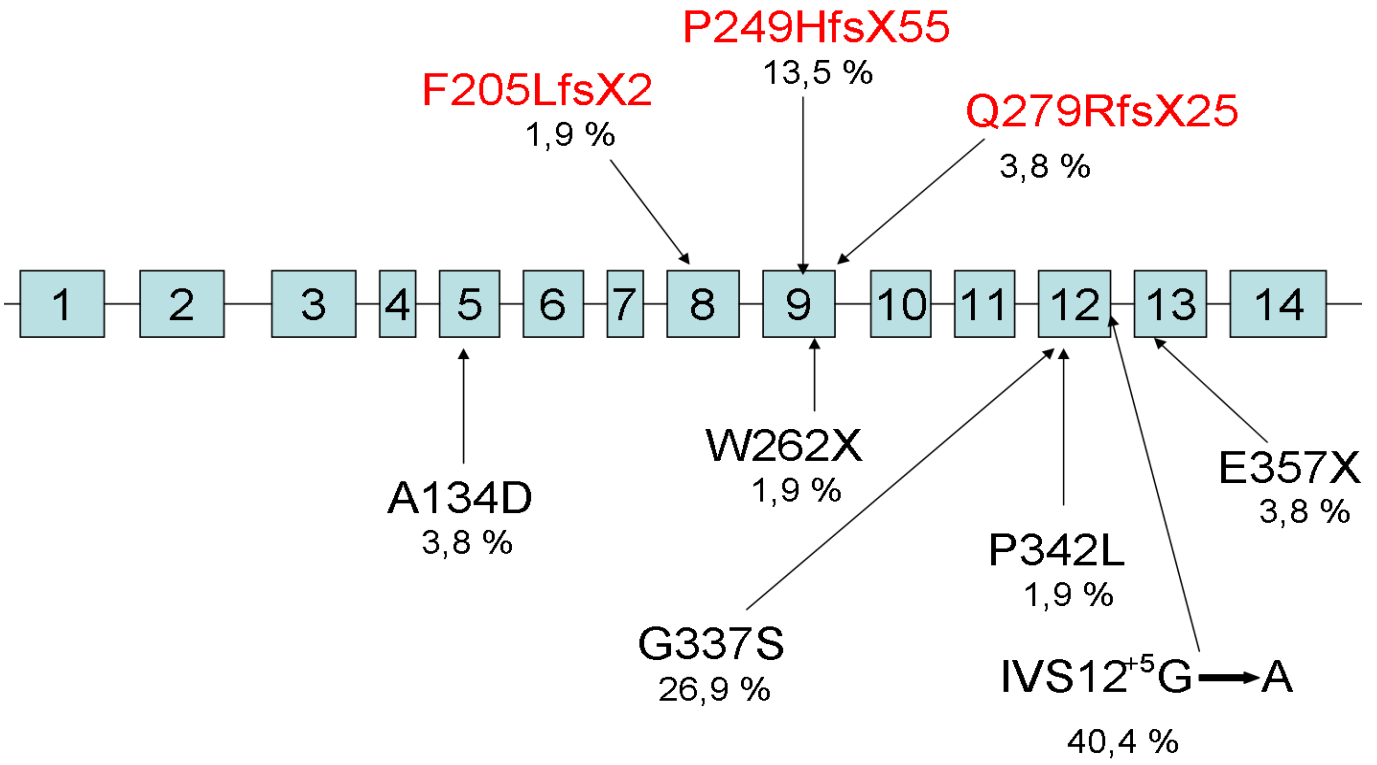

A
B
Phe205LeufsX2
Pro249HisfsX55
C
Gln279ArgfsX25
Human
Mouse
D

Figure 3


P249HfsX55
13,5 %
F205LfsX2
1,9 %
Q279RfsX25
3,8 %
1
2
3
4
5
6
7
8
9
10
11
12
13
14
A134D
3,8 %
W262X
1,9 %
E357X
3,8 %
P342L
1,9 %
G337S
26,9 %
IVS12+5G→A
40,4 %

Publication II

J Mol Med (Berl). 2005 May;83(5):406-10. Epub 2005 Mar 10

Tyrosinaemia type I--de novo mutation in liver tissue suppressing an inborn splicing defect.

Bliksrud YT, Brodtkorb E, Andresen PA, van den Berg IE, Kvittingen EA.

Publication III

Submitted 10 April 2012

Fumarylacetoacetate inhibits the initial step of the base excision repair pathway; implication for the pathogenesis of tyrosinaemia type I

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Fumarylacetoacetate inhibits the initial step of the base excision repair pathway; implication for the pathogenesis of tyrosinaemia type I

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Abstract

Hereditary tyrosinaemia type 1 (HT1) is an autosomal recessive disease caused by a deficiency in the human fumarylacetoacetate hydrolase (FAH), which is the last enzyme in the catabolic pathway of tyrosine. Several reports suggest that intracellular accumulation of intermediates of tyrosine catabolism such as fumarylacetoacetate (FAA) and succinylacetone (SA) is important for the pathogenesis in liver and kidney of HT1 patients. In this work we examined the effect of FAA and SA on DNA glycosylases initiating base excision repair (BER), which is the most important pathway for removing mutagenic DNA base lesions. *In vitro* assays monitoring DNA glycosylase activity of Ogg1, Neil1 and Neil2 demonstrated that FAA but not SA inhibited base removal strongly. These DNA glycosylases initiate excision of a broad range of mutagenic oxidative base lesions. Further, FAA and SA showed no inhibitory effect on the activity of uracil DNA glycosylase, Ung2. These data indicate that inhibition of DNA glycosylases oxidative base lesions by FAA in HT1 patients increase mutagenesis, suggesting an important mechanism for development of hepatocarcinoma and somatic mosaicism. .

Introduction

Hereditary tyrosinaemia type I [(HT1) MIM 276700] is a disease of autosomal recessive inheritance. It is caused by deficiency of fumarylacetoacetase [(FAH) E.C. 3.7.1.2], the last enzyme of tyrosine degradation. The condition leads to accumulation of the metabolites fumarylacetoacetate (FAA) and succinylacetone (SA), Fig.1A. HT1 is characterized by hypophosphatemic rickets due to renal tubular dysfunction and progressive liver disease with pronounced regeneration (Mitchell et al. 2001). The assumption of increased mutagenesis in hepatocytes of HT1 patients is supported by the high incidence of hepatocellular carcinoma, and by the frequent phenomenon of point mutation reversion in liver nodules (Bliksrud et al. 2005). FAA contribute to genomic instability in HT1 hepatocytes being an alkylating agent and by generating glutathione adducts leading to increased oxidative stress (Jorquera and Tanguay 1997). Less is known about the effect of FAA and SA on DNA repair enzymes such as DNA glycosylases that recognize and initiate repair of numerous mutagenic base lesions in the genome.

Base excision repair (BER) is the most important pathway for repair of DNA base damage, abasic sites and single strand breaks. The BER pathway is initiated by a DNA glycosylase removing the damaged base, Fig. 1B (I). The resulting abasic site is further processed by an AP-lyase activity associated with bifunctional DNA glycoslyases or by an AP-endonuclase, Fig. 1B (II). The suger-phosphate residue at the nicked abasic site is removed by 5' or 3' phosphodiesterase activities and repair synthesis is completed by DNA polymerase and DNA ligase, Fig. 1B (III). DNA-glycosylases are classified as mono or bifunctional based on their reaction mechanism. The monofunctional DNA glycosylases attack the N-glycosylic bond at C1 carbon of sugar, creating a free base and an intact abasic site. The bifunctional DNA glycosylases are associated with a β-elimination activity that

incises the phosphate backbone 3' of the abasic site. Structurally the DNA glycosylases consist of five superfamilies (reviewed in Dalhus et al 2009 FEMS). The helix-hairpin-helix (HhH) and the helix-two-turn-helix (H2TH) family are termed after their distinctive DNA-binding motifs. The human 8-oxoguanine (8-oxoG) DNA glycosylase, Ogg1, and human EndonucleaseIII, Nth1, belong to the HhH family (Bjørås et al, EMBO, 1997; PNAS; Aspinwall et al, PNAS, 1997), whereas Neil1, 2 and 3 belong to the H2TH. Nth1 and Neil1, 2 and 3 display overlapping substrate affinities removing a broad range of oxidative base lesions, including formamidopyrimidines, 5-hydroxypyrimidines and hydantoins (Hazra et al, JBC 2002, Hazra et al, PNAS, 2002; Morland et al, NAR 2002; Liu M, PNAS, 2010). The third and the fourth classes of DNA glycosylases are the uracil DNA glycosylase (Ung) and the alkyladenine DNA glycosylase (Aag). In addition to alkylated bases the Aag family removes deaminated purines (hypoxanthine and xanthine) and ethenoadducts such as ethenoadenine.

In this report we analyzed the effect of FAA and SA on the activity of four human DNA glycosylases (Ogg1, Neil1, Neil2 and Ung2). FAA inhibited all DNA glycosylases removing oxidative base lesions, suggesting that inhibition of BER of mutagenic base lesions play a significant role for development of cancer and somatic mosaicism in liver of HT1 patients.

Materials and methods

Enzyme purification

Human Ogg1 was expressed and purified as previously described (Dalhus et al. 2011).
Purified recombinant human Ung2 , containing the core catalytic domain (lacking 84 amino

acids at the N-terminal), was a kind gift from Professor Geir Slupphaug. Plasmids for purification of Neil1 (pET22 Neil1) and Neil2 (pET22Neil2) were a kind gift from Professor Sankar Mitra. BL21 Codon Plus RIL cells (Stratagene) containing plasmids of Neil1 and Neil2 were grown in LB-medium supplemented with sorbitol (0.5 M), betain (2.5 mM). Protein expression was induced when the cell density reached an OD_{600} of 1.0 by adding 1mM IPTG. Induced cells were grown for 2 (Neil 1) and 4 hr (Neil 2) prior to harvesting by centrifugation. Cell pellets were resuspended in 300 mM NaCl, 50 mM Na_2HPO4/NaH_2PO4 (pH 8.0) and 10 mM 2-mercaptoethanol (2-ME) (buffer A). Crude extracts were prepared by sonication, and the extracts were applied to nickel NTA-agarose columns pre-equilibrated with buffer A. Unbound proteins were removed by extensive washing with buffer A supplemented with 50 mM imidazole. The proteins were eluted with 300 mM imidazole in buffer A. Fractions containing Neil1 or Neli2 were pooled and dialyzed against 50 mM NaCl, 10 mM MES (pH 6.0) and 10 mM 2-ME (buffer B), and applied on a Hitrap SP column (GE Lifesciences). The proteins were eluted with a salt gradient to 2 M NaCl in buffer B. Protein fractions were identified by SDS-PAGE gel, pooled, and concentrated. Protein solutions for assays were supplemented with 20 % glycerol prior to storage at -70°C (Neil1) and -20°C (Neil2).

DNA glycosylase activity assay

The DNA glycosylase assays were performed in a reaction buffer of 50 mM MOPS, pH 7.5, 1mM ethylenediaminetetraacetic acid, 5 % glycerol, 1mM dithiothreitol with 10 fmol ^{32}P end-labeled duplex oligonucleotide substrate and enzyme as indicated in a total volume of 10 µl at 37°C for 30 min. Increasing concentrations of the metabolites FAA or SA were added to the reaction mixtures as indicated. The DNA glycosylase assay was terminated by addition of

NaOH to a final concentration of 0.1 M, incubated for 20 min at 70°C and neutralized with HCl. The reaction products were separated on a 20 % PAGE and quantified by phosphor imaging (Typhoon™, software: Image Quant TL Version 2003.02 Amersham Biosciences).

DNA substrates

The single stranded oligonucleotides were ^{32}P-end labeled at the 5′-end using T4 polynucleotide kinase (MBI Fermantas) and [γ32P]ATP (GE Healthcare). The following DNA-strands were used substrates containing 8-oxoG, 5-hydroxycytosine (5-ohC) and uracil (U): 5'- GGCGGCATGACCC[8-oxoG]GAGGCCCATC-3'; 5' GCATGCCTGCACGG [5-ohdC] CATGGCCAGATCCCCGGGTACCGAG-3'; 5′-GCTCATGCGCAG [U] CAGCCGTACTCG-3'. The oligonucleotides were hybridized to their complementary strands to generate the double-stranded substrates 8-oxoG:C, 5-ohC:G and U:A, respectively. The ^{32}P-labeled DNA substrates were then purified on a 20 % native polyacrylamide gel, the radiolabeled bands were excised from the gel, eluted in H_2O and stored at 4°C.

Others

FAA was produced as previously described (RAVDIN and CRANDALL 1951) and stored at -70°C. SA was ordred from Sigma Aldricht, eluted in H_2O and stored at -20°C. Bradford protein assay (Bio-Rad) was used to measure protein concentration.

Statistics

A nonparametric test (Passing & Bablok) and Spearman's rank correlation were used in regression analyses (Excel Analyse-it, v2.21).

Results

DNA glycosylase activity was assayed on duplex oligonucleotides containing one single base lesion at a defined position in the sequence. An 8-oxoG containing oligo substrate was used to monitor Ogg1 activity, whereas 5-ohC was used to monitor Neil1 and Neil2 activity. Ung2 activity was monitored using an oligoe containing uracil. The DNA glycosylase activities was analyzed by denaturing polyacrylamide gelelectrophoreses as exemplified in Fig. 2a. The intensity of the cleaved substrate (8-oxoG) band is fading with increasing concentrations of FAA, demonstrating that the DNA glycosylase (Ogg1) is inhibited by FAA. Figures 2b-d summarize DNA glycosylase assays with Ogg1, Neil1 and Neil2, respectively. The diagrams in figures 2b- d show an initial upper platau ("A"), an apparently linear falling segment and a final lower plateau ("B"). The intensity of the product bands, read as light intensity (LI) of the storage phosphor screen, was normalized to the positive control (the sample without the actual test substance, [C] = 0) for each electrophoresis gel; and then plotted against increasing [C]. Each column shows the mean value of the replicates with 95%-CI . To evaluate the observed descending part of the curve and to calculate [C_{50}] that corresponds to 50% inhibition, A and B were calculated as the median levels from all gels of the initial plateau and final plateau, respectively. The LI values from the intervening concentrations were used in a non-parametric regression (Passing & Bablok) $LI_i = a + b \cdot \log[C]_i + \varepsilon_i$; i = 1,…n. The estimated regression

coefficients â and $\hat{b}$ are presented with 95% confidence intervals together with Spearman's correlation coefficient. From the estimated coefficients the [C_{50}] that corresponds to 50% inhibition was calculated: (A-B)/2 = â + $\hat{b}$·log[C_{50}]. The plateaus "A" and "B" were calculated for each gel as the mean value of the positive and negative control respectively and the samples visually judged at approximately the same level. In sum, the results presented in figures 2b-d showed strong inhibition of Ogg1, Neil1 and Neil2 activity, respectively, with increasing FAA-concentrations. The [C_{50}] values for inhibition of Ogg1, Neil1 and Neil2 by FAA were calculated to, 1000, 6, and 32 nM, respectively. In contrast, FAA showed no significant inhibition of uracil removal by Ung2 (data not shown). Next, DNA glycosylase assays with increasing concentrations of SA showed no inhibition of Neil1, Neil2, Ogg1 and Ung2 (data not shown). It thus appears that FAA exhibit a strong inhibitory effect on DNA glycosylases removing a broad range of mutagenic DNA base lesions, suggesting an important mechanism for cancer development and somatic mosaicms in liver of HT1 patients.

Discussion

Oxidative DNA modifications are potent premutagenic lesions formed spontaneously at high frequencies in the genomes of aerobic organisms and BER is the major DNA repair pathway that corrects oxidative base damage. In human cells, BER of oxidative base lesions is initiated by at least seven different DNA glycosylases, which have redundant activity. In this report we demonstrated that FAA strongly inhibit three different human DNA glycosylases (Neil1, Neil2 and Ogg1) removing numerous oxidative base lesions. In contrast, FAA showed no effect on the human Ung2 glycosylase, which remove uracil in DNA. Moreover, SA shows no inhibition of any of the DNA glycosylases examined. These data indicate that impairment of

DNA glycosylases initiating BER of oxidative lesions by FAA increase mutagenesis and, consequently, lead to cancer development in HT1 patients.

One of the most common mutagenic adducts that result from oxidative DNA damage is 8-oxoG (Shibutani, Takeshita, and Grollman 1991) Ogg1 is the major DNA glycosylase for removal of 8-oxoG opposite C and Neil1 function as a backup activity (Bjoras et al. 1997;Morland et al. 2002). Further, Neil1 and Neil2 remove oxidized pyrmimidins, including 5ohC, 5ohU and di-hT, which are mutagenic lesions(Feig, Sowers, and Loeb 1994). Consequently, we suggest that inhibition of Ogg1, Neil1 and Neil2 by FAA in liver cells of HT1 patients increase mutagenesis.

Numerous DNA glycosylase deficient mouse models have been developed to investigate the impact of endogenous genotoxic stress for cancer development and other diseases. Single mutants with deficiencies in DNA glycosylases removing oxidative base lesions such as *neil1*$^{-/-}$, *ogg1*$^{-/-}$, *nth1*$^{-/-}$ or *myh*$^{-/-}$ showed no or modest increase in cancer development(Chan et al. 2009;Xie et al. 2004), However, tumor formation increased several fold in the double mutants *neil1*$^{-/-}$ *nth1*$^{-/-}$ and *ogg1*$^{-/-}$ *myh*$^{-/-}$ demonstrating that redundancy in repair of endogenous oxidative DNA base damage is important to prevent tumor formation. By example, Ogg1 Myh deficient mice started to develop tumors at the age of two months (Xie et al. 2004). In this work we showed that FAA strongly inhibited DNA glycosylases excising oxidative damage, suggesting that reduced capacity to initiate BER results in accumulation of oxidative DNA base damage and increased mutagenesis in HT1 patients. In addition endogenous oxidative stress is elevated in hepatocytes of HT1 patients (Jorquera and Tanguay 1997), supporting that increased accumulation of oxidative DNA base lesions, impaired repair and increased mutagenesis is a major cause of hepatocellular carcinoma and somatic mosaicms in liver.

Dyk at al used a comet assay to examine if BER was inhibited in cells treated with SA or p-hydroxyphenylpyruvate (van Dyk et al. 2010). However, it is unclear from this work whether DNA glycosylase activities are affected by SA or p-hydroxyphenylpyruvate. In a recent paper, Dyk showed reduced gene expression of Ogg1 in lymphocytes from two HT1 patients (van Dyk and Pretorius 2012) indicating that down-regulation of DNA glycosylases at the transcriptional level could impair BER of oxidative damage.

In sum, our data demonstrate that FAA is a very efficient inhibitor of DNA glycosylases removing a broad range of mutagenic base lesions, suggesting an important contribution to the pathogenesis of HT1.

Acknowledgments. We thank Lars Mørkrid for help with statistical analyses and Pernille Strøm Andersen and Mari Ytre-Arne for technical assistance with protein purification.

Figure Legends

Fig. 1 (a) Pathway of tyrosin degradation. Tyrosinaemia type I (HT1) is an enzyme deficiency of the last step of tyrosine degradation. Blockage of fumarylacetoacetate hydrolase (FAH) leads to accumulation of fumarylacetoacetate (FAA) and maleylacetoacetate which are converted to succinylacetoacetate and succinylacetone. **(b)** The Base Excision Repair (BER) pathway. BER is initiated by a DNA glycosylase which identifies a base lesion (I) and creates an abasic site (II). The abasic site is removed by phosphodiesterases/AP-lyases, and repair is completed by DNA polymerase and DNA ligase (III).

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Fig. 2 (a) DNA glycosylase assay with 8-oxoG:C duplex DNA and human Ogg1. 10 fmol 5' end-labeled 8oxoG:C duplex DNA was incubated with 1.6 fmol human Ogg1. Substrate and product of the DNA glycosylase reaction was separated on a denaturing polyacrylamide gel and visualized by Image Quant. Lane 1: Positive control with Ogg1 and without FAA. Lane 2 -8: Samples with Ogg1 and increasing concentrations of FAA (10^{-1}-10^{5} nM). Lane 9: Negative control (without Ogg1). The Ogg1 activity decreased with increasing concentrations of FAA. **(b)** 8-oxoG:C activity was measured with 1.6 fmol human Ogg1for seven different concentrations of FAA as indicated with eight replicates. The columns show the mean value of 8 replicates with 95%-CI. The total number of samples including controls was 80. 23 samples in the descending area of the curve were used in the regression analyses with the following results: a= 1,21 (CI: 1,03 – 1,48), b= -0,12 (CI: -0,21 – 0,06). Spearman correlation coefficient: -0,69 (CI: -0,86 – -0,39). 2-tailed p (t-approximation, corrected for ties): 0,0002. LOG [C_{50}]: 2,99. **(c)** DNA glycosylase assay with 5-ohC:G duplex DNA and human Neil1. 10 fmol 5' end-labeled 5-ohC:G duplex DNA was incubated with 20 fmol human Neil1 and increasing concentrations FAA. Enzyme activity was measured for seven

different concentrations of FAA as indicated with 8 replicates. The columns show the mean value of the replicates with 95%-CI. The total number of samples including controls was 72. 33 samples in the descending area of the curve were used in the regression analyses with the following results: a= 0,93 (CI: 0,86 – 1,00), b= -0,08 (CI: -0,12 – 0,05). Spearman correlation coefficient: -0,51 (CI: -0,72 – -0,20). 2-tailed p (t-approximation, corrected for ties): 0,0027. LOG [C_{50}]: 0,79. **(d)** DNA glycosylase assay with 5-ohC:G duplex DNA and human Neil2. 10 fmol 5' end-labeled 5-ohC:G duplex DNA was incubated with 400 fmol human Neil2 and increasing concentrations FAA. Enzyme activity was measured for seven different concentrations of FAA as indicated with 8 replicates. The columns show the mean value of the replicates with 95%-CI. The total number of samples including controls was 72. 2 samples were excluded for technical reasons. 43 samples in the descending area of the curve were used in the regression analyses with the following results: a= 0,99 (CI: 0,91 – 1,07), b= -0,11 (CI: -0,16 – 0,08). Spearman correlation coefficient: -0,56 (CI: -0,74 – -0,31). 2-tailed p (t-approximation, corrected for ties): < 0,0001. LOG [C_{50}]: 1,51.

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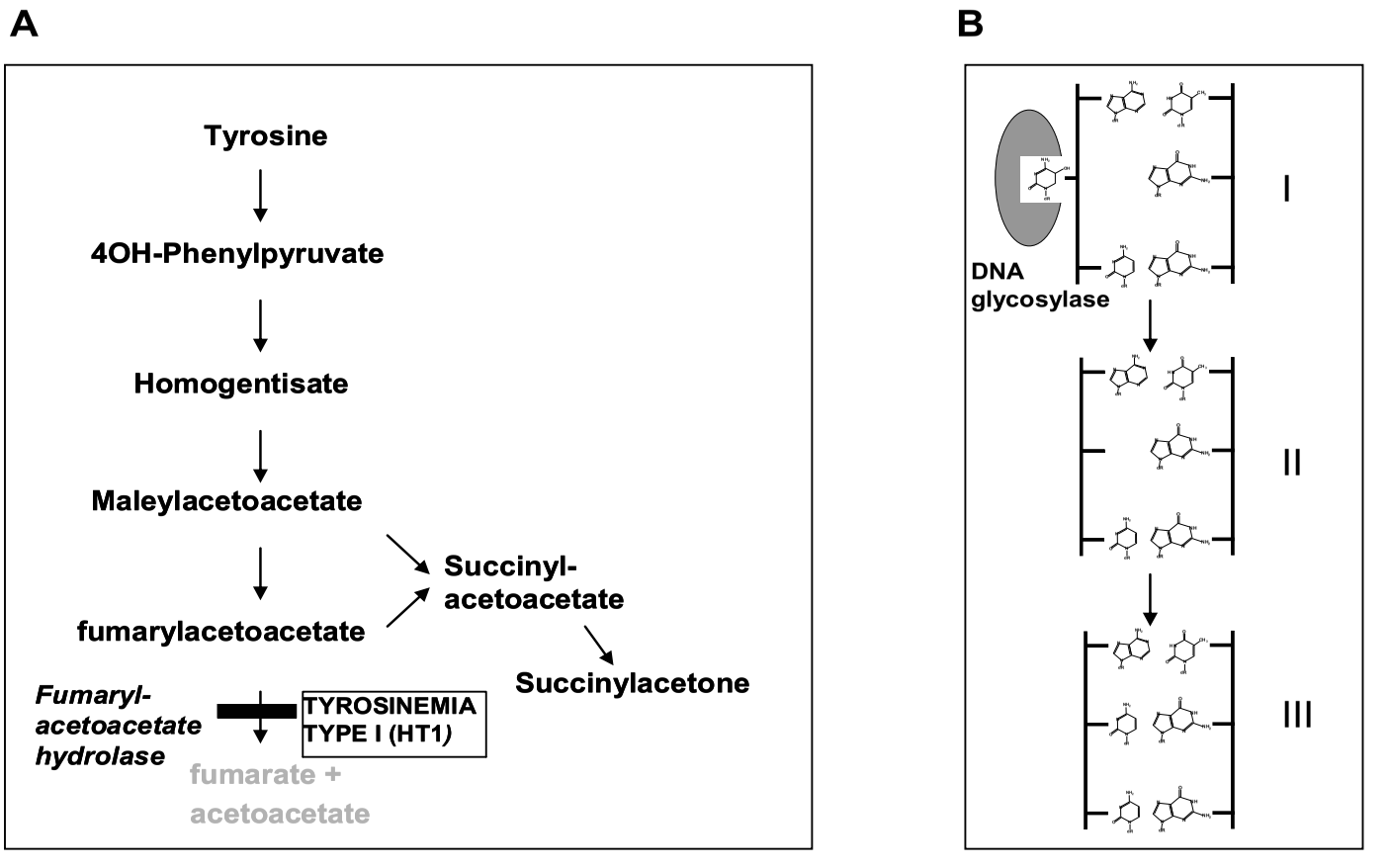
Figure 1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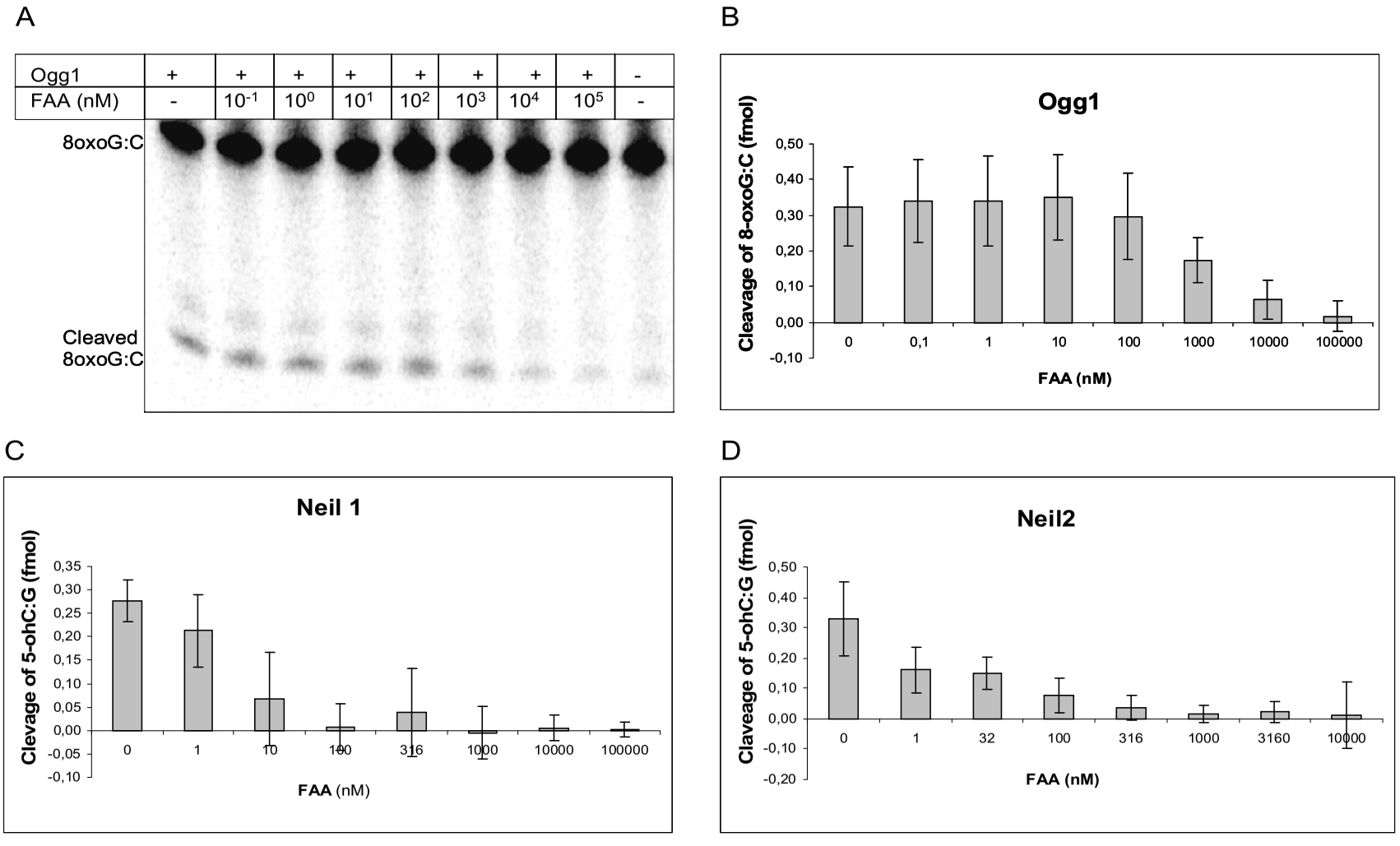
Homogentisate
Maleylacetoacetate
fumarylacetoacetate
Succinyl-
acetoacetate
Succinylacetone
Fumaryl-
acetoacetate
hydrolase
TYROSINEMIA
TYPE I (HT1)
fumarate +
acetoacetate
B
DNA
glycosylase
I
II
III

Figure 2


A
Ogg1
FAA (nM)
8oxoG:C
Cleaved 8oxoG:C
B
Ogg1
Cleavage of 8-oxoG:C (fmol)
FAA (nM)
C
Neil 1
Clevage of 5-ohC:G (fmol)
FAA (nM)
D
Neil2
Claveage of 5-ohC:G (fmol)
FAA (nM)