DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
97

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97

POLYMORPHIC GLUTATHIONE S-TRANSFERASES — BIOLOGY AND ROLE IN MODIFYING GENETIC SUSCEPTIBILITY TO SENILE CATARACT AND PRIMARY OPEN ANGLE GLAUCOMA

GUNNAR TASA

TARTU UNIVERSITY
PRESS

Department of Human Biology and Genetics, Institute of General and Molecular Pathology, University of Tartu, Estonia

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Opponents: Professor Toomas Veidebaum, Ph.D.,
Estonian Institute for Health Development
Director of Research Centre
Estonia

Professor Mihkel Zilmer, Ph.D and Dr.Med
Department of Biochemistry
University of Tartu

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The dissertation is based on the following publications:

I. **Tasa G.**, Juronen E., Uusküla M., Viikmaa M., Mikelsaar AV. Phenotyping of glutathione S-transferase M1 in the Estonian population by ELISA using GSTM1A and GSTM1B specific monoclonal antibodies. Clinica Chimica Acta 1995; 239: 191–196.

II. **Tasa, G.**, Juronen, E., Viikmaa, M., Tiidla, A., Pärlist, P., Uusküla, M., Kalev, I. and Mikelsaar, A.-V. (1996) Distribution of glutathione S-transferase T1 phenotypes in the Estonian population. Gene Geography 1996; 10: 181–189.

III. Juronen, E., **Tasa, G.**, Uusküla, M., Pooga, M. and Mikelsaar, A.-V. Purification, characterization and tissue distribution of human class Theta glutathione S-transferase T1-1. Biochem. Mol. Biol. Int. 1996; 39: 21–29.

IV. Juronen, E., **Tasa G.**, Veromann, S., Parts, L., Tiidla, A., Pulges, R., Panov, A., Soovere, L., Koka, K. and Mikelsaar, A.-V. Polymorphic glutathione S-transferases as genetic risk factors for senile cortical cataract in Estonians. Invest. Ophthalmol. Vis. Sci. 2000; 41: 2262–2267.

V. Juronen, E., **Tasa G.**, Veromann, S., Parts, L., Tiidla, A., Pulges, R., Panov, A., Soovere, L., Koka, K. and Mikelsaar, A.-V. Polymorphic glutathione S-transferase M1 is a risk factor of primary open-angle glaucoma among Estonians. Exp. Eye Res. 2000; 71:447–452.

ABBREVIATIONS

CDNB	1-chloro-2,4-dinitrobenzene
DCIP	2,6-dichlorophenol indophenol
DCM	dichloromethane
ELISA	enzyme-linked immunosorbent assay
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase
GSTA	glutathione S-transferase class alpha
GSTM	glutathione S-transferase class mu
GSTO	glutathione S-transferase class omega
GSTP	glutathione S-transferase class pi
GSTT	glutathione S-transferase class theta
GSTZ	glutathione S-transferase class zeta
IEF	isoelectric focusing
MTT	3-[4,5-dimethylthiaxol-2-yl]-2,5-diphenyltetrazolium bromide
PCR	polymerase chain reaction
POAG	primary open angle glaucoma
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TMB	3,3',5,5' tetramethylbenzidine

1. INTRODUCTION

The pathways of detoxification have long been classified as either phase I or phase II. Phase I enzymes catalyze the oxidation, reduction and hydrolysis of a substrate. Phase II enzymes conjugate substrates with various endogenous moieties, such as glucuronide, glutathione and sulfate to produce hydrophilic products, which are excreted easily from the cells (Williams 1959).

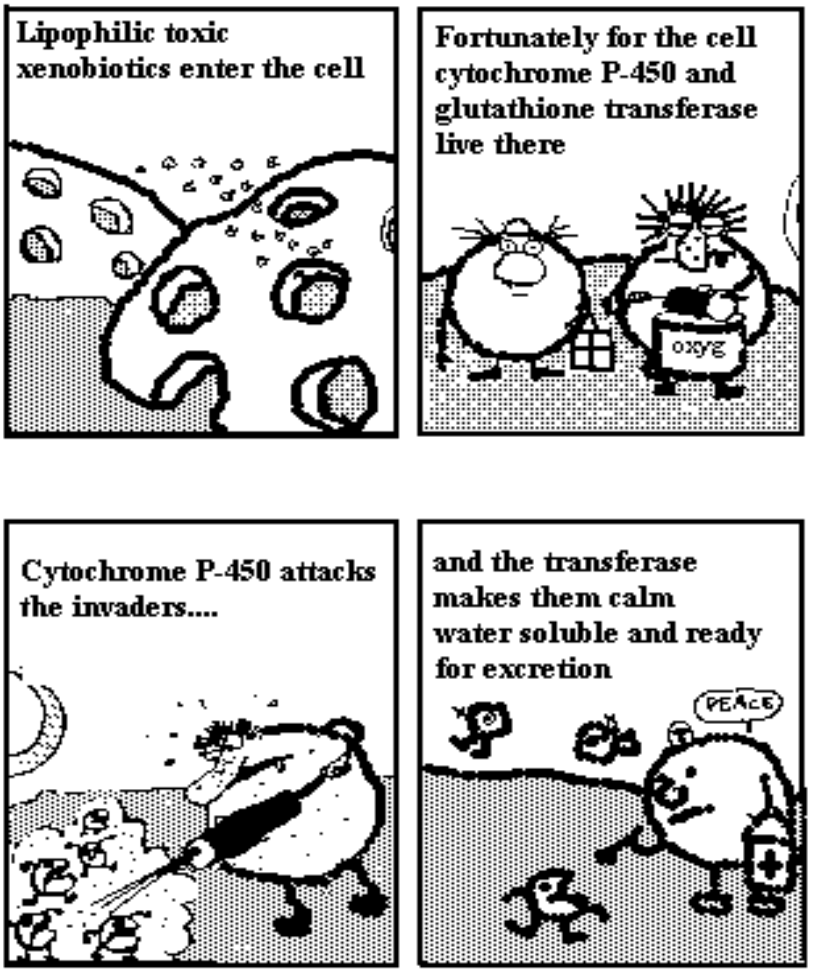

Figure 1. Detoxification of xenobiotics (Morgenstern 1983).

Detoxification enzymes are involved in detoxification reactions of certain endogenous as well as exogenous compounds. Numerous studies have demonstrated that multiple allelism at loci encoding detoxifying enzymes is the basis of inter-individual variation in detoxification metabolism. Differences in genetic susceptibility to diseases can be partly attributed to inter-individual variation in metabolic activity.

The glutathione S-transferases take a prominent place among phase II detoxifying enzymes. GSTs play critical roles in providing protection against electrophiles and products of oxidative stress, by catalyzing the formation of glutathione conjugates and by eliminating peroxides.

Idependently of catalytic activity in intracellular glutathione metabolism, some glutathione S-transferases modulate the stress-activated signals by suppressing apoptosis signal-regulating kinase 1 (Cho *et al.* 2001). Some of GSTs play a regulatory role in the mitogen-activated protein kinase pathway that participates in cellular survival and death signals via protein-protein interactions with c-Jun N-terminal kinase 1 and apoptosis signal-regulating kinase, which are activated in response to cellular stress (Townsend *et al.* 2003).

GSTs are divided into two distinct super-family members: the membrane-bound microsomal and cytosolic family members. Genetic variation is described for most of the cytosolic GSTs and some of them have a functional importance. Polymorphism in GST genes has been linked to several diseases, including different types of cancers, neurodegenerative diseases and asthma.

Detoxification mechanisms have special importance in the eye, where oxidative damage can result in a number of molecular changes that contribute to the development of glaucoma, cataract or other eye diseases. As GSTs are thought to be important in protection of the eye from oxidative damage, it is reasonable to study the role of polymorphic GSTs in modifying susceptibility to glaucoma and cataract.

The present study was carried out in order to describe distribution of polymorphic GSTs in Estonian population and to reveal the possible association between GSTs and eye diseases.

2. REVIEW OF LITERATURE

2.1. Glutathione S-transferases

2.1.1. Definition

Glutathione S-transferases (EC 2.5.1.18) were first identified in 1961 (Booth *et al.* 1961). The glutathione S-transferases are a group of multifunctional proteins that detoxify many different endogenous and exogenous compounds catalyzing the conjugation of electrophilic substances to tripeptide glutathione (γ-glutamylcysteinylglycine) (Mannervik 1985). The common feature of the enzymic conjugation reaction is the attachment of the sulphydryl group of GSH to an organic electrophile (Fig. 2).

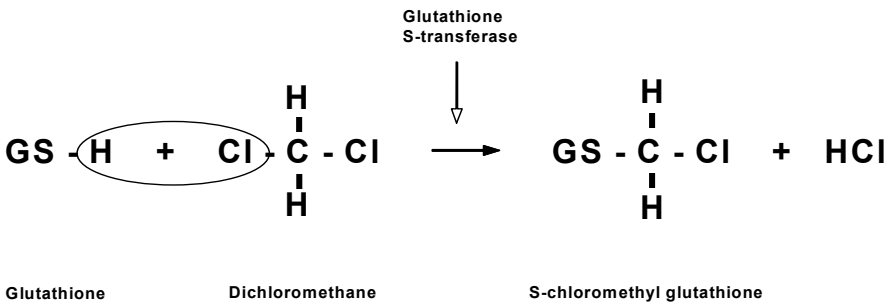

Figure 2. Conjugation of dichloromethane with glutathione.

2.1.2. Main classes of GSTs

The human GSTs are classified based on homology of amino acid sequences. Members of the same class share 75%–95% protein sequence identity and members of different classes have 25%–30% protein sequence identity (Pearson *et al.* 1993).

Human glutathione S-transferases are divided into two classes — cytosolic and microsomal GSTs. Cytosolic or soluble GSTs have been described mainly in cytoplasm, but are also present in the nucleus and the mitochondrion, where they may play an important role in defense against chemical and oxidative stress. Group of microsomal GSTs consists of six proteins, two of which are involved in the production of leukotrienes and prostaglandin E. Other microsomal GSTs catalyze the conjugation of glutathione to electrophiles and the reduction of lipid hydroperoxides. Microsomal GSTs are localized to the endoplasmic reticulum and

outer mitochondrial membrane, protecting the membranes from oxidative stress (Raza *et al.* 2002).

Among soluble GSTs expressed in tissue cytosols, following gene families are known (Hayes *et al.* 2000, Strange *et al.* 2001):

alpha class on chromosome 6	zeta class on chromosome 14
mu class on chromosome 1	sigma class on chromosome 4
theta class on chromosome 22	kappa class on chromosome 7
pi class on chromosome 11	omega chi) class on chromosome 10

2.1.3. Genetic polymorphisms in cytosolic glutathione S-transferases

GST Alpha gene family.

The Alpha gene cluster consists of 5 genes: GSTA1, GSTA2, GSTA3, GSTA4 and GSTA5, as well as 7 pseudogenes (Morel *et al.* 2002). GSTA1 gene is genetically polymorphic with two alleles — *GSTA1*A* and *GSTA1*B*, which are formed by the combination of three SNP-s in the proximal promoter of the gene at positions –567, –69, and –52. Expression of *GSTA1*A* has been demonstrated to be greater than *GSTA1*B*.

GST Mu gene family.

GSTM1 gene and clinical significance of its polymorphisms have been intensively investigated during many years. Polymorphism at glutathione S-transferase M1 locus and allelic variants were first described by Board (1981), using starch gel electrophoresis. The pattern obtained by Board (1981) for glutathione S-transferase Mu-class enzyme from liver was characteristic for a polymorphic dimeric protein, encoded by three alleles with a common null allele.

GST Mu class genes are located on chromosome 1p13.3 and they are arranged in a 20 kb cluster in following order (Xu *et al.* 1998):

5'-GSTM4-GSTM2-GSTM1-GSTM5-GSTM3-3'

There are three alleles in GSTM1 locus: *GSTM1*0*, *GSTM1*A* and *GSTM1*B*. *GSTM1*0* allele corresponds to a gene deletion and homozygotes express no protein (Seidegard *et al.* 1988). *GSTM1*A* and *GSTM1*B* differ by one base in exon 7. The catalytic properties of the enzymes encoded by these alleles are similar. A negative glutathione S-transferase M1 phenotype (GSTM1 null) and the three positive phenotypes (GSTM1 A, GSTM1 B and GSTM1 A,B) are the result of homo and heterozygotic combinations of the *GSTM1*0*, *GSTM1*A* and *GSTM1*B* alleles (Board 1981). GSTM1 has been of special interest in molecular-epidemiological studies since in Caucasian populations about half of individuals tested have homozygous gene deletion of GSTM1 and are therefore assumed to be

at greater risk when exposed to carcinogens because of reduced detoxification ability compared with those who are GSTM1 positive.

Another polymorphic gene in Mu gene cluster is GSTM3. Allelic variation of GSTM3 was discovered by Inskip *et al.* (1995). GSTM3 gene has two alleles — *GSTM3*A* and *GSTM3*B*. A 3 bp deletion in intron 6 of GSTM3*B creates a recognition motif for the YY1 transcription factor and therefore expression of GSTM3 alleles may be differently regulated. *GSTM3*B* and *GSTM1*A* are in linkage disequilibrium. (Inskip *et al.* 1995).

Recently a genetic polymorphism was identified also in GSTM4 locus by Liloglou *et al.* (2002). There is a C-T polymorphism in intron 6 of the GSTM4 gene.

GST Theta gene family.

First hints about the existence of a polymorphic glutathione S-transferase Theta came from Peter *et al.* (1989), demonstrating that erythrocytes from only 60% of individuals ("conjugators") catalyse the conjugation of methyl chloride with glutathione while the remaining 40% ("non-conjugators") lack this activity. There are two genes in GST theta family — GSTT1 and GSTT2, which are located on chromosome 22 and are separated by about 50 kb (Coggan *et al.* 1998). Like GSTM1, the GSTT1 locus has a deleted allele — *GSTT1*0* and persons having the deletion in both chromosomes express no protein (Pemble *et al.* 1994). The combinations of functional (*GSTT1*1*) and non-functional (*GSTT1*0*) alleles are thought to give three phenotypes: GSTT1 +/+, GSTT 1+/– and GSTT1 –/– (Warholm *et al.* 1994). About 20% of Caucasians are homozygotes for a gene deletion of GSTT1 and are probably less protected against environmental carcinogens.

GST Pi gene family.

Genetic polymorphism of GSTP1 was described in 1997 (Ali-Osman *et al.* 1997). The three alleles of GSTP1 result from A→G and C→T transitions at nucleotides 313 and 341. The transitions change codon 104 from ATC (Ile) in *GSTP1*A* to GTC (Val) in *GSTP1*B* and *GSTP1*C* and change codon 113 from GCG (Ala) to GTG (Val) in *GSTP1*C* (Ali-Osman *et al.* 1997). Both amino acid changes are in the electrophile-binding active site, resulting in functionally different GSTP1 proteins (Ali-Osman *et al.* 1997).

GST Zeta gene family.

Two single nucleotide polymorphisms have been found at coding region of GSTZ1 gene: A→G at nucleotide 94 and A→G at nucleotide 124. These nucleotide changes result in Lys-32 Glu and Arg-42 Gly substitutions respectively in protein sequence (Board *et al.* 1997). Three alleles can be found as a result of combination of the two SNP-s:

1) *GSTZ1*A* with nucleotide A at position of 94 and A at position of 124

2) *GSTZ1*B* with nucleotide A at position of 94 and G at position of 124
3) *GSTZ1*C* with nucleotide G at position of 94 and G at position of 124.

Recently a new allele of human GSTZ1, characterized by a Thr82Met substitution has been identified by Blackburn *et al.* (2001). The new allele is termed *GSTZ1*D* and it has haplotype Glu32/Gly42/Met82. Like GSTZ1b-1b and GSTZ1c-1c, the GSTZ1d-1d isoform has low activity with dichloroacetic acid as compared to GSTZ1a-1a.

GST Omega gene family.

Class Omega has been discovered by analysis of the expressed sequence tag database and sequence alignment (Board *et al.* 2000). Functional analysis determined that GSTO1 lacks activity with most GST substrates but has uniquely high thiol transferase activity. Board *et al.* (2000) suggested that the wide expression and conserved sequence of GSTO1 indicates that it may have a significant housekeeping function.

An investigation of the genomic organization of human GSTO1 identified a second actively transcribed member of the Omega class (GSTO2). Both GSTO1 and GSTO2 are composed of six exons and are separated by 7.5 kb on chromosome 10q24.3 (Whitbread *et al.* 2003). Two non-synonymous polymorphisms have been described in GSTO 1–1 gene by Toshiko *et al.* (2003): 419C/A and 650C/A resulting in amino acid alterations Ala140Asp and Thr217Asn, respectively. 140Ala-217Thr isoform of GSTO1 has highest thioltransferase activity, whereas activity was decreased to 75% in the case of 140Asp-217Thr isoform and to 40% in the case of 140Ala-217Asn isoform. Whitebread *et al.* (2003) described an additional polymorphism, deletion of residue E155, that appears to contribute towards increased enzymatic activity.

To the best of my knowledge no validated polymorphisms have been described for the Kappa family of GST's (Morel *et al.* 2004) and for the Sigma class GST's (Kanaoka *et al.* 2002).

2.2. Distribution of gene frequencies of some polymorphic glutathione S-transferases in different ethnic groups

Not all of the results cited below came from investigations where description of gene frequencies of polymorphic glutathione S-transferases in different ethnic groups was the primary objective. In some cases data from case-control studies, designed for elucidating the role of GST's in modifying genetic susceptibility to different diseases, was used. It is obvious that control groups of case-control studies can be considered as a source of information about the distribution of gene frequencies in the normal population only with certain reservations.

Distribution of GSTM1 variants in some populations is given in Table 1. GSTM1 null phenotype is quite equally distributed in different Caucasian and Asian populations with frequency of about 50%, while its frequency in Nigerians is only 22%. The distribution of two positive alleles varies greatly between East Asian and African populations — Chinese and Japanese are characterized by low occurrence of GSTM1A phenotype and high frequency of GSTM1B phenotype, while the frequency proportions in Nigerians are contrary.

Table 1. The distribution of GSTM1 variants in some populations.

Population	n	GSTM1 null (%)	GSTM1 A (%)	GSTM1 B (%)	GSTM1 A,B (%)	Reference
Nigerian	69	21.7	71.0	5.8	1.4	Zhao *et al.* 1994
Indian	43	34.9	34.9	16.2	14	Board 1981
Finnish	142	43.7		56.3		Hirvonen *et al.* 1993
English	225	42.0		58.0		Zhong *et al.* 1991
	153	52.0	26.0	16.0	6.0	Zhao *et al.* 1994
French	56	43.0	43.0	9.0	5.0	Laisney *et al.* 1984
	45	53.3		46.7		Groppi *et al.* 1991
Japanese	168	47.7	8.2	41.2	2.9	Harada *et al.* 1987
	84	47.6		52.4		Harada *et al.* 1992
Russian	100	49.0	41.0	7.0	3.0	Afanasyeva *et al.* 1990
Sweden	248	54.0		46.0		Seidegard *et al.* 1985
German	145	55.2		44.8		Brockmöller *et al.* 1992
Chinese	96	58.3	10.4	29.3	2	Board 1981
Caucasian from Australia	40	65.0	15.0	20.0	0	Board 1981

Homozygous deletion of **GSTT1** is most prevalent in some Asian populations with frequency up to 60%. In Caucasian populations the frequency of GSTT1 negative individuals ranges from 10–25%. Distribution of GSTT1 phenotypes in some ethnic groups can be found in Table 2.

Table2. GSTT1 polymorphism in different ethnic groups.

Population	n	GSTT1 negative (%)	GSTT1 positive (%)	Reference
Mexican-American	73	9.7	90.3	Nelson *et al.* 1995
Swedish 1	208	11.1	88.9	Warholm *et al.* 1994
Swedish 2	78	18.0	82.0	Jakobsson *et al.* 1995
US Caucasian – New England	185	15.7	84.3	Nelson *et al.* 1995
US Caucasian – nationwide	257	23.7	76.3	Nelson *et al.* 1995
Indian	158	16.0	84.0	Lee *et al.* 1995
African-American	119	21.8	78.2	Nelson *et al.* 1995
Malays	167	38.0	62.0	Lee *et al.* 1995
Korean	103	60.2	39.8	Nelson *et al.* 1995
Chinese 1	45	64.4	35.6	Nelson *et al.* 1995
Chinese 2	187	58.0	42.0	Lee *et al.* 1995

Most frequent **GSTP1** isoform is Ile/Ile, having frequency of approximately 50% in Caucasian populations and 60–70% in Asian populations (Table 3).

Table 3. GSTP1 isoform frequencies in different populations.

Population	n	Ile/Ile (%)	Ile/Val (%)	Val/Val (%)	Reference
Spanish	201	50	39	11	To-Figueras *et al.* 2002
Finnish	481	55	38	7	Mitrunen *et al.* 2001
Northern Euro-pean Caucasians	297	46	40	14	Layton *et al.* 1999
Brazilian	591	50	38	12	Rossini *et al.* 2002
Chinese 1	119	71	29	1	Wang *et al.* 2003
Chinese 2	38	63	34	3	Wang *et al.* 2003
Korean	110	67	30	3	Pae *et al.* 2003

GSTM3 genotypes are also quite similarly distributed in different Caucasian population with prevailing A/A genotype (Table 4).

Table 4. Distribution of GSTM3 genotypes in some Caucasian populations.

Population	n	A/A (%)	A/B (%)	B/B (%)	Reference
Spanish	202	66	31	3	To-Figueras *et al.* 2002
Finnish	480	75	23	2	Mitrunen *et al.* 2001
Northern European Caucasians	295	75	20	5	Layton *et al.* 1999

2.3. Role of polymorphic glutathione S-transferases in modifying genetic susceptibility to diseases

Most biological processes are catalysed by means of enzymes and study of these in health and disease is important for our understanding of both biology and disturbed function (Hayes *et al.* 1991). Individuals with genotypes that decrease activity of detoxification enzymes have been assumed to be at risk when exposed to elevated levels of carcinogens and toxic chemicals compared with those who have higher levels of detoxification enzymes (Board 1981, Warholm *et al.* 1994).

Association studies offer powerful approach to identify genetic variants that influence susceptibility to common diseases (Lohmueller *et al.* 2003).

2.3.1. Neoplastic diseases

Lung cancer.

Lung cancer was the first disease, where association was extensively sought between polymorphic glutathione S-transferases and a pathological condition. Seidegard *et al.* (1986) found in smokers a greater proportion of the GSTM1 positive individuals among controls as compared to lung cancer patients. Up to now very many studies have been performed on different populations trying to establish links between polymorphic expression of different GSTs and lung cancer risk but the results have been conflicting (Hayes *et al.* 2000) ranging from complete absence of association (London *et al.* 1995) to strong genetic predisposition by GST's (Pinarbasia *et al.* 2003). Efforts to determine whether the relationship between a certain GST subtype and lung cancer may differ according to histological type, history of exposure to tobacco, subjects' ethnic background, gender or by function of other biotransformation enzymes have also produced conflicting results (Houlston 1999). The results of meta-analyses allow to conclude

that among GST's, GSTM1 locus has strongest association with lung cancer. GSTM1 deficiency can be considered as a moderate risk factor for lung cancer (Nazar-Stewart *et al.* 2003). The effect is most evident among heavy smokers. McWilliams *et al.* (1995) has calculated that GSTM1 null phenotype accounts for approximately 17% of lung cancer cases because of the high prevalence of GSTM1 deficiency.

Head and neck cancers.
Association between polymorphic glutathione S-transferases and squamous cell carcinoma of head and neck was first reported by Trizna *et al.* (1995). Results of twenty five studies are reviewed by Lohmueller *et al.* (2003) on that subject and the conclusion drawn by the authors was that lack of the GSTM1 gene can be considered as moderate genetic risk factor of head and neck cancers.

Breast cancer.
Genetic factors are involved in one-fourth of breast cancer cases (Lichtenstein *et al.* 2000). As germline mutations in high-penetrance cancer susceptibility genes account for only up to 5%, relatively common genes with moderate effect are likely to account for a much higher proportion of breast cancer cases (Johnson-Thompson *et al.* 2000). Current evidence supports some role for oxidative metabolites, in particular catechol oestrogens, in the initiation of breast cancer and therefore the potential role of polymorphic genes encoding enzymes involved in conversation of oestrogen metabolites is hypothesed. The studies conducted to date on polymorphic estrogen metabolizing enzymes and breast cancer risk have yielded contrasting results (Mitrunen *et al.* 2003). Some of them suggested an association between polymorphic GST's, especially with GSTM1 null genotype, while others found no association (Ruano-Ravina *et al.* 2003, Mitrunen *et al.* 2003). Evidently more studies are needed before any firm conclusions can be drawn.

Haematological conditions.
Myelodysplastic syndrome is the clonal proliferative disorder of bone marrow that often progress to acute myeloid leukaemia. Association study by Chen *et al.* (1996) revealed that GSTT1 null genotype increases the risk to develop myelodysplastic syndrome. The frequency of GSTT1 null genotype reported by Chen *et al.* (1996) among myelodysplastic syndrome cases was considerably higher than in controls (46% vs 16%) and the genotype was found to confer a 4.3-fold risk of myelodysplastic syndrome disorder. The initial finding of association between GST's and myelodysplastic syndrome was later confirmed by the study of Sutton *et al.* (2004).

Yuille *et al.* (2002), studying patients with chronic lymphocytic leukaemia found, that frequency of both GSTM1 and GSTT1 null genotypes and the GSTP1-Ile allele was higher in patients than in controls. The risk of chronic lymphocytic

leukaemia associated with possession of all 3 high-risk genotypes was increased 2.8-fold.

Voso *et al.* (2002) demonstrated that GST deletions predicted poor response to chemotherapy and shorter survival among patients of acute myeloid leukaemia.

Study by Takanashi *et al.* (2003) revealed that the GST double null genotype is more predictive than any other parameter of early relapse of childhood B-precursor acute lymphoblastic leukaemia.

2.3.2. Non-neoplastic diseases

Carless *et al.* (2002) examined the role of GSTM1, GSTT1, GSTP1 and GSTZ1 gene polymorphisms in susceptibility to **solar keratoses**. No significant differences were detected in GSTP1 or GSTZ1 allele or genotype frequencies, however, a significant association was found between GSTM1 genotypes and solar keratoses. GSTM1 null individuals were found to have approximately 2-fold increase in risk for solar keratoses development and a significantly higher increase in risk in conjunction with high outdoor exposure.

Lee *et al.* (2001) investigated whether polymorphisms in GSTM1 and GSTT1 loci can affect the likelihood of developing **aplastic anaemia**. They found that the incidence of GSTM1 and GSTT1 gene deletions was significantly higher for aplastic anaemia patients than for healthy controls. Among the aplastic anaemia patients, 17.5% had chromosomal abnormalities at the time of diagnosis, and all aplastic anaemia patients with chromosomal abnormalities showed GSTT1 gene deletions. The initial results reporting positive association were later replicated also by Sutton *et al.* (2004).

Based on the results of many investigations demonstrating that exposure to pesticides or herbicides increases **Parkinson disease** risk up to 4-fold and that glutathione S-transferases metabolize them, Menegon *et al.* (1998) pursued the hypotheses that polymorphisms in GST loci may be associated with susceptibility to Parkinson disease. Indeed, association was found with the GST isoform expressed in the blood-brain barrier — GSTP1.

Fraser *et al.* (2003) hypothesized that risk of **systemic lupus erythematosus** associated with sun exposure is modulated by GSTM1, GSTT1, and GSTP1 genotypes. They genotyped 243 DNA samples form cases and 298 samples from control persons and found, that GSTM1 homozygous null genotype may modify the effect of occupational sun exposure on the risk of systemic lupus erythematosus in Caucasians.

2.4. Cataract

2.4.1. Definition and prevalence

Cataract is defined as a progressive increase in lens opacification which deteriorates the quality of the retinal image, reducing visual acuity and if untreated, leads ultimately to blindness. Age-related cataract is the leading cause of visual impairment worldwide and it is estimated that globally more than 20 million people are bilaterally blind from this condition (Lawrenson 2003). Overall, 30% of persons of 65 years and over (Desai *et al.* 1999) have been found to have visually impairing cataract in one or both eyes. It has been estimated that some degree of lens opacity is present in 50% of those over 60 years.

2.4.2. Morphology of the eye

The anatomy of the eye (Shun-Shin 1999) is shown in Figure 3.

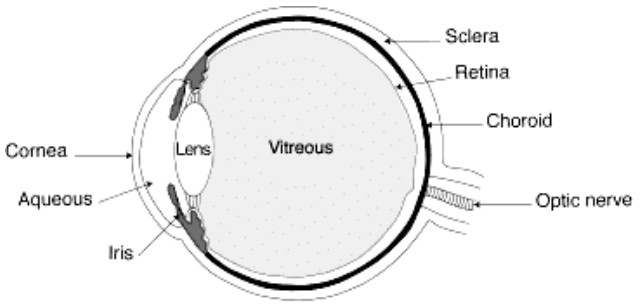

Figure 3. Anatomy of the eye.

The lens helps to focus light on to the retina. Structure of the lens is shown diagrammatically in Figure 4.

Lens continues to grow throughout life, adding new layers to its outside. The outer layers, which are added subsequently, are known as the cortex . The central portion of the lens is called the nucleus and represents the part of the lens that was present at birth. The whole body sits within a membrane called the capsule. Cell growth takes place in a layer of epithelial cells at the front of the lens just inside the anterior capsule.

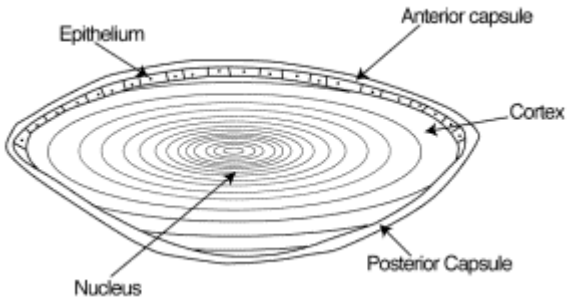

Figure 4. Structure of the lens.

2.4.3. Classification of cataract

Classification of cataract based on localization and aetiology (Chylack *et al.* 1993):

1. Classification by anatomic location
   a) cortical
   b) nuclear
   c) posterior subcapsular
   d) mixed
2. Classification by aetiology.
   a) age-related cataract
   b) congenital cataract
   c) traumatic cataract
   d) cataract associated with intraocular disease (uveitis, glaucoma, retinal detachment, retinal degenerations, persistent hyperplastic primary vitreous, aniridia, high myopia)
   e) cataract associated with systemic diseases
      – metabolic disorders (diabetes, galactosaemia, hypoparatyroidism)
      – skin diseases (atopic dermatitis, congenital ectodermal dysplasia, Wilson syndrome, Fabry syndrome, Refsum syndrome)
      – connective tissue disorders (myotonic dystrophy, Marfan syndrome)
      – renal disease (Alport syndrome, Lowe syndrome)
      – central nervous system (neurofibromatosis II, Sjogren syndrome)
   f) cataract associated with noxious agents
      – ionising radiation (X-ray, ultraviolet)
      – drug-induced (steroids, chlorpromazine).

2.4.4. Aetiology of cataract

Most cataract cases are likely to have multifactorial aetiology. Some of the factors having influence over cataract formation are presented in Figure 5 and are discussed below.

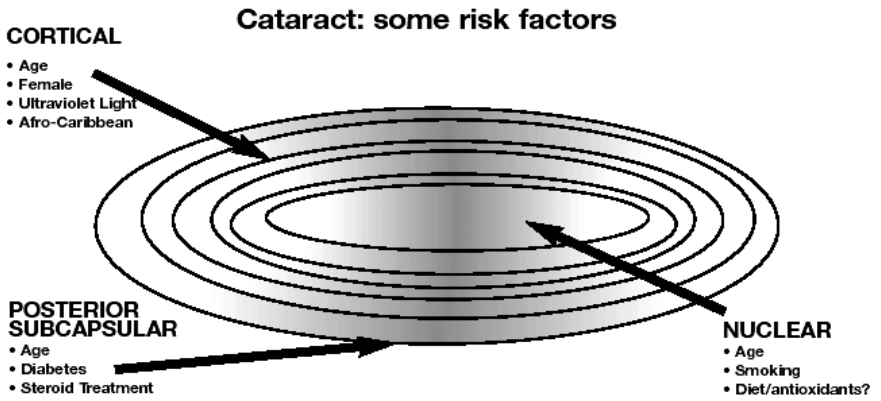

Figure 5. Diagrammic representation of some of the more important risk factors of cataract (Hammond 2001).

1) Genetic background.
Twin and family studies support the view that cataract formation is strongly influenced by genetic factors. Klein *et al.* (1992) found a significant sibling correlation in both nuclear and cortical cataract. Twin studies by Hammond *et al.* (2000) have been shown that genetic contribution for nuclear and cortical cataract was 48% and 59% respectively. Therefore about half of the variability of cataract formation is explained by genetic factors. Age explained 38% of the variability, while environment about 14%.

2) Age.
Age is a strong risk factor for cataract (Klein *et al.* 1992). The prevalence of visually impairing cataract rises steadily with age (Desai *et al.* 1999): 16% in the 65 to 69 year age group; 24% in persons of 70 to 74 years of age; 42% in those 75 to 79 years of age; 59% in 80 to 84 years and 71% in persons of 85 years or more.

3) Smoking.
A strong association has been found between nuclear cataract and smoking. The risk appears to be cumulative. Flayde *et al.* (1989) reported that smokers of more than 25 cigarettes a day were three times as likely to develop cataracts as non-smokers. The precise mechanism by which lens damages by smoking is not clear. However, smokers seem to have an impaired ability to cope with oxidative stress in

general, and several constituents of cigarette smoke are capable of causing chemical modification of lens proteins.

4) Exposure to UV-light.
Measurements of the exposure of individuals in cohort and case-control studies have shown that UV light is a major risk factor for cortical cataract (Lawrenson 2003). Taylor *et al.* (1988) calculated that doubling exposure to UV-light increased the risk of cortical cataract by 60%.

5) Female gender.
Excess of cataract patients among women has been shown in many studies (Mitchell *et al.* 1997). The overall prevalence ratio (females : males) was found to be 1.22. That phenomenon can be explained by the effect of oestrogen.

6) Diabetes.
Sperduto *et al.* (1984) found cataract in 19% of diabetics compared to 12% in non-diabetics. There is also in vitro and in vivo evidence of the causation of cataract by elevated glucose levels and osmotic changes (Veromann *et al.* 2003).

7) Treatment with steroid drugs.
The cataractogenic influence of steroid drugs has been well described. Steroid drugs are mostly linked to posterior subcapsular cataract.

8) Alcohol consumption.
Alcohol consumption has been found to be cataract risk factor (Harding *et al.* 1989). Harding *et al.* (1991) demonstrated that heavy beer drinking is associated with a two-fold increase of cataract risk.

9) Strong dehydration.
Case-control studies in India showed that severe diarrhoea and dehydration, resulting in confinement to bed for at least three days, carried a three to four-fold risk for developing cataract in later life (Hammond 2001).

10) Socio-economic factors.
An excess of cataract has been found in rural population and among persons with lower education in terms of years at school. Also the non-professionals have higher rate of cataract than professionals (Leske *et al.* 1991). Probably these factors can be explained by other factors such as smoking, diet, exposure to sunlight and alcohol consumption.

11) Antioxidants.
Oxidation of lens proteins is associated with cataract formation. High levels of antioxidants, such as vitamins can be protective. The Lens Opacities case-control study in Boston (Leske *et al.* 1991) found that regular intake of multivitamins is protective of all types of cataract. A study in the United States using data of 50 000 persons determined that risk of cataract formation is 45% lower in women taking vitamin C supplements for at least 10 years.

12) Myopia and glaucoma.
Although myopia and glaucoma have been reported as strong risk factors in Oxfordshire case-control studies (Harding *et al.* 1993), further research is necessary on that subject before firm conclusions can be drawn.

2.4.5. Pathogenesis of age related cataract

The pathogenesis of age-related cataract is both multifactorial and highly complex. It is also likely that aetiological differences exist between each morphological sub-type. The lens protein content is surprisingly high — 33% (Harding *et al.* 1991) and there is a consensus of opinion that posttranslational modification of lens proteins plays a central role in all forms of cataract. These chemical changes accumulate over a number of years (Spector 1995, Ottonello *et al.* 2000). Reactive oxygen species such as peroxide, superoxide and hydroxyl radicals are thought to be a major cause of protein modification. The healthy lens is normally well equipped with antioxidants, e.g. glutathione, ascorbate and catalase, which protect lens proteins against reactive oxygen species. Among them glutathione is the most important antioxidant in lens (Lawrenson 2003). The fact that lens cells and proteins are very long-lived means that a cataract may result from an event that affected the eye many years earlier or from chronic low-level exposures.

2.5. Glaucoma

2.5.1. Definition and prevalence

The glaucomas are a range of disorders that have in common a characteristic optic neuropathy with associated visual field loss. Irreversible damage of optic nerve is the basic condition. Uncontrolled glaucoma is characterized by a loss of peripheral vision and development of tunnel vision. Elevated intraocular pressure is a common feature but not necessarily required for the glaucomas.

Glaucomas are the second commonest cause of blindness in the world, and the most common cause of irreversible blindness (Khaw *et al.* 2004). Although the number of people suffering from glaucomas varies in different populations and reports, it is estimated that approximately seventy million people are affected worldwide (Quigley 1996, Borras *et al.* 2003).

2.5.2. Classification

Glaucoma can be classified into primary, secondary and developmental glaucomas. (Kroese *et al.* 2002). Three major subtypes of glaucomas are primary open angle glaucoma (POAG), primary acute closed angle glaucoma and congenital glaucoma (Ray *et al.* 2003).

2.5.3. Open angle glaucoma — definition and epidemiology

Definition.
Open-angle glaucoma is an asymptomatic, progressive optic neuropathy characterized by enlarging optic disc cupping and visual field loss (Distelhorst *et al.* 2003).

Epidemiology of POAG.
POAG is the most prevalent form of glaucomas with approximately 33.1 million sufferers around the world (Quigley 1996). Approximately 3% of blindness in Caucasian population is caused by the primary open angle glaucoma (Quigley *et al.* 1997). The overall prevalence of POAG is about 3% of people aged over 45 years. The prevalence rises with age to approximately 9% in 80 to 90 year olds (Cockburn 2000).

2.5.4. POAG as a multifactorial disease

No single factor has been identified as a cause of primary open−angle glaucoma, thus POAG is a multifactorial disorder (Quigley *et al.* 1994). Risk factors for primary open angle glaucoma as listed by Khaw *et al.* (2004) are:

1) Level of intraocular pressure.
Elevated intraocular pressure is a strong, modifiable risk factor for open-angle glaucoma, but it is not diagnostic. Some patients with glaucoma have normal intraocular pressure (i.e., normal-pressure glaucoma), and many patients with elevated intraocular pressure do not have glaucoma (Distelhorst *et al.* 2003).

2) Age.
Patients at increased risk for open-angle glaucoma include blacks older than 40 years and whites older than 65 years (Distelhorst *et al.* 2003).

3) African-Caribbean origin
In the black American population, POAG prevalence is estimated to be six times as high in certain age groups compared to whites (Racette *et al.* 2003).

4) Thin corneas

5) Large cup to disc ratio

6) Severe myopia

7) Family history
Risk indicators of open-angle glaucoma correlate highly in families, and the patterns are consistent with the hypothesis of genetic determinants of these factors. Heritability estimates were 0.36, 0.55, 0.57, and 0.48 for intraocular pressure, optic cup diameter, optic disc diameter, and cup-to-disc ratio, respectively (Klein *et al.* 2004). First degree relatives to glaucoma cases have 8–10 times increased risk of developing the disease (Wolfs *et al.* 1998).

2.5.5. Genes associated with glaucoma

A number of genes have now been identified as possible factors in many cases of glaucoma. These include the discovery of three genes — myocilin, optineurin and CYP1B1 (Ray *et al.* 2003).

Recently **OPTN gene** that encodes optineurin protein has been identified as one of the genes responsible for adult-onset POAG (Rezaie *et al.* 2002). Rezaie et al found the presence of mutations in approximately 17% of patients with POAG. Mutations in **MYOC gene** that encodes myocilin protein were first associated with juvenile primary open angle glaucoma (Stone *et al.* 1997). Fingert *et al.* (1999) found that myocilin mutations were observed in a significant percentage of adult-onset POAG patients. Defects in this gene occur in 3–6% of patients with adult−onset and juvenile open−angle glaucoma. Myocilin is a sticky protein and

persons with mutated gene appear to overproduce it, which can clog the trabecular meshwork. Myocilin is also known as a stress protein and is overproduced during periods of stress.

2.6. Glutathione S-transferases and eye

The blood-aqueous barrier and its scavenging system preserve the clarity of the lens of the eye. This barrier consists mainly of the arylhydrocarbon hydroxylase and the glutathione S-transferase systems and GSTs are considered to be key enzymes, protecting the eye from toxic chemicals and electrophiles (Sekine *et al.* 1995). Moreover, in several studies linkage between cigarette smoking and increased risk of developing cataracts has been shown (Christenv *et al.* 1992). Based on this knowledge involvment of GSTs in cataract formation has been hypothesized.

Study performed to identify retinal proteins that are the targets of serum autoantibodies in patients with glaucoma demonstrated, that patients had significantly higher titers of anti-GST antibody as compared to the controls (Yang *et al.* 2001). That provides evidence suggesting that persons expressing GSTM1 are at increased risk of developing auto-antibodies against the protein that has been linked to an increased risk of developing glaucoma.

2.7. Ability of association studies to find out genetic factors of common diseases

Case–control studies are the most commonly used methods to seek potential associations between genetic polymorphisms and common diseases. However, association studies are plaqued by the impression that they are not consistently reproducible. Quite often the first reported association fails to be replicated in other populations. The inconsistency may be due to false positive results, false negative results or can reflect the true variability in association among different populations. False positive or negative results can be caused by ethnic admixture, population stratification (i.e. inclusion of individuals from a heterogeneous genetic background) or by a small sample sizes that could lack power to detect modest genetic effects. Recently a meta-analysis study was performed by Lohmueller *et al.* (2003), summarizing data from different loci and from large number of publications to investigate the ability of association studies to find genetic factors of common diseases. The study leads to encouraging conclusion, that there are many common variants in the human genome with modest but real effects on

common disease risk, and properly performed association studies will convincingly identify such variants.

One of the factors influencing results of association studies is the accuracy of phenotyping and/or genotyping results. A meta analysis has indicated a stronger relationship between GSTM1 phenotype and risk to lung cancer than between GSTM1 genotype and the risk (McWilliams *et al.* 1995). It is possible that genotype measurements do not correspond completely with function due to regulatory or post-transcriptional influences on enzyme expression or function. Genotype is a convenient but sometimes imperfect marker for function, resulting in misclassification of GST status and attenuated estimates of risk.

Therefore, proper methodological basis should be used for association studies and they should be conducted among well-defined study groups of a homogenous origin.

3. AIMS OF THE STUDY

The general aim of the present work was to investigate polymorphic glutathione S-transferases in order to evaluate possible associations between certain genotypes of the detoxification enzymes and genetic susceptibility to glaucoma and cataract.

Based on this the present study was aimed:

1) to create proper methodological basis suitable for large-scale population screening
2) to describe gene frequencies of polymorphic glutathione S-transferases in Estonian population
3) to perform association study in order to seek potential link between polymorphic glutathione S-transferases and senile cataract
4) to study possible association between polymorphic GSTs and open angle glaucoma.

4. MATERIALS AND METHODS

4.1. Isolation of GSTM1 and GSTT1 proteins

The aim of the protein purification was to isolate the proteins for producing monoclonal antibodies against polymorphic glutathione S-transferases.

4.1.1. GSTM1 isolation

GSTM1 proteins were isolated from liver samples, obtained at autopsy within 24 hours of death. The GSTM1 phenotype was identified using polyacrylamide isoelectric focusing followed by specific enzyme staining as described below. Liver cytosols having only GSTM1a-1a or GSTM1b-1b homodimeric proteins were used for enzyme purification. Both homodimeric GSTM1 isoenzymes were isolated separately by two-step liquid chromatography methods: glutathione agarose affinity chromatography and chromatofocusing with PBE94 gel (Pharmacia, Uppsala, Sweden) as described by Kashiwada *et al.* (1991) and Strange *et al.* (1992). N,N'-disuccinimidylcarbonate activated glutathione agarose was made by Jüri Parik (Department of Evolutionary Biology, Institute of Molecular and Cell Biology, University of Tartu). The identity of chromatography peaks was confirmed by isoelectric focusing and enzymic activity assays towards CDNB. The purity of enzymes was established by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, performed according to Laemmli (1970).The protein concentrations were determined by the Bradford method, using mouse IgG as a standard (Bradford 1976).

4.1.2. GSTT1 isolation

GSTT1 protein was isolated from liver tissues and erythrocytes. Liver tissues expressing GSTT1–1 were found using activity measurement towards dichloromethane as described below. Individuals being "conjugators" were found using determination of ability of erythrocytes to conjugate methyl chloride with glutathione as described below. Protein isolation was performed by Erkki Juronen as published (Juronen *et al.* 1996).

4.2. Electrophoresis of proteins

4.2.1. Isoelectric focusing

Isoelectric focusing was performed to find GSTM1 positive liver samples for glutathione S-transferase M1 isolation, as well as for isoelectric point determination of purified GSTM1 and GSTT1 proteins.

Acrylamide/N,N'-Methylenebisacrylamide gel (T=4%, C=4%), containing 2% of Ampholines (80% of Ampholine 3.5/10, 10% of Ampholine 4/6.5 and 10% of Ampholine 4/8) was used for analytical isoelectric focusing. Electrophoresis was performed in a Multiphor II Electrophoresis Unit at +4°C, using gel size of 125 * 260 mm. Two different isoelectric focusing protocols were employed: 1) IEF in ultrathin polyacrylamide layers (360 μm), when a quick separation of proteins could be performed and 2) IEF with thick polyacrylamide gels (2 mm), if the large sample volumes were used or retaining of enzymic activity during electrophoresis was essential. Running conditions were as follows: 1) for fast electrophoresis, 15 minutes prefocusing at 10 W and 45 minutes focusing at 14 W and 2) for IEF in thick gels, 20 minutes of prefocusing at 2 W, followed by the overnight focusing at 1 W.

After IEF of purified GSTs, the focused proteins were visualized by silver staining. GSTM1 phenotypes of focused liver cytosols were detected by a specific histochemical staining (Board 1981). In brief, the localization of enzyme activity was revealed by coating of IEF gel with an agarose, containing CDNB (2 mM) as a substrate for GSTs and reduced glutathione (2.2 mM) as a co-substrate. After incubation of 45 minutes at 37°C, gel was covered with a second agarose layer containing 2,6-dichlorophenol indophenol (0.1 mM) and 3-[4,5-dimethylthiaxol-2-yl]-2,5-diphenyltetrazolium bromide (0.1 mM). The formation of red formazane was prevented in the places where GSH had been conjugated to CDNB by GSTs and enzymes were visualized as colourless bands (Figure 6).

Isoelectric point determination was performed using protein pI standards from Pharmacia. Silver-stained gels were scanned by Model SL-2D/1D UV/VIS densitometer (Biomed Instruments, Inc., Fullerton, CA) and the data analyzed using the manufacturer software.

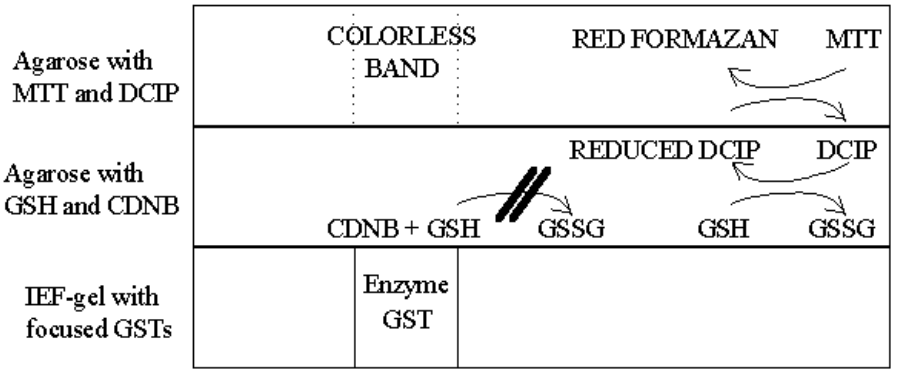

Figure 6. Specific staining of GSTs in IEF gels. Formazan is formed only in places where the reduced glutathione has not been used for conjugation with CDNB by the action of glutathione S-transferases. GSH – reduced glutathione; GSSG – oxidised glutathione; DCIP – 2,6-dichlorophenol indophenol; MTT – 3-[4,5-dimethylthiaxol-2-yl]-2,5-diphenyltetrazolium bromide; CDNB – 1-chloro-2,4-dinitrobenzene.

4.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for molecular weight determination and evaluation of purity of isolated GSTs. SDS-PAGE was carried out according to Laemmli (1970).

4.3. Immunoblotting

Proteins were run in 15% SDS-PAGE using the Laemmli system or focused using thick polyacrylamide as described above, and then transferred onto nitrocellulose filters (45-µm pore size; Schleicher&Schüll, Dassel, Germany) using a semidry blotting apparatus (Kyhse-Andersen 1984). After electroblotting the nonspecific binding sites were blocked for 30 minutes with 0.15M phosphate-buffered saline, pH 7.4 (PBS), containing 0.05% Tween 20 and 0.05% casein. The blots were then incubated overnight with a monoclonal antibody hybridoma supernatant at a 1:5 dilution in PBS-Tween, followed by a peroxidase-conjugated goat anti-mouse IgG antibody (LabAs, Ltd., Tartu, Estonia) at a 1:1000 dilution. Each incubation step was followed by washing four times (5 minutes each) with PBS-Tween. Staining was performed with mixed 4-chloro-1-naphthol and 3,3'-diaminobenzidine (Sigma, St.Louis, MO) chromomeric substrate solution in PBS until bands were detected (Young 1989).

4.4. Determination of enzymatic activity

4.4.1. Activity of GSTs towards CDNB

Activity of glutathione S-transferases with 1-chloro-2,4-dinitrobenzen (Sigma, St.Louis, MO) was determined by monitoring changes in absorbance at 340 nm in a DU-5 spectrophotometer (Beckman Instruments, Inc., Fullerton) as described by Habig *et al.* (1974). A complete assay mixture without enzyme served as a control. The concentration of 1 mM was used for glutathione (Sigma, St.Louis, MO) and 1-chloro-2,4-dinitrobenzen. The amount of enzyme used, resulted in an absorbance change of less than 0.05 per minute. Assays were performed at 25°C in 50mM Tris-HCl, pH 6.5. Activity was expressed per mg of protein and calculated by the equation:

$$SA = \Delta ABS / \Delta\varepsilon * C,$$

where SA is the specific activity, ΔABS is the change of absorbance per minute, Δε is the extinction coefficient (9.6 for 1-chloro-2,4-dinitrobenzen) and C is the amount of protein in the assay mixture.

4.4.2. Activity of Theta-class GSTs towards methyl chloride

Activity of Theta class enzyme from erythrocytes towards methyl chloride was measured by Warholm *et al.* (1994) and carried out in the Department of Toxicology, National Institute of Occupational Health, Solna, Sweden. In brief, after preincubation of 2 ml of cytosol from erythrocytes with 3 ml of PBS in sealed head space vials at 37°C for 30 minutes, 1.7 ml air from the gas phase of each vial was withdrawn and substituted by an equal volume of 1% methyl chloride in air. The reaction was followed for 3 hours. At 0, 30, 60, 120 and 180 minutes, 0.3 ml from the gas phase was withdrawn and the concentration of methyl chloride was analysed by gas chromatography. A varian gas chromatograph, model 3700, equipped with a Tenax TA 35/50 mesh column (2m*3mm) and a flame ionization detector, was used at 100°C with nitrogen (30 ml min^{-1}) as a carrier gas. In control incubations the erythrocyte cytoplasm was substituted by water. As a crude measure of haemoglobin content of the blood samples the absorbance at 415 nm was determined. Activity was calculated by dividing the activity (expressed as the "slope" per ml of sample) with the absorbance, determined at 415 nm.

4.4.3. Activity of Theta-class GSTs towards dichloromethane

An assay designed for determination of Theta class activity from liver samples (Bogaards *et al.* 1993) was modified and adapted for measurement of GSTT1 activity present in erythrocytes. Activity towards dichloromethane was determined as follows: 300µl of 0.5M Tris-HCl, pH 7.4, 120µl of 100mM reduced glutathione, 480µl of lysed and centrifuged erythrocytes diluted previously 1:1 with water and 300µl of 1.6M dichloromethane in water, in a total volume of 1.2 ml were incubated at 37°C for three hours. During the incubation formaldehyde was formed via glutathione S-transferase mediated enzymatic reaction (Fig. 7). Chemical blanks consisted of incubations without sample and after incubation the blank was transferred into a tube containing the sample. The reaction was terminated by cooling the vials with ice water. Subsequently 1 ml of the incubation mixture was transferred to a clean microcentrifuge tube and protein was precipitated by addition of 333µl of 20% trichloroacetic acid. After incubation at +4°C for 10 minutes and centrifugation at 12000g for 10 minutes, 600µl of the supernatant was decanted and the amount of formaldehyde, which was formed during the enzymatic reaction, quantified by adding of 300µl of Nash reagent (86µl acetic acid, 62µl acetylacetone and 4.6g ammonium acetate in a volume of 10 ml) and subsequent incubation at 60°C for 30 minutes. Absorbance at 414nm was measured in Nunc (Roskilde, Denmark) microwell plates using Twinreader Plus (Labsystems, Helsinki, Finland). Calibration standards were solutions of 0–0.1mM formaldehyde in water. Enzymatic activity was expressed as formaldehyde formation, normalized with protein content (nmol/min/mg of protein).

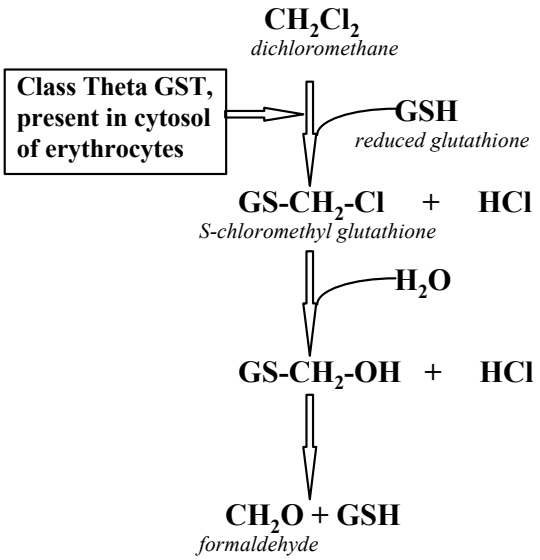

Figure 7. Conjugation of dichloromethane with glutathione by GST and formation of formaldehyde.

To measure the conjugating activity towards dichloromethane of purified GSTT1–1 or theta-class GST enzyme present in liver, essentially the same method was used as described above except that amount of the sample was smaller (for liver 250µl and for purified GSTT 1–1 50µl) and the missing volume was substituted with water.

4.5. Production of monoclonal antibodies against GSTM1-1 and GSTT1-1 proteins

Using purified GSTM1-1 and GSTT1-1 proteins monoclonal and polyclonal antibodies were produced by Erkki Juronen (Department of Human Biology and Genetics, Institute of General and Molecular Pathology, University of Tartu) as published (Juronen *et al.* 1994; Juronen *et al.* 1996).

4.6. ELISA methods for phenotyping of polymorphic GST-s

4.6.1. ELISA for phenotyping of GSTM1

Out of 16 Mabs obtained against GSTM1 isoforms, three were chosen for performing ELISA using whole blood: Mab 11F12 — specific for GSTM1 subunit 1a, Mab 1H8 — specific for subunit 1b and Mab 10G12 — recognizing an epitope present in both allelic forms of GSTM1.

The allele specific detection of GSTM1 isoforms was performed as follows. The wells of two different microplates (Nunc Maxisorp, Denmark) were coated at 4°C overnight with 100µl of purified Mabs 11F12 (GSTM1a specific) and 1H8 (GSTM1b specific) in 0.15mol/l phosphate buffered saline, pH 7.4 (PBS) at a concentration of 5µg/ml, followed by washing twice with PBS, containing 0.05% Tween 20 (PBS-Tween) and blocking with PBS-Tween, containing 0.05% casein for 30 min. Next, 100µl of whole blood containing 0.1% Triton X-100 was added to the wells coated with different Mabs, and the plates were incubated for 60 min on the shaker at room temperature. After that, the plates were washed 5 times with PBS-Tween and 100µl of peroxidase conjugated Mab 10G12 (diluted 1:2000 in PBS-Tween) was added to the wells. The plates were incubated for 60 min on the shaker, followed by washing 5 times with PBS-Tween. The peroxidase activity bound to the plates was assayed for 10 min by adding 100µl of TMB substrate solution (3,3',5,5'-tetramethylbenzidine/H_2O_2 in 0.1mol/l acetate-citrate buffer, pH 4.5). The reaction was stopped by adding 50µl of 3mol/l sulfuric acid and the absorbance read at 450 nm with a TwinReader Plus (Labsystems, Finland).

4.6.2. ELISA for phenotyping of GSTT1

Out of six hybridoma clones producing specific monoclonal antibodies against GSTT1-1, Mabs 4G1 and 2D8 were selected for determining GSTT1 in whole blood. Study population was first tested using ELISA differentiating between GSTT1 negative and positive individuals and in the second step ELISA measuring the GSTT 1-1 concentration in whole blood was applied for quantification of the enzyme in GSTT1 positive individuals.

ELISA differentiating between GSTT1 negative and positive individuals.
100µl of whole blood diluted 1:10 with 0.1% Triton X-100 was added into the wells of ELISA microplates, previously coated with purified Mab 4G1, washed and blocked using the concentrations, temperatures and incubation times described above for GSTM1 detection with ELISA. After incubation of the plates with diluted blood for 60 min on a shaker at room temperature and subsequent washing steps, 100µl of peroxidase conjugated Mab 2D8 (diluted 1:2000 in PBS-Tween) was added to the wells. The peroxidase activity bound to the plates was assayed for 10 min by adding 100µl of TMB substrate solution (3,3',5,5'-tetramethylbenzidine/H_2O_2 in 0.1 M acetate-citrate buffer, pH 4.5) and the absorbance read at 450 nm with a TwinReader Plus.

Determination of GSTT1-1 concentrations in whole blood.
The GSTT1-1 concentration in whole blood was measured by ELISA as described above, except that the blood was diluted 1:100 with 0.1% Triton X-100. The enzyme standard ranging from 15 to 1000 ng/ml was prepared by adding purified GSTT1-1 into the blood of GSTT1 negative individuals. The haemoglobin content was determined in diluted blood samples by measuring haemoglobin absorbance at 414 nm, and GSTT1-1 concentrations were expressed as µg/ml/abs 414.

4.7. Genotyping of polymorphic glutathione S-transferases

4.7.1. GST M1 genotyping using PCR differentiating between GSTM1 positive and negative individuals

The PCR method is essentially the same as described by Hirvonen *et al.* (1993). In brief, the set of three primers was used. Primer P1 anneals to the 5' region of exon 4 of GSTM1, giving with primer P3, annealing to the 3' region of exon 5 of GSTM1, a 231-bp product in GSTM1 positive individuals. Primer P1 hybridizes also to the 5' region of exon 4 of GSTM4 gene and gives with primer P2, which is

complementary to the 3' region of exon 5 of GSTM4, a 158-bp product in all persons. Primes sequences were as follows:

P1:5'- CGC CAT CTT GTG CTA CAT TGC CCG -3'
P2:5'- ATC TTC TCC TCT TCT GTC TC -3'
P3:5'- TTC TGG ATT GTA GCA GAT CA -3'.

The amplification reaction was carried out in a total volume of 50µl in the presence of 1.5mM magnesium chloride, 10mM Tris-HCl, pH 8.3, 50mM potassium chloride, 0.1% (w/v) gelatine, 0.2 mM of each dNTP, 2µM of primers P1 and P3, 1µM of primer P2 and 1.5 U Taq polymerase. Thirty cycles were performed using 60s at 94°C for denaturation, 90s at 50°C for annealing and 60s at 72°C for primer extension. After amplification the aliquots were run on a 2% agarose gel, stained with ethidium bromide, and photographed on Polaroid type 667 film.

4.7.2. GSTM1 genotyping using PCR allowing identification of GSTM1 A, B and null polymorphisms

Identification of GSTM1 A, GSTM1 B, GSTM1 A,B and GSTM1 null polymorphisms by PCR was performed using primers, described by Fryer *et al.* (1993):
I6; a common GSTM1-specific primer to intron 6:
5'- GCT TCA CGT GTT ATG AAG GTT C -3'
E7A; *GSTM1*A* allele specific primer to exon 7:
B 5'- TTG GGA AGG CGT CCA AGC GC -3'
E7B; *GSTM1*B* allele specific primer to exon 7:
5'- TTG GGA AGG CGT CCA AGC AG -3'
HG 1; fist primer annealing to β-globine gene:
5'- CTG CCC TAC TTG ATT GAT GGG -3'
HG 2; second primer annealing to β-globine gene:
5'- CTG GAT TGT AGC AGA TCA TGC -3'

Each DNA sample was amplified using two allele-specific amplification reactions. To amplify a 132-bp fragment of *GSTM1*A* allele and a 268-bp fragment of β-globine gene (as an internal control) the PCR reaction was carried out in a total volume of 50µl in the presence of 1.5 mM magnesium chloride, 0.2 mM of each dNTP, 10 mM Tris-HCl, pH 8.3, 50 mM potassium chloride, 0.1% (w/v) gelatine, 0.5µM of primers I6 and E7A, 0.3 µM of β-globine primers HG1 and HG2 and 1.5 U Taq polymerase. The above described mixture was also used for *GSTM1*B* amplification except that primer E7A was replaced with primer E7B. After initial denaturation (90s at 95°C) 30 cycles of denaturation (60s at 95°C), primer annealing (30s at 57°C) and elongation (120s at 72°C) followed by a final

extension step (10 min at 72°C) were performed, in a Techne Programmable Dri-Block PHC-1. Using the described protocol no digestion of PCR product with restriction endonuclease was needed, as done in the original method. GSTM1 polymorphisms were directly detected after the DNA fragments were resolved by electrophoresis in 2.1% agarose containing ethidium bromide (0.5 μg/ml).

4.7.3. GSTT1 genotyping

Primers T1 and T2, used for detection of GSTT1 polymorphism were described by Pemble *et al.* (1994). In addition, to confirm the presence of amplifiable DNA, primers complementary to CYP2D6 gene (Hirvonen *et al.* 1993) were added to the PCR reaction. Primer sequences were as follows:

T1: 5'- TTC CTT ACT GGT CCT CAC ATC TC -3'
T2: 5'- TCA CCG GAT CAT GGC CAG CA -3'
CYP2D6 A: 5'- TGC CGC CTT CGC CAA CCA CT -3'
CYP2D6 B: 5'- TAT GCA AAT CCT GCT CTT CC -3'

PCR reactions were performed in 20μl of 75 mM Tris-HCl, pH 9.0, containing 20 mM $(NH_4)_2SO_4$, 0.01% Tween20, 1.2 mM $MgCl_2$, 0.2 mM dNTP, 1 μM GSTT1 primers, 2μM CYP2D6 primers and 0.4 U Taq polymerase. After initial denaturation at 94°C for 1.5 min 32 amplification cycles, 94°C for 1 min and 69°C 1 min, with a final extension 69°C for 2 min were performed. The PCR products were analysed electrophoretically on a 1.8% agarose gel.

4.7.4. GSTM3 genotyping

Genotyping of GSTM3 locus was performed essentially as described by Inskip *et al.* (1995) except that electrophoresis was carried out under the denaturing conditions.
Primer sequences used for amplification were as follows:

GSTM3F: 5'-CCT CAG TAC TTG GAA GAG CT-3'
GSTM3R: 5'-CAC ATG AAA GCC TTC AGG TT-3'

Digestion of amplicons with MnlI (recognition sequence CCTC) was carried out overnight at 37ºC. Digested PCR products were incubated with equal volume of formamide at 56ºC before subjected to electrophoresis in 15% of polyacrylamide gel (C=4.8%), containing 6.4 M urea.

4.7.5. GSTP1 genotyping

A-G polymorphism at codon 105 in exon 5 and C-T polymorphism at codon 114 in exon 6 of the GSTP1 gene were detected by the RFLP analysis of PCR amplified DNA. Both exons were amplified separately and thereafter analyzed.

To generate a 463 bp DNA fragment containing the fifth exon of the GSTP1 gene, primers P1E5A (5'- TGT GTG GCA GTC TCT CAT CCT T -3') and P1E5B (5' -TAC TTG GCT GGT TGA TGT CCC A -3') were used. The reaction was carried out in a total volume of 20µl in the presence 1.5 mM $MgCl_2$, 10 mM Tris-hydrochloric acid, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.2 mM of each dNTP, 0.75 µM of each primer and 0.4 units Taq-polymerase (Fermentas). After initial denaturation at 95ºC for 1 min, 32 cycles of 30 s at 94ºC, 30 s at 56ºC and 45 s at 72ºC were performed. The amplified DNA was then digested using enzyme Alw26I (Fermentas), that has a recognition sequence GTCTC. Digested amplicons were separated by electrophoresis in a 12.5% polyacrylamide gel (C=4.8%), containing 33mM Tris-sulfate and 7% of glycerol. The gels were stained with ethidium bromide and photographed under UV-light.

The DNA fragment from the GSTP1 gene extending from intron 5 to intron 6 and encompassing codon 114 in exon 6 was amplified using oligonucleotides P1E6A (5'- TGG CAG CTG AAG TGG ACA GGA TT-3') and P1E6B (5'- ATG GCT CAC ACC TGT GTC CAT -3'). The amplification reaction was carried out as described above, except that the primer concentrations were 1 µM, and the following cycling conditions were used: initial denaturation at 95ºC for 1 min followed by 32 cycles of 94ºC for 45 s and 69ºC for 45 s. PCR products were digested with either AciI (CCGC) or Cac8I (GCNNGC) (both from New England Biolabs).

4.8. Study populations

Collection of samples, phenotyping and genotyping of polymorphic GST-s as well as publication of the results was carried out throughout of several years. Therefore in earlier publications number of tested individuals was considerably lower than in latest articles. The common criterion in selection of individuals for testing of polymorphic GSTs in population and association studies was that both parents of them, as well as grandparents had to be of Estonian nationality.

Samples collected for performing of population genetic studies were obtained from several parts of Estonia and number of persons tested ranged from 307 to 673 in different studies.

Patients for POAG and cataract studies as well as matched control individuals were recruited from two ophthalmic clinics that provide outpatient care. All persons were interviewed to obtain data on smoking habits.

Patients suffering from POAG had a typical visual field defect and pathological cupping of the optic disc with an intraocular pressure higher than 20mmHg.

In patients with cataract the disease status was determined by the lens examination in transient and side illumination using a biomicroscope and ophthalmoscope, and classified into nuclear (n=77), cortical (n=155), posterior subcapsular (n=120) and mixed type (151) of opacities. Any patients with secondary cataracts were excluded, e.g. those due to trauma, diabetes and other known causes.

The case group for POAG study consisted of 250 patients and for cataract study of 503 patients with senile cataract. The control group comprised of 202 unrelated volunteers without cataract, glaucoma or uveitis. The mean age of the glaucoma group was 70.7±9.1 years (ranging from 44 to 91 years), for cataract patients 72±8.2 years (ranging from 47 to 93 years) and 65.7±6.9 years (ranging from 43 to 90 years) for control group. Proportion of women among patients with glaucoma, among persons suffering from cataract and among control group was 64.4%, 69.4% and 72.3% respectively.

4.9. Processing and storage of samples

Tissue samples for enzyme purification or immunoblotting were obtained less than 24 hours post mortem and stored at –80°C until required. Homogenization of the samples was performed using 2 volumes of ice-cold 20mM sodium phosphate buffer, pH 6.4, containing 2mM EDTA, 2mM 2-mercaptoethanol and 25μM phenylmethylsulfonyl fluoride (PMSF). To disrupt all the cell membranes the soluble supernatant, obtained after centrifugation (20.000g for 20 minutes at +4°C) was twice frozen and thawed. The cytosol was then centrifuged again (20.000 g for 20 minutes at +4°C) and filtered through a plug of glass wool to remove floating lipids.

10 ml of umbilical cord blood from newborns and 10–20 ml of venous blood from adults was collected in EDTA as an anticoagulant. Genomic DNA from 5 ml of blood was isolated either by the standard phenol extraction procedure (Sambrook *et al.* 1989) or by Boom *et al.* (1990) and stored at –20°C. Erythrocytes for GSTT1 activity measurement were prepared by centrifugation (800g, 10min) and removal of plasma and white blood cells. The red blood cells were then washed twice in PBS and stored at –80°C until measurement. Whole blood for ELISA was stored at –20°C.

4.10. Statistical methods

To assess the agreement of the observed genotype proportions with the expected frequencies Hardy-Weinberg equation was used for all the loci and study groups.

In association studies relative risks for the polymorphic GST pheno- and genotypes were evaluated by calculation of odds ratios (OR), 95% confidence intervals (CIs) and CI-based p-values according to Mantel and Haenszel (1959). OR was defined as the odds of case patients having a susceptible phenotype or genotype divided by the odds of controls having a susceptible phenotype or genotype. The limit of statistical significance was set at $p=0.05$. The Bonferroni correction of the p-value for multiple testing has been used (Matthews and Farewell 1996).

5. RESULTS

5.1. Characterization of biological properties of glutathione S-transferase T1

5.1.1. Enzymic activity of GSTT1-1

The purification of GSTT 1-1 from liver as described (Juronen *et al.* 1996) yielded an enzyme having quite high values of activity, unlike to the data published so far where all the purification schemes have resulted an inactive protein.

The activity of GSTT1-1 isolated from liver, was high enough to characterize the enzymic properties. Specific activities of GSTT1-1, GSTM1a-1b and liver cytosols towards model substrates of GSTs are presented in table 5.

Table 5. Specific activities of GSTT1-1, GSTM1-1 and liver cytosols towards CDNB and DCM.

Enzyme/cytosol	Specific activity	
	CDNB (μmol/min per mg)	DCM (nmol/min per mg)
Liver cytosols from different persons	0.1–0.4	0.05–0.4
GSTT1-1	0.165	1609
GSTM1-1	10.4	not determined

CDNB – 1-chloro-2,4-dinitrobenzen; DCM – dichloromethane

The activity of GSTT1-1, purified from erythrocytes decreased rapidly during the last stages of the purification, making the characterization of its enzymatic properties impossible

5.1.2. Molecular weight and isoelectric point of GSTT1-1

The SDS-PAGE analysis of Theta class GSTs isolated from liver and erythrocytes showed that both enzymes migrated as single bands. The electrophoretic mobility of the two enzymes was identical. Also the Western blot analysis of cytosols, prepared from liver tissues and red blood cells gave the immunochemical staining exactly in the same place using Mab 1A2. The M_r value of the isolated GSTT1 subunit was 25300 as estimated with a comparative electrophoretic study on the same gel with M_r marker proteins as well as the GSTM1-1 subunit protein, isolated from human liver cytosol (Fig.8).

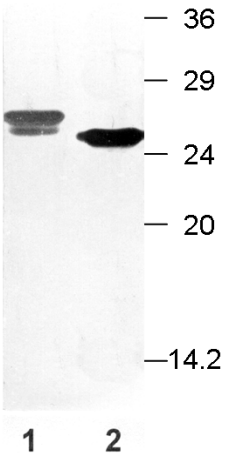

Figure 8. SDS-PAGE of the isolated GSTM1-1 and GSTT1-1 enzymes from liver, silver-stained. Lane 1: GSTM1-1, lane 2: GSTT1-1.

The pI value for hepatic GSTT1-1, determined by the isoelectric focusing method, was 6.64 (Fig.9). Comparative isoelectric focusing of red blood cell's cytosols and hepatic cytosols, followed by immunoblotting revealed exactly the same isoelectric point for GSTT1-1 in erythrocytes. No differences in pI values were observed in 10 different immunoblotted liver and red blood cell cytosols, indicating the presence of only one isoform of GSTT1-1.

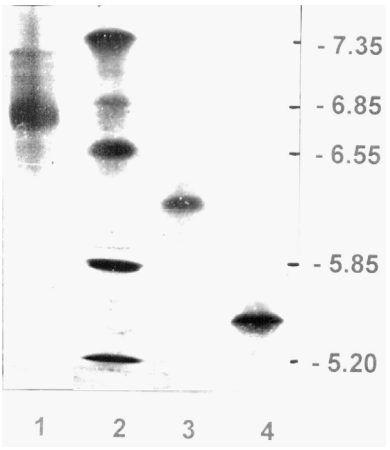

Figure 9. Isoelectric focusing of isolated human hepatic GSTT1-1 and GSTM1-1 isoenzymes. Lane 1: GSTT1-1; lane 2: pI calibration standards; lane 3: GSTM1a-1a (pI=6,2); lane 4: GSTM1b-1b (pI=5,7). Repeated freezing and thawing of the purified GSTT 1-1 enzyme resulted in substitution of the initial single IEF band with a ladder.

5.1.3. Tissue distribution of GSTT1-1

Immunoblot analysis of various tissue cytosols, carried out using 8–25% gradient SDS-PAGE and anti-GSTT1-1 monoclonal antibody 1A2, suggested that GSTT 1-1 is widely expressed (Fig.10).

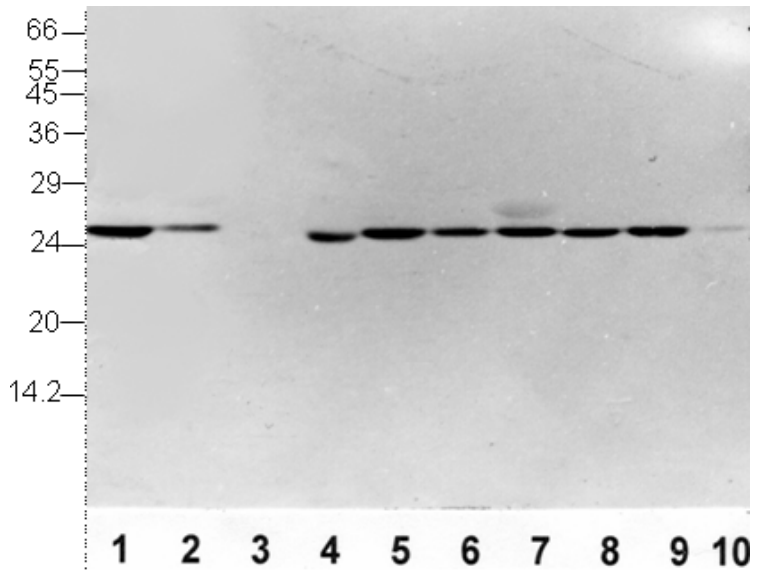

Figure 10. Immunoblot analysis of GSTT1-1 in the cytosols of various human tissues. Protein content was 300 μg per lane. Lane 1: liver; lane 2: erythrocytes; lane 3: pancreas; lane 4: lung; lane 5: kidney; lane 6: brain; lane 7: skeletal muscle; lane 8: heart muscle; lane 9: small intestine; lane 10: spleen.

5.2. Methodology for phenotyping and genotyping of polymorphic GSTs

5.2.1. GSTM1 phenotyping by isoelectric focusing

Overall 39 liver cytosols, previously assayed for presence of glutathione S-transferase activity towards CDNB, were focused and histochemically stained as described in materials and methods. Different GSTM1 phenotypes could be easily determined (Fig. 11).

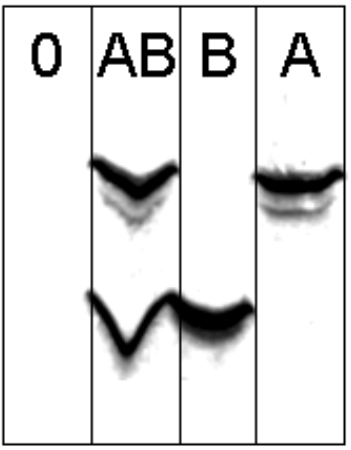

Figure11. Phenotyping of GSTM1 isoforms

The numbers and frequencies of GSTM1 phenotypes obtained are presented in table 6.

Table 6. Phenotype frequencies of GSTM1, obtained by isoelectric focusing of liver cytosols.

	number	frequency
GSTM1 A	11	0.282
GSTM1 B	8	0.205
GSTM1 AB	3	0.077
GSTM1 0	17	0.436
	39	1.000

5.2.2. GSTM1 phenotyping using ELISA

A single substitution Lys→Asp in amino acid position 173 of GSTM1 molecule has changed the immunological properties of protein so that monoclonal antibodies are able to distinguish between GSTM1a and GSTM1b isoforms. ELISA plates coated with GSTM1-1 isoenzyme-specific monoclonal antibodies 11F12 and 1H8 gave a positive reaction with lysed whole blood containing GSTM1a-1a and GSTM1b-1b enzyme respectively (OD >2.0 at 450 nm). Heterozygotic individuals (GSTM1a-1b) were positive with both Mabs, and blood from individuals lacking the GSTM1-1 protein did not give any reaction (OD <0.1 at 450 nm).

5.2.3. GSTM1 genotyping by PCR

1) **PCR differentiating between GSTM1 positive and negative individuals**. The PCR protocol yielded a 158 bp fragment, consistently found in each person and served as an internal control, and a 231 bp fragment, present only in GSTM1 positive individuals (Fig. 12).

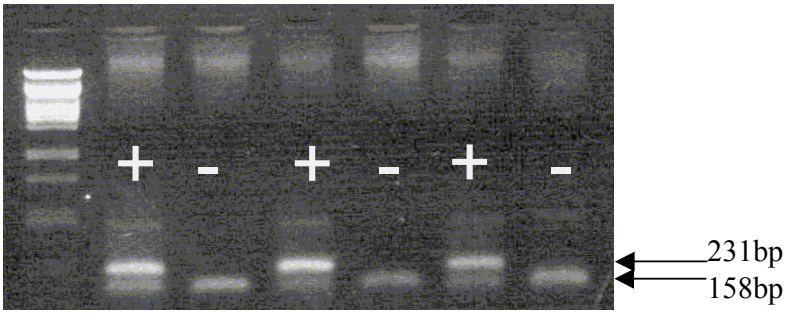

Figure 12. Genotyping of GSTM1, 231 bp fragment is present in GSTM1 positive and absent in GSTM1 negative individuals.

2) **Genotyping assay to detect GSTM1 null, GSTM1 A, GSTM1 B and GSTM1 A/B polymorphisms** allowed amplification of 268 bp DNA fragment of β-globine gene as an internal control in every individual, whereas the polymorphic 132 bp fragment could only be seen in GSTM1 positive persons. Using two allele-specific

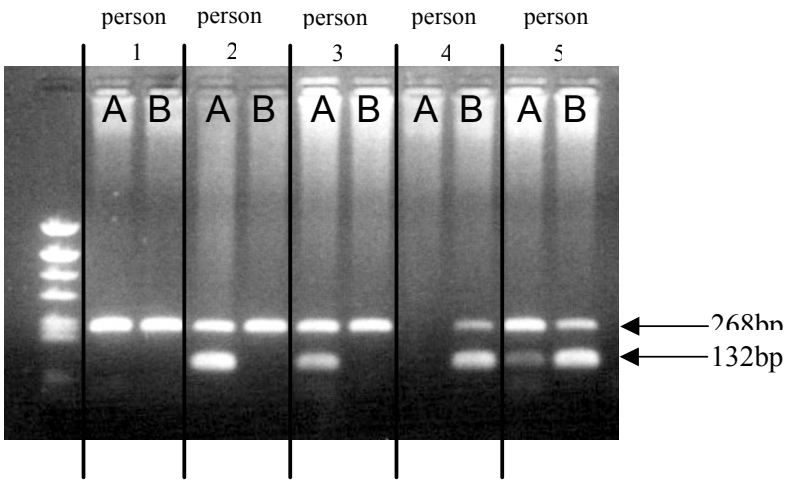

Figure 13. Genotyping of GSTM1 locus with primers allowing to identify GSTM1A, B and null individuals. DNA from each person was genotyped in two different reactions, one identifying the presence of A and another presence of B allele. Person 1 has GSTM1 null genotype. Persons 2 and 3 have A allele. Person 4 has B-allele but as the control band is missing, nothing can be said about the A-allele. Person 5 is AB heterozygote.

amplification reactions for every person, one with a *GSM1*A* specific primer and the another with a *GSTM1*B* specific primer, discrimination between GSTM1 A, GSTM1 B and GSTM1 A,B phenotypes could be easily performed after electrophoresis without any digestion, unlike described in the original protocol (Fig. 13).

5.2.4. Comparison of different methods for detection of GSTM1 polymorphism

Altogether 449 individuals from Estonia were tested, using ELISA for GSTM1 detection from whole blood. DNA from 353 subjects was examined with PCR discriminating between GSTM1 positive/negative genotypes and DNA from 116 subjects with PCR detecting GSTM1 A, B, A,B and null polymorphisms. Apart from two of the 353 subjects investigated with different methods, genotyping and phenotyping assays gave the same result (discrepancy 0.6%). In one noncongruent case PCR genotyping revealed the presence of *GSTM1*B,* but expression of the enzyme in blood was not confirmed with ELISA. Another discrepancy was characterized with negative GSTM1 genotype and positive ELISA result (GSTM1 A/B phenotype). In the latter case specific GSTM1-1 enzymatic activity towards chloro-dinitrobenzene (CDNB) was measured and not detected after capturing the enzyme from whole blood with different GSTM1-1 specific Mabs. One can conclude that this positive ELISA result was affected by nonspecific interfering factors.

5.2.5. GSTT1 phenotyping using activity determination

1) **Activity of Theta class GST from erythrocytes towards methyl chloride**. Lysed erythrocytes were used for phenotyping of GSTT1 using activity determination towards methyl chloride, i.e. using method generally accepted for GSTT1 phenotyping. The pattern obtained indicates the presence of three different phenotypes — nonconjugators, conjugators with intermediate activity and conjugators with high activity (Fig. 14). Phenotyping towards methyl chloride served as a starting point for all of our GSTT1-1 studies, including adopting the dichloromethane assay for measuring GSTT1 activity from erythrocytes.

2) **Activity of Theta class GST from erythrocytes towards dichloromethane**. Phenotyping assay for measuring of GSTT1 activity towards dichloromethane was developed using red blood cells from subjects with known GSTT1 phenotypes, previously determined with the methyl chloride testing. The originally described

dichloromethane assay (Bogaards *et al.* 1993) was modified as described in materials and methods and adapted for using cytosols derived from erythrocytes.

Glutathione S-transferase activity in erythrocytes towards dichloromethane was measured in 76 individuals. Results of activity determination revealed the presence of three groups of individuals: one having no GSTT1 activity in erythrocytes (phenotype GSTT1 null), the second having intermediate activity (phenotype GSTT1 +/–) and the third group with high activity towards dichloromethane (phenotype GSTT1 +/+). The activity of 3.5 nmol/min/mg of protein was chosen as the cut-off value to differentiate between intermediate and high activity. The mean activity of GSTT1 towards dichloromethane in persons having phenotype GSTT1 +/+ was more than twice as high as in persons with GSTT1 +/– phenotype (5.08 vs. 1.92).

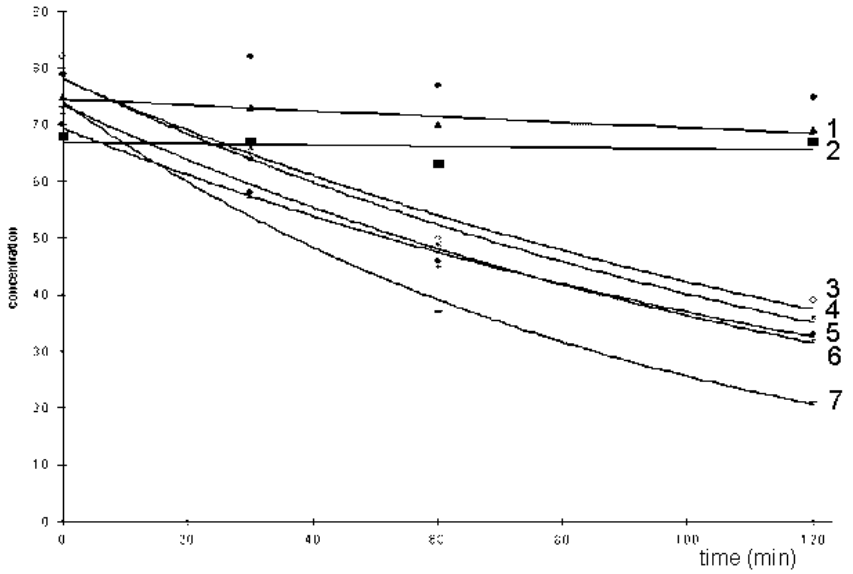

Figure 14. GSTT1 activity determination towards methyl chloride. Persons nr 1 and 2 have no activity, nr 3–6 have intermediate activity and person nr 7 has high activity.

5.2.6. GSTT1 phenotyping with ELISA

Altogether 673 persons of Estonian nationality were tested using ELISA differentiating between GSTT1 positive and negative phenotypes. Subjects expressing the GSTT1 in erythrocytes gave a positive reaction with the ELISA, while the blood from GSTT1 negative individuals failed to give any reaction.

The same sample, except 154 newborns, was used to measure the GSTT1 protein concentration in whole blood. The distribution of the GSTT1 concentration

in 519 people studied was trimodal (Fig. 15). In the first group of individuals no detectable amount of GSTT1-1 was found (phenotype GSTT1 null). The second group had GSTT1-1 in concentrations ranging from 12.3 to 68.1 μg/ml/abs414 (phenotype GSTT1 +/–) and the third group consisted of individuals having GSTT1-1 concentrations in whole blood from 70 to 141.9 μg/ml/abs 414 (phenotype GSTT1 +/+). The measurement of GSTT1 concentrations in whole blood revealed that mean concentration of the enzyme in GSTT1 +/+ individuals (97.85) is more than twice higher as in GSTT +/– subjects (41.37). The described finding is consistent with the results of activity determination and gives evidence of the gene-dosage dependent expression of GSTT1.

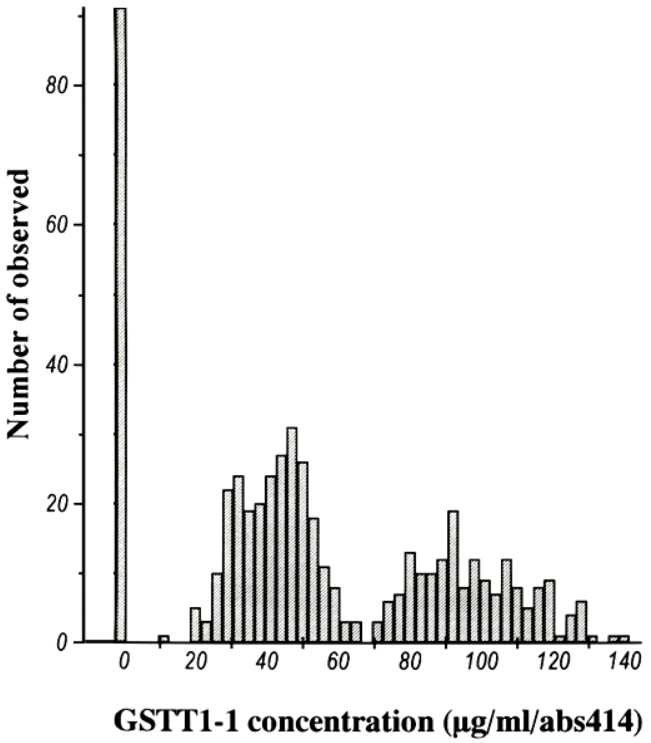

Figure 15. Distribution of GSTT1-1 concentrations in whole blood of 519 individuals.

5.2.7. GSTT1 genotyping with PCR

The original method for GSTT1 genotyping by PCR (Pemble *et al.* 1994) was modified by simplifying the amplification protocol and adding primers complementary to CYP 2D6 gene. Amplification of the CYP 2D6 gene fragment, present in all individuals served as an internal control, excluding the possibility of a false interpretation due to failure in the PCR reaction. Amplification yielded the constant 363 bp DNA fragment and polymorphic 480 bp DNA fragment, present only in subjects with GSTT1 positive genotype (Fig. 16).

5.2.8. Comparison of phenotyping and genotyping methods, used for determination of GSTT1 polymorphism

673 subjects were studied using GSTT1-ELISA differentiating between GSTT1 positive/negative phenotype and 519 of them also with quantitative GSTT1-ELISA. Activity of GSTT1 was measured in 76 individuals and GSTT1 genotype established by PCR in 133 persons.

No discrepancies were observed when the results obtained with different methods were compared. In all the 133 cases investigated with genotyping by PCR and phenotyping by ELISA, the presence or absence of the GSTT1 gene coincided with the positive (GSTT1 +/+ and GSTT1 +/–) and negative (GSTT1 null) phenotypes respectively.

In all 76 cases studied with both of the quantitative phenotyping methods, results were essentially the same: people with no GSTT1 activity, intermediate or high activity had respectively no GSTT1 protein in whole blood or had it in intermediate or high concentrations. The correlation coefficient between the two quantitative values characterizing GSTT1 expression in erythrocytes — enzymatic activity and GSTT1 concentration — was very high: 0.914.

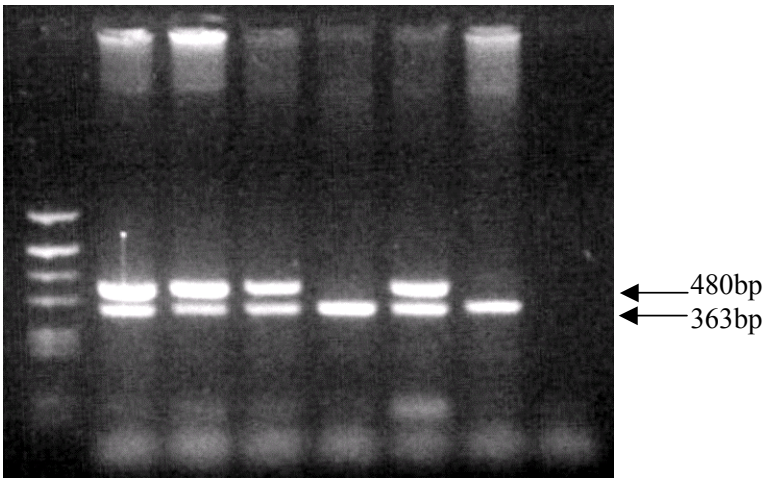

Figure 16. GSTT1 genotyping. The 363 bp CYP2D6 fragment is present in all individuals and the 480 bp fragment is present only in GSTT1 positive individuals

5.2.9. Genotyping of GSTM3

A 3 bp deletion in B-allele of GSTM3 gene resulted in a loss of a recognition sequence for restriction endonuclease Mnl I. The longest DNA fragment seen after digestion was 138 bp for B-allele and 132 bp for A-allele (Fig. 17).

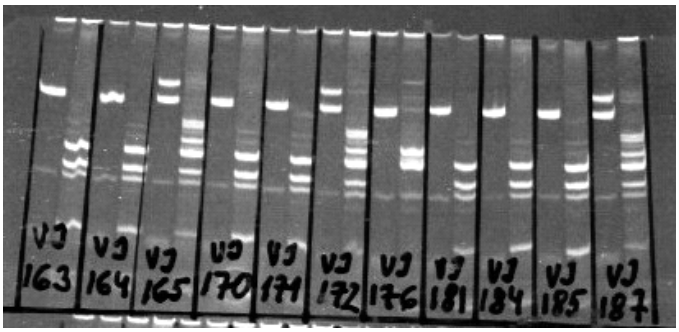

Figure 17. Genotyping of GSTM3. Amplicon from the A-allele (wild type) is cut into the 132bp, 104bp and 37bp fragments. PCR product from the B-allele (3bp deletion) is cut into 138bp and 132bp fragments. As specific heteroduplecs were seen in heterozygotic individuals, making easier to interpret the genotype. Both digested and undigested amplicons from every individual were subjected to electrophoresis. Example of patterns of DNA fragments in AB, BB and AA genotypes are seen in persons encoded as VJ172, VJ176 and VJ181 respectively.

5.2.10. Genotyping of GSTP1

463 bp DNA fragment containing the fifth exon of the GSTP1 gene and A-G polymorphism was digested with restriction endonuclease Alw26I. The DNA amplified from the *GSTP1*A* (ATC at codon 105) was cut into the 17 bp and 446 bp fragments. DNA derived from alleles *GSTP1*B* and *GSTP1*C* (GTC at codon 105) had an additional recognition site for the Alw26I and was digested into 17 bp, 221 bp and 225 bp segments (Fig. 18).

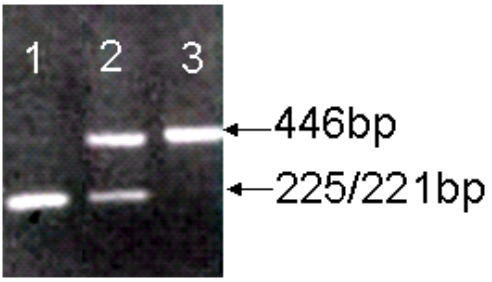

Figure 18. Genotyping of the fifth exon of GSTP1 gene. Line 1 – GTC/GTC at codon 105, line 2 – GTC/ATC at codon 105, line 3 – ATC/ATC at codon 105.

The amplicon containing exon 6 of GSTP1 gene and C-T polymorphism was digested with either AciI or Cac8I (both from New England Biolabs). Amplified DNA from the *GSTP1*A* and *GSTP1*B* (GCG at codon 114) was cut into two fragments by AciI (158 bp and 174 bp) and three fragments by Cac8I (49 bp, 110 bp and 173 bp). The PCR product from the *GSTP1*C* (GTG at codon 114) left undigested by AciI and cut into 49 and 283 bp fragments when Cac8I was used (Fig. 19).

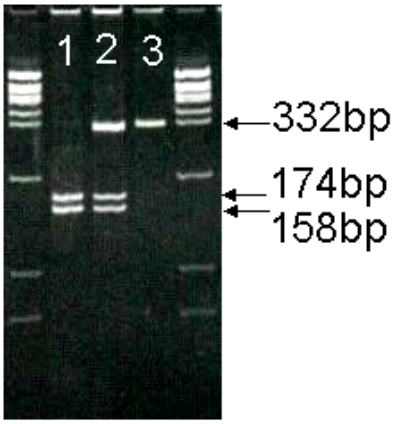

Figure 19. Genotyping of the sixth exon of GSTP1 gene with AciI. Line 1 – GCG/GCG at codon 114, line 2 – GCG/GTG at codon 114, line 3 – GTG/GTG at codon 114.

5.3. Polymorphic glutathione S-transferases in Estonian population

5.3.1. GSTM1 polymorphism in Estonian population

GSTM1 polymorphism was determined in 307 unrelated individuals of Estonian nationality. Numbers and frequencies of GSTM1 phenotypes in Estonians are presented in Table 7. Frequencies of GSTM1 alleles were calculated assuming Hardy-Weinberg equilibrium and were found to be as follows: *GSTM1*0* = 0.68, *GSTM1*A* = 0.2107 and *GSTM1*B* = 0.1137. The expected distribution of GSTM1 genotypes was in a good correlation with that observed ($\chi^2 = 0.51$, df = 1 and P = 0.47).

Table 7. Frequency of GSTM1 polymorphisms in Estonian population.

Phenotype	Genotype	Observed	Expected	
		No	Frequency	
A	A-A	99	0.322	0.045
	A-0			0.284
B	B-B	49	0.160	0.013
	B-0			0.154
A/B	A-B	17	0.055	0.048
Null	0-0	142	0.463	0.457
Total		307		

5.3.2. GSTT1 polymorphism in Estonian population

Overall 673 subjects of Estonian nationality were tested using phenotyping assay differentiating between GSTT1 positive and negative individuals. The proportion of individuals lacking or expressing GSTT1-1 in whole blood was 18% and 82% respectively.

The same sample except 154 newborns was used for studying GSTT1 polymorphism with assay enabling to detect all the three GSTT1 phenotypes. 33% of individuals expressed GSTT 1-1 protein in high concentrations being therefore GSTT1 heterozygotes and 49% had intermediate concentrations of GSTT1 protein being homozygotes for the positive allele. Frequency for the GSTT1 positive (*GSTT1**+) and deleted allele (*GSTT1**-) was calculated to be 0.577 and 0.423 respectively. The distribution of GSTT1 phenotypes in the population studied is in concordance with Hardy-Weinberg's equilibrium ($\chi^2 = 0.02$, Df=1, p = 0.88). The frequencies of GSTT1 phenotypes and alleles in Estonian population are presented in Table 8.

Table 8. Distribution of GSTT1 phenotypes and alleles in Estonian population.

	phenotype or allele	frequency (%)
GSTT1 phenotypes	GSTT1 +/+	33.1
	GSTT1 +/–	49.1
	GSTT1 –/–	17.8
GSTT1 alleles	*GSTT1*+*	57.7
	GSTT1–*	42.3

5.3.3. GSTM3 polymorphism in Estonian population

Number of individuals genotyped for determining of gene frequencies in Estonian population was 366. Frequency for the *GSTM3*A* allele and allele with 3bp allele (*GSTM3*B*) was calculated to be 0.825 and 0.175 respectively (Table 9). The distribution of GSTT1 phenotypes in the population studied is in concordance with Hardy-Weinberg's equilibrium ($\chi^2 = 0.005$, Df=1, p = 0.94).

Table 9. Distribution of GSTM3 genotypes and alleles in Estonian population.

	genotype or allele	frequency (%)
GSTM3 genotypes	GSTM3 AA	68.0
	GSTM3 AB	29.0
	GSTM3 BB	3.0
GSTM3 alleles	*GSTM3*A*	82.5
	*GSTM3*B*	17.5

5.3.4. GSTP1 polymorphism in Estonian population

Data about distribution of GSTP1 gene frequencies comes from genotyping of control group (n=202) formed for studying of genetics of eye diseases. So the data can be considered as a source of information about the distribution of gene frequencies in the normal population only with certain reservations.

Population meets the criterion of Hardy-Weinberg's equilibrium for the locus ($\chi^2 = 1.37$, Df=3, p = 0.71). Data is presented in table 10.

Table 10. Distribution of GSTP1 genotypes and alleles.

	phenotype or allele	frequency (%)
GSTP1 genotypes	GSTP1 AA	42.6
	GSTP1 BB	6.4
	GSTP1 CC	0.5
	GSTP1 AB	29.7
	GSTP1 AC	14.9
	GSTP1 BC	5.9
GSTP1 alleles	*GSTT1*A*	64.8
	*GSTT1*B*	24.3
	*GSTT1*C*	10.9

5.4. Polymorphic glutathione S-transferases in patients with senile cataract

Distribution of the GSTM1, GSTT1, GSTM3 and GSTP1 pheno- and genotypes in the cataract patients and the control individuals are shown in Table 11.

Most significant association with polymorphic GSTs was found in subgroup of cortical cataract patients. Proportion of the GSTM1 positive individuals among the subgroup of patients with cortical opacity was significantly higher (60.6%) than in the controls (45.0%) with odds ratio of 1.88 (95% CI 1.23–2.94; p=0.004).

The second strongest association with the genetic marker and the disease was found in GSTP1 locus. Frequency of two alleles out of three present in the locus was different in cortical cataract patients with higher prevalence of *GSTP1*A* allele (73.2% vs. 64.9%; p=0.017) and decreased incidence of *GSTP1*B* allele (15.8% vs. 24.3%; p=0.006). Individuals having *GSTP1*A* allele (genotypes AA, AB or AC) were overrepresented among cases as compared to the controls (95.4% vs. 87.1%; OR=3.1; 95% CI 1.31–7.35; p=0.007). At the same time the number of persons being either hetero- or homozygotes for the *GSTP1*B* allele was decreased among patients with cortical cataract (30.5% vs. 42.1%; OR=0.6; 95% CI 0.39–0.94; p=0.026). Of the 155 patients suffering from cortical cataract, 0.6% were identified as carriers of the GSTP1 BB genotype, a portion which was significantly lower than the 6.4% for the controls, with an odds ratio of 0.09 (95% CI 0.01–0.73; p=0.006).

The frequency of GSTT1 positive individuals as well as persons having GSTM3AA genotype was also increased among patients, but the tendency did not reached to the level of significance.

Table 11. GST pheno- and genotype frequencies in senile cataract patients and control individuals.

	Cataracts					
GSTs	Posterior subcaps. (n=120)	Cortical (n=155)	Nuclear (n=77)	Mixed (n=151)	All cases (n=503)	Controls (n=202)
GSTM1 positive	62 (51.7%)	94 **(60.6%)**[a)]	36 (46.8%)	71 (47.0%)	263 (52.3%)	91 **(45.0%)**
GSTM1 null	58 (48.3%)	61 (39.4%)	41 (53.2%)	80 (53.0%)	240 (47.7%)	111 (55.0%)
GSTT1 positive	101 (84.2%)	136 (87.7%)	69 (89.6%)	124 (82.1%)	430 (85.5%)	166 (82.2%)
GSTT1 null	19 (15.8%)	19 (12.3%)	8 (10.4%)	27 (17.9%)	73 (14.5%)	36 (17.8%)
GSTM3 AA	89 (74.2%)	111 (71.6%)	60 (77.9%)	113 (74.8%)	373 (74.2%)	140 (69.3%)
GSTM3 AB	28 (23.3%)	41 (26.5%)	14 (18.2%)	33 (21.9%)	116 (23.1%)	60 (29.7%)
GSTM3 BB	3 (2.5%)	3 (1.9%)	3 (3.9%)	5 (3.3%)	14 (2.8%)	2 (1.0%)
GSTP1 AA	56 (46.7%)	79 (51.0%)	42 (54.5%)	75 (49.7%)	252 (50.1%)	86 (42.6%)
GSTP1 AB	40 (33.3%)	42 (27.1%)	18 (23.4%)	39 (25.8%)	139 (27.6%)	60 (29.7%)
GSTP1 AC	11 (9.2%)	27 (17.4%)	9 (11.7%)	21 (13.9%)	68 (13.5%)	30 (14.9%)
GSTP1 BB	6 (5.0%)	1 **(0.6%)**[b)]	4 (5.2%)	4 (2.6%)	15 **(3.0%)** [c)]	13 **(6.4%)**
GSTP1 BC	6 (5.0%)	5 (3.2%)	4 (5.2%)	10 (6.6%)	25 (5.0%)	12 (5.9%)
GSTP1 CC	1 (0.8%)	1 (0.6%)	0 (0.0%)	2 (1.3%)	4 (0.8%)	1 (0.5%)

Significant differences from the control group:
[a)] OR=1.88; 95% CI=1.23 – 2.88; p=0.004
[b)] OR=0.09; 95% CI=0.01 – 0.73; p=0.006
[c)] OR=0.45; 95% CI=0.21 – 0.96; p=0.034

Considering the possible additive effect of different GST loci, the frequencies of combined pheno- and genotypes were also observed. The risk of cortical opacities associated with the GSTM1 positive phenotype increased in carriers of the combined GSTM1 positive and GSTT1 positive phenotypes (OR=1.99; 95% CI 1.30–3.11; p=0.002) as well as in carriers of the combined GSTM1 positive and

GSTM3 AA genotype (OR=2.38; 95% CI 1.51–3.73; p<0.001). The highest risk of cortical cataract (OR=2.56) was observed in patients having all three susceptible genotypes (GSTM1 pos/GSTT1 pos/GSTM3 AA) (Table 12).

Table 12. Combined effect of GSTM1 positive status with other polymorphic GSTs investigated in patients with cortical cataract.

Genotypes or phenotypes	Cases (n=155)	Controls (n=202)	OR	95% CI	p
GSTM1 pos	94 (60.6%)	91 (45.5%)	1.88	1.23 – 2.94	0.004
GSTM1 pos/ GSTT1 pos	82 (52.9%)	73 (36.1%)	1.99	1.30 – 3.11	0.002
GSTM1 pos/GSTM3 AA	68 (43.9%)	50 (24.8%)	2.38	1.51 – 3.73	<0.001
GSTM1 pos/GSTT1 pos/GSTM3 AA	60 (38.7%)	40 (19.8%)	2.56	1.59 – 4.11	<0.001

Gene frequencies of polymorphic GSTs in patients with other types of cataract were similar to those in the cortical cataract group, but less expressed and did not meet the criterion of significance.

The distribution of the GSTM1 A, GSTM1 B and GSTM1 A/B phenotypes was also studied, but no effect of *GSTM1*A* and *GSTM1*B* alleles on any type of cataract was detected.

5.5. Polymorphic glutathione S-transferases in patients with primary open-angle glaucoma

The distribution of polymorphisms in GSTM1, GSTT1, GSTM3 and GSTP1 loci in the primary open-angle glaucoma patients and control individuals is presented in Table 13.

The only significant difference between the glaucoma cases and controls was observed in the distribution of GSTM1 positive and null phenotypes. The proportion of GSTM1 positive persons was significantly higher in the glaucoma group (60.0%) as compared to the controls (45.0%) with odds ratio of 1.83 (95% CI 1.26 – 2.66; p=0.002). Subdivision of the glaucoma patients according to a GSTM1 A, GSTM1 B and GSTM1 A/B phenotype (frequencies of 35.2%, 17.2% and 7.6% vs. 28.7%, 12.4% and 4.0% in healthy individuals) revealed no significant differences between the cases and control group.

Table 13. GST's in primary open-angle glaucoma patients and control individuals.

GSTs	POAG cases (n=250)	Controls (n=202)
GSTM1 positive	150 (60.0%)*	91 (45.0%)
GSTM1 null	100 (40.0%)	111 (55.0%)
GSTT1 positive	213 (85.2%)	166 (82.2%)
GSTT1 null	37 (14.8%)	36 (17.8%)
GSTM3 AA	170 (68.0%)	140 (69.3%)
GSTM3 AB	70 (28.0%)	60 (29.7%)
GSTM3 BB	10 (4.0%)	2 (1.0%)
GSTP1 AA	113 (45.9%)	86 (42.6%)
GSTP1 AB	71 (28.9%)	60 (29.7%)
GSTP1 AC	32 (13.0%)	30 (14.9%)
GSTP1 BB	15 (6.1%)	13 (6.4%)
GSTP1 BC	12 (4.9%)	12 (5.9%)
GSTP1 CC	3 (1.2%)	1 (0.5%)

Statistically significant difference from the control group:
* – OR=1.83; 95% CI=1.26 – 2.66; p=0.002

The predisposing effect of the GSTM1 positive phenotype to glaucoma was even more clearly expressed in smokers. Among individuals with positive smoking history 62.7% of patients with glaucoma were classified as GSTM1 positives, while the proportion of GSTM1 positive persons in control group was only 33.3% (OR=3.36; 95% CI 1.49 – 7.56; p=0.012).

The frequency of the GSTT1 positive phenotype was only slightly and not significantly higher in glaucoma group as compared to controls. Also the presence of the GSTT1 positive allele in GSTM1 positive individuals had no major impact on the disease risk.

No differences between patients and control individuals in the frequencies of GSTP1 genotypes or alleles of GSTP1 gene were found.

6. DISCUSSION

6.1. Biological properties of GSTT1-1

1. Enzymatic activity.
Like described earlier GSTT1-1 isolated from liver has high activity towards dichloromethane (1609 nmol/min per mg of protein) as compared to the enzymes from other classes of GSTs. Also GST theta class enzymes were first defined as GST's lacking activity towards model substrate for GST's – CDNB, some activity of purified GSTT1-1 towards CDNB was detected (0,17 μM/min per mg of protein). The ability of GSTT1-1 enzyme to conjugate CDNB with glutathione is approximately 50 times less as compared to GSTM1-1 enzyme (10 μM/min per mg of protein), but is noteworthy as in other theta class enzyme — GSTT2-2 the activity towards CDNB could not be detected.

2. Molecular weight.
The Mr of the GSTT1-1 was fount to be 25300. As shown for the GSTT2-2 (Tan *et al.* 1995), the SDS-PAGE estimated GSTT1-1 subunit molecular mass (25300) is lower than the calculated molecular mass (31400) (Pemble *et al.* 1994). The M_r value found for GSTT1-1 is quite similar to those of GSTT2-2 (M_r 25100) and GSTP1-1 (M_r 24800).

3. Isoelectric point.
The pI value for hepatic GSTT1-1, determined by the isoelectric focusing method, was 6.64. Unlike to GSTM1-1 only single band was detected, supporting the two allele model for the GSTT1 locus. The value obtained with IEF was lower than the pH value that was used to elute the GST1-1 enzyme out from the chromatofocusing column (7,3). The discrepancy between two values found with different methods is probably caused by the fact that also other characteristics of the protein besides pI have effect on elution of GSTT1-1 during chromatofocusing. Therefore the pI value of GSTT1-1 found using isoelectric focusing method is more precise. No differences in pI values were observed in 10 different immunoblotted liver and red blood cell cytosols, indicating the presence of only one isoform of GSTT1-1.

4. Tissue distribution of GSTT1-1.
Immunoblot analysis of various tissue cytosols, carried out using 8–25% gradient SDS-PAGE and anti-GSTT1-1 monoclonal antibody 1A2 suggested that most tissues investigated expressed a detectable amount of GSTT1-1. In pancreas the enzyme was not detected, which was probably caused by high sensitivity of GSTT1-1 to proteolytic enzymes. GSTT1-1 was not detected in the immunoblotted lysate of isolated human lymphocytes. Lysates from erythrocytes and spleen proved to contain lower concentrations of this enzyme than the other lysates.

6.2. Methodological basis for performing of population studies

To elucidate the role of polymorphic glutathione S-transferases in modifying genetic susceptibility to diseases it is essential in the first step to create proper methodological basis for performing large-scale epidemiological investigations and obtain data about ethnic distribution of phenotype and gene frequencies. Both genotyping and phenotyping methods are available for the studies of GST's.

Methods available for GSTM1 phenotyping have been:

1) measurement of glutathione transferase activity towards tritiated trans-stilbene oxide in mononuclear leucocytes (Seidegard *et al.* 1985);
2) immunological detection of GSTM1 in whole blood using Mukit (Biotrin International, Dublin, Ireland) and
3) electrophoresis of tissue extracts followed by a specific staining (Board 1981, Harada *et al.* 1987).

The last approach enables to identify all four GSTM1 phenotypes but requires large amount of samples and is not therefore applicable for screening of patients from hospitals. Assay of GSTM1 activity in lymphocytes and immunological detection with Mukit, though suitable for screening, do not differentiate the *GSTM1*A* and *GSTM1*B* alleles from each other.

The most frequently used protocols for genotyping of GSTM1 enable to identify GSTM1 positive and GSTM1 negative genotypes. Freyer et al modified the genotyping of GSTM1 by introducing a restriction site into the amplified DNA, which allowed after incubation with restriction endonuclease HaeII and electrophoresis, identification of the GSTM1 null, GSTM1 A, GSTM 1 B and GSTM1 A,B polymorphisms. Also primer sequencies have been published for detection of *GSTM1*0* allele (Kerb *et al.* 1999) enabling to distinguish *GSTM1*A/GSTM1*0* and *GSTM1*B/GSTM1*0* heterozygotes from *GSTM1*A* and *GSTM1*B* homozygotes, no epidemiological studies have been published that would use the methodology.

Based on monoclonal antibodies 11F12 and 1H8 that were specific to GSTM1 subunit 1a and 1b ELISA test was developed that allows to perform large scale association studies. The ELISA test enables to phenotype individuals with high degree of accuracy as was seen when the results obtained with different methods were compared. In two cases out of 353 results of ELISA phenotyping were not in concordance with outcome from other methods (discrepancy 0.6%). One noncongruent case was probably caused by a rare mutation that led to lack of expression, as the gene was present but the enzyme could be detected in blood neither with ELISA nor with activity measurement. Another discrepancy can be explained by the effect of nonspecific interfering factors, as both gene and enzymatic activity were absent but ELISA yielded positive result with both

monoclonal antibodies. The results are in good correlation with previous studies where only 2 cases among 272 PCR and ELISA (Mukit, Biotrin, Ireland) tests were inconsistent (0,7%) (Brockmöller *et al.* 1993).

The GSTM1 ELISA test developed is the only phenotyping test that can be used for large scale population studies and is able to differentiate between GSTM1 A, B, AB and null individuals. As compared to other methods ELISA test is the quickest and the cheapest. Obviously the most reliable results can be obtained when both genotyping and phenotyping tests are used.

Phenotyping of GSTT1 is usually performed by measuring of enzymatic activity towards methyl halogens. As methyl halogens are gases, it is inconvenient to use these substrates for determination of GSTT1 activity in population studies. PCR-based genotyping of GSTT1 enables to detect whether the individual is genotypically positive or negative but discrimination between the positive/negative heterozygotes and positive homozygotes has not been possible.

The highly specific monoclonal antibodies developed by us against GSTT1 enabled to work out an ELISA test for GSTT1 phenotyping. Unlike genotyping by PCR, ELISA can distinguish between individuals being either GSTT1 +/– heterozygotes or GSTT1 +/+ homozygotes. Similar discrimination can be performed by measuring of GSTT1 activity, but compared to ELISA it is both more time consuming and expensive. Considering full accordance of ELISA results with PCR and activity determination one can conclude that the ELISA test is a reliable way to measure GSTT1 concentration in whole blood.

6.3. Population genetics of polymorphic GSTs

Data about distribution of GSTM1 gene frequencies has been described in different populations including many Caucasian, African (Zhao *et al.* 1994) and Oriental populations (Harada *et al.* 1987, Board 1981). Phenotype frequencies of GSTM1 in Estonians are close to those published earlier for other Caucasians, but different from Nigerians ($\chi^2 = 36.25$, df = 3, $P < 0.001$), Chinese ($\chi^2 = 24.88$, df = 3, $P < 0.001$) and Japanese ($\chi^2 = 55.81$, df = 3, $P < 0.001$). The distribution of two positive GSTM1 phenotypes varies greatly between East Asian and African populations — Chinese and Japanese are characterized with low occurrence of GSTM1 A (10% and 8% respectively) and high frequency of GSTM1 B (29% and 41% respectively), while the frequency proportions in Nigerians are contrary, 6% for GSTM1 B and 71% for GSTM1 A

Like in GSTM1 locus the frequency of GSTT1 negative phenotype varies greatly between different populations. In Estonian population about 18% of individuals lacked the GSTT1 gene and protein. The percentage found for Estonians is in the range found also for other Caucasians. The lowest fraction of GSTT1 negative individuals has been found in Mexican-Americans (9,7%) and

highest in oriental populations (64.4% in Chinese). While in Caucasians and Africans the proportion of GSTT1 negative individuals does not exceed 24%, Asians are clearly different from all other ethnic groups with a frequency of people homozygous for GSTT1 deletion of more than 60%.

Although not confirmed statistically it might be that the GSTT1 negative phenotype is less frequent in older age groups. A similar influence of age to gene frequencies was described by Chenevix-Trench *et al.* (1995), who found comparing unselected controls with geriatric patients, that the homozygous GSTT1 null genotype was less common among older individuals. The tendency of smaller proportions of GSTT1 negative individuals in older age groups may be the result of predisposition of them to various carcinomas.

The distribution of GSTM3 and GSTP1 genotypes is similar to those published for other Caucasian populations.

6.4. Association of polymorphic GSTs with susceptibility to senile cataract

Biochemical, toxicological and epidemiological studies have led to the conclusion that polymorphic GSTs are involved in the metabolism as well as induction of many known and suspected endogenous and exogenous compounds (Wormhoudt *et al.* 1999). Oxidative damage is considered to be one of the major risk factors for the development of senile cataract. As GSTs are involved in metabolism of wide range of xenobiotics including those that can cause oxidative damage and based on the fact that allelic variants of GSTs have different ability to conjugate substances to glutathione, the role of GST polymorphism in cataractogenesis was hypothesed.

The results of the present study indicate that polymorphic glutathione S-transferases may be involved in cataract formation. In persons with the GSTM1 positive phenotype a 2-fold increase in risk to develop cortical cataract was found. Another locus that associated with cortical cataract was GSTP1, where the carriers of the *GSTP1*A* allele conduced to a 3-fold higher risk of developing cortical cataract and *GSTP1*B* allele had an opposite effect — protective against the disease. Although individuals with GSTT1 positive phenotype and persons with GSTM3 AA genotype were only moderately overrepresented among the patients and the finding was not statistically significant, the presence of these two GST variants in GSTM1 positive individuals increased additively the risk of cortical opacity development. Therefore it is reasonable to suppose that the presence of the GST T1-1 enzyme and GSTM3 AA genotype also makes individuals more susceptible to cortical cataract. The disease risk was highest in persons having all three predisposing genotypes – GSTM1 positive, GSTT1 positive and GSTM3 AA.

No effect of GSTM1, GSTM3, GSTT1 and GSTP1 genes on susceptibility to other types of cataract could be seen.

The possible influence of GSTs to cataract formation is also supported by the distribution of GSTs in the lens. A study by Huang *et al.* (1993) has shown that class Mu and Pi isoenzymes are abundantly expressed in the human lens. The highest GST activity in the human lens was detected in the peripheral and equatorial cortex, while quite low activity was present in the posterior cortex and no activity could be detected in the nucleus and lens epithelium. The described activity distribution in the human lens is in a good accordance with the results of present study, where association between GSTs and cataract formation was clearly expressed in cases of cortical cataract. These results support the theory that senile cataract is a multifactorial disease and different mechanisms are involved during formation of lens opacities.

Unlike hypothesed in the beginning of the study no excess of GST deficient phenotypes was found among patients but contrary, overrepresentation of expressing phenotypes was found. Although GSTs are generally considered to function as detoxifying enzymes, they may also be involved in activation of toxic compounds (Sherratt *et al.* 1997). The exact molecular mechanisms, by which the expressing genotypes of GSTM1 and GSTT1 loci lead to increased cortical cataract risk, remain unclear, but one can hypothese that GST's can take part in activation and formation of some toxic metabolites derived from nutrition, drug metabolism or environmental pollution. The toxic metabolites formed can induce changes in the protein structure, thus favouring aggregation of lens proteins and promoting the development of cataract.

The role of GSTM3 locus in cataractogenesis has two possible explanations, either the effect comes from linkage disequilibrium between the GSTM1 and GSTM3 loci or from different biological effect of two GSTM3 alleles. Although polymorphism occurs in the sixth intron of the GSTM3 gene, three base pair deletion in the *GSTM3*B* allele creates a recognition motif for a transcription factor and possibly the expression of the *GSTM3*A* and *GSTM3*B* alleles or even the GSTM1 gene is regulated differently.

Among glutathione S-transferases expressed in lens GST P1-1 isoenzyme is the most prevalent. In the present study overrepresentation of the *GSTP1*A* allele, as well as individuals with GSTP1 A genotype (AA, AB or AC) was found among cortical cataract patients. Also frequency of individuals having *GSTP1*A* allele in both chromosomes was higher in patients with cortical opacities (51% vs. 42.6%), the difference did not reach the level of significance. In contrast to *GSTP1*A* allele, a protective role of *GSTP*B* allele against cortical cataract was observed. The protective role was dose dependent being higher in persons with two copies of the allele. A different effect of the *GSTP1*A* and *GSTP1*B* alleles on disease susceptibility may be caused by different catalytic properties of the corresponding isoenzymes. The previous studies have shown that the two different GSTP1-1 isoenzymes with isoleucine or valine at position 105 (corresponding to the

*GSTP1*A* and *GSTP1*B* allele products respectively), differ significantly from each other in respect of catalytic properties. The mechanism by which the *GSTP1*A* allele conduces to increased risk of cortical cataract is probably different from the predisposing mechanisms of GSTM1, GSTT1 and GSTM3 loci, as no additive effect was observed of the GSTM1, GSTT1 and GSTM3 polymorphisms on genetic predisposition caused by *GSTP1*A* allele.

The different influence of particular allelic forms of some polymorphic GSTs may be caused by their different ability to resist the oxidative damage and function in the conditions of oxidative stress. As a matter of fact the GST activity is significantly decreased in cataractous lenses as compared to normal age-matched lenses (Rao *et al.* 1983).

The results of present study are in accordance with what is known about the differences in frequency distribution of GSTM1 gene variants in different ethnic groups. Both the risk of developing cortical cataract and frequency of predisposing GSTM1 positive phenotype are considerably higher among individuals of African origin.

Besides the present study two other publications are available where association between GSTs and cataract were investigated. In Japanese study an association between homozygous deletion of GSTM1 and cataract was found while no association was discovered in an Italian study (Alberti *et al.* 1996). The apparent discrepancy could be explained by false positive as well as false negative results or can reflect the true variability between different populations.

One strong side of the present study favouring the conclusion about the importance of GSTs in modifying genetic predisposition to senile cataract is the finding that predisposing genotypes have an additive effect.

6.5. Association of polymorphic GSTs with susceptibility to open angle glaucoma

The present study was the first attempt to investigate the possible association between polymorphic GST genotypes and adult-onset primary open-angle glaucoma. Like in cataract study, contrary to the initial hypothesis of protective role of GSTs approximately a 2-fold increased risk of POAG associated with the GSTM1 positive phenotype (OR=1.83) was found. The study suggests that the GSTM1 positive phenotype may be a genetic risk factor for development of primary open-angle glaucoma.

Recently a similar study was conducted in Sweden (Jansson *et al.* 2003) and no association was found between GSTM1 locus and POAG.

There are several reasons which induce to think that GSTM1 gene may be involved in modifying genetic susceptibility to POAG.

First, the POAG risk found in the present study to be in association with the GSTM1 expressing phenotype is higher among smokers as compared to non-smokers. By the data Wilson *et al.* (1987) the cigarette smokers are at an increased risk of developing glaucoma and at he same time glutathione S-transferases Mu class enzymes are involved in the metabolism of polyaromatic hydrocarbons and other combustion products found in cigarette smoke (Ketterer *et al.* 1992). Therefore it is reasonable to assume that predisposing effect to the disease is increased when both risk factors are present.

Evidence supporting the role of GSTs in formation of POAG comes also from the studies of autoimmunity. Yang *et al.* (2001) have shown that anti-GST antibody was present in sera from patients in significantly higher titers as compared to the controls and the related antigen belonged to Mu class of GST.

In addition to the present study involvement of detoxification enzymes in ethiopathogenesis of glaucoma has been described also for cytochrome P450 1B1 (CYP1B1), where several mutations in the P450 1B1 gene have been found to be responsible for formation of some forms of primary congenital glaucoma (Stoilov *et al.* 1997).

Some supportive evidence favouring towards predisposing role of GSTM1 locus comes also from the fact that predisposing GSTM1 1 positive phenotype can be found much more frequently in populations of African origin, where also the incidence of POAG is higher as compared to Caucasian populations. Undoubtedly more extensive studies in various ethnic groups are required to establish the role of GSTM1 and other GSTs in the interethnic difference of POAG incidence.

Glaucoma is a disease often caused by an increase in intraocular pressure due to a decreased facility of outflow of aqueous humour through the trabecular meshwork. Obstruction of trabecular meshwork by macromolecules is considered to be one of the mechanisms that lead to decreased outflow. Supposedly during development of POAG some polymorphic GST isoenzymes expressed in eye take part in activation of endogenous and exogenous compounds including those present in cigarette smoke. These toxic metabolites may induce changes in the structure of proteins present in the aqueous humour, thus contributing to modification or aggregation of proteins and promoting the development of glaucoma.

7. CONCLUSIONS

1. Unlike believed earlier GSTT1-1 isoenzymes show detectable activity towards model substrate for glutathione S-transferases – CDNB. The M_r value of the GSTT1 is 25 300 and isoelectric point 6.64. GSTT1-1 is expressed in many organs and tissues including liver, erythrocytes, lung, kidney, brain, skeletal muscle, heart muscle, small intestine and spleen.

2. ELISA test developed for phenotyping of GSTM1-1 produces reliable results as could be seen in validation experiments. The ELISA worked out is the only phenotyping test for large scale population studies having ability to differentiate between GSTM1 A, B, AB and null individuals.

3. ELISA based phenotyping of GSTT1-1 produces highly accurate results as decided by comparison of ELISA outcome with results from other phenotyping and genotyping methods. Test is quantitative enabling to measure GSTT1 concentration in tissues. Unlike most of the methods used for GSTT1 studies ELISA test makes possible to detect all three phenotypes.

4. Frequency distribution of GSTM1, GSTT1, GSTM3 and GSTP1 alleles and genotypes in Estonian population is similar to those described for other Caucasian populations.

5. Polymorphic glutathione S-transferases are involved in cataract formation. Persons with the GSTM1 positive phenotype have a 2-fold increase in risk to develop cortical cataract and carriers of the *GSTP1*A* allele have three times higher probability to develop cortical cataract. Although GSTT1 positive phenotype and GSTM3 AA genotype had only moderate effect on disease susceptibility and were not proven to be independent risk factors, they increased additively the risk of cortical opacity development in GSTM1 positive individuals. The disease risk was highest in persons having all three predisposing genotypes — GSTM1 positive, GSTT1 positive and GSTM3 AA.

6. GSTM1 positive phenotype is also a genetic risk factor for primary open-angle glaucoma. The POAG risk associated with GSTM1 expressing phenotype is increased in case of smoking.

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9. SUMMARY IN ESTONIAN

Polümorfsed glutatioon S-transferaasid — bioloogia ning roll primaarse avatud nurgaga glaukoomi ja vanaduskatarakti geneetilise vastuvõtlikkuse modifitseerimisel

Sissejuhatus probleemi

Detoksifikatsiooniensüümid jaotatakse esimese ja teise faasi ensüümideks. Esimese faasi ensüümid katalüüsivad oksüdeerimist, redutseerimist ja hüdrolüüsi. Teise faasi ensüümid muudavad substraadid hüdrofiilseks läbi konjugatsiooni erinevate substraatidega. Glutatsioon S-transferaasid (GST) on teise faasi detoksifikatsiooni-ensüümid, mis konjugeerivad substraate tripeptiid glutatiooniga. Inimese GST-d jaotatakse mikrosomaalseteks ja tsütosoolseteks. Tsütosoolsed GST-d jagunevad omakorda vastavalt aminohappelise järjestuse homoloogilisusele kaheksasse klassi — alfa, müü, teeta, pii, zeeta, sigma, kappa ja omega. GST geenides on kirjeldatud mitmeid geneetilisi polümorfisme, millest osa omavad ka funktsionaalset tähendust.

Geneetilisest polümorfismist tulenevalt omavad GST isovormid erinevat võimet kaitsta kudesid mitmete kahjulike endo- ja eksogeensete toksiinide ning oksüdatiivse stressi vastu, millest lähtuvalt on läbi viidud arvukalt uuringuid leidmaks võimalikku seosest haigusvastuvõtlikkuse ja GST-de vahel.

Kõige enam on molekulaarepidemioloogilistes uuringutes huvi pakkunud kaks glutatioon S-transferaasi — GSTM1 ja GSTT1. Neis esineva polümorfismi aluseks on geeni täielik deletsioon. Isikutel, kellel esineb deletsioon mõlemas kromosoomis, puudub vastav ensüüm täielikult. Need kaks ensüümi on olnud ka antud töö uurimisobjektideks. GSTM1 lookuses esineb ka funktsionaalne ühe-nukleotiidne asenduspolümorfism, mistõttu ensüüm omab kahte homodimeerset ja ühte heterodimeerset alavormi, vastavalt GSTM1a-1a, GSTM1b-1b ja GSTM1a-1b. Lisaks polümorfismidele GSTM1 ja GSTT1 lookustes on antud töös uuritud ka ühenukleotiidseid asenduspolümorfisme GSTP1 geenis ja määratud kolme aluspaari suurust deletsiooni GSTM3 geenis.

Detoksifikatsiooniensüümidel on oluline roll täita ka silmas, kus kahjustavad faktorid võivad viia muutuste tekkeni valkudes, mis aastate jooksul kumuleerudes võivad omakorda olla aluseks katarakti, glaukoomi või ka mõne teise silmahaiguse tekkele.

Käesoleva töö käigus on püstitatud hüpotees polümorfsete GST-de rollist primaarse avatud nurgaga glaukoomi ja vanaduskatarakti etiopatogeneesis. Töö läbiviimiseks on esmalt välja töötatud metoodiline arsenal polümorfismide kirjeldamiseks, mille käigus on saadud ka lisainformatsiooni mõnede seni kirjeldamata GSTT1-1 ensüümi bioloogiliste omaduste kohta. Valideeritud metoodilist baasi on kasutatud populatsiooni- ja molekulaarepidemioloogiliste uuringute läbiviimiseks.

Töö ülesanded

1) Luua ja valideerida metoodiline baas polümorfsete GST-de kirjeldamiseks.
2) Uurida GST-de geenisagedusi eestlaste populatsioonis.
3) Viia läbi assotsiatsiooniuuring leidmaks võimalike seoseid polümorfsete glutatioon S-transferaaside ja vanaduskatarakti vahel.
4) Viia läbi assotsiatsiooniuuring leidmaks võimalikke seoseid polümorfsete glutatioon S-transferaaside ja primaarse avatud nurgaga glaukoomi vahel.

Kasutatud metoodikad ja uurimistulemused

GSTM1 fenotüpeerimise läbiviimiseks töötati välja alleelispetsiifiline ELISA. ELISA väljatöötamist alustati GSTM1 puhastamisest maksakeost. GSTM1 isoleerimise eelselt selekteeriti välja sobivad isikud läbi fenotüpeerimise, kasutades isoelektrilist fokusseerimist koos sellele järgneva reaktsiooniga ensüümi aktiivsusele. GSTM1 isoleeriti maksa tsütosoolidest läbi glutatsioon-agaroos affinsuskromatograafia ja kromatofokusseerimise. Saadud fraktsioonides määrati GST-de olemasolu läbi ensümaatilise aktiivsuse mõõtmise substraadi CDNB suhtes. Monoklonaalsete antikehade saamiseks viidi läbi immuniseerimine maksakoest isoleeritud GSTM1 valguga. ELISA baseerus kolmel monoklonaalsel antikehal. Neist 11F12 on spetsiifiline 1a alavormile, 1H8 on spetsiifiline 1b alavormile ja 10G12 tunneb ära epitoope mõlemas alavormis. Saadud test valideeriti läbi 449 isiku võrdluse kahe genotüpeerimissüsteemiga, millest üks eristab GSTM1 positiivseid isikuid negatiivsetest ja teise abil saab positiivsete hulgas vahet teha ka GSTM1 A, B ja AB isikute vahel. Valideerimisel leiti, et välja töötatud ELISA test võimaldab isikuid usaldusväärselt fenotüpeerida GSTM1 suhtes.

GSTT1 fenotüpeerimiseks töötati samuti välja ELISA test. Väljatöötamise esimeses etapis leiti sobivad isikud läbi fenotüpeerimise. Erütrotsüütides ekspresseeruva ensüümi määramiseks mõõdeti erütrotsüütide võimet siduda GSTT1 substraati metüülkloriidi. Maksakoest määrati tsütosooli aktiivsust diklorometaani suhtes. GSTT1-1 isoleerimisel saadud fraktsioonides tõestati GSTT1 olemasolu samuti läbi ensüümi aktiivsuse mõõtmise diklorometaani suhtes. Isoleeritud GSTT1-1 valku kasutati ensümaatiliste omaduste kirjeldamiseks, molekulmassi määramiseks SDS-polüakrüülamiidforeesil, isoelektrilise täpi määramiseks isoelektrilisel fokusseerimisel ja ekspressiooni tuvastamiseks erinevates kudedes läbi immunobloti. Isoleeritud GSTT1 ensüümi abil töötati välja monoklonaalsed antikehad ja ELISA test. ELISA test valideeriti läbi selle, et 76-l isikul mõõdeti erütrotsütaarse GSTT1 aktiivsus diklorometaani suhtes ja 133-l viidi läbi genotüpeerimine. Valideerimise tulemusena leiti, et ELISA test GSTT1 fenotüpeerimiseks on usaldusväärne fenotüpeerimissüsteem ja võimaldab kindlaks teha kõik kolm GSTT1 fenotüüpi.

GSTM3 ja GSTP1 lookuste osas kasutati polümorfismide määramiseks genotüpeerimist läbi PCR koos sellele järgneva restriktsioonanalüüsiga.

Populatsiooniuuringu tarvis kasutati isikuid, kelle puhul oli kindlaks tehtud nende põlvnemine vaid Eesti päritolu esivanematest kahe põlvkonna ulatuses.

Molekulaarepidemioloogilise uuringu tarvis koguti kliiniline info ja vereproovid 250-lt primaarse avatud nurgaga glaukoomi diagnoosiga patsiendilt ja 503-lt vanaduskatarakti diagnoosiga isikult. Kontrollgrupp formeeriti 200-st isikust, kellel oli kontrollitud katarakti ja glaukoomi mitteesinemine.

Vanaduskatarakti diagnoosiga patsientidel leiti kõige tugevam seos GSTM1 lookuse osas. Tuvastati oluline GSTM1 positiivsete isikute osakaalu tõus kortikaalse kataraktiga isikute hulgas (60,6% vs 45%; p=0,004). Samuti olid üleesindatud *GSTP1*A* alleeliga isikud. Ilmnes ka haigusriski tõusu potentseeriv efekt, kui GSTM1 positiivsele staatusele lisandus GSTM3 AA genotüüp ja/või GSTT1 positiivne fenotüüp.

Primaarse avatud nurgaga glaukoomi patsientide hulgast leiti GSTM1 isikute üleesindatus võrrelduna kontrollgrupiga (60% vs 45%; p=0,002). Assotsiatsioon glaukoomi ja GSTM1 vahel oli tugevam, kui arvesse võeti ka suitsetamist.

Järeldused

1. Vastupidiselt varem arvatule omab GSTT1-1 ensüüm aktiivsust GST-de mudelsubstraadi CDNB suhtes. GSTT1-1 molekulmass määratuna SDS-foreesil oli 25300 ja isoelektriline täpp määratuna läbi isoelektrilise fokusseerimise 6,64. GSTT1-1 ekspresseerub paljudes kudedes, sealhulgas maksas, erütrotsüütides, kopsus, neerudes, ajus, skeletilihaskoes, südamelihases, peensooles ja põrnas.
2. Välja töötatud ELISA test GSTM1 fenotüpeerimiseks on usaldusväärne vahend GSTM1 fenotüpeerimiseks ja on maailmas ainus test, mis võimaldab suuremahulisi kliinilisi kontingete fenotüpeerides vahet teha mitte ainult GSTM1 positiivsetel ja negatiivsetel isikutel, vaid ühtlasi ka identifitseerida GSTM1 A, B ja AB isikuid.
3. Välja töötatud ELISA test GSTT1 fenotüpeerimiseks on usadusväärne fenotüpeerimissüsteem ja on sealjuures kvantitatiivne. Erinevalt enamusest kasutuses olevatest GSTT1 määramismeetoditest võimaldab antud ELISA kindlaks teha kõik kolm GSTT1 fenotüüpi.
4. Genotüüpide, fenotüüpide ja alleelide sagedusjaotus GSTM1, GSTT1, GSTM3 ja GSTP1 lookustes Eesti populatsioonis ei erine oluliselt sellest, mis on eelnevalt kirjeldatud teiste europiidsete populatsioonide osas.
5. Polümorfsed GST-d on seotud kataraktogeneesiga. Risk saada katarakt on GSTM1 positiivsetel inimestel tõusnud kaks korda ja *GSTP1*A* alleeliga isikutel kolm korda. GSTM3 AA genotüüp ja GSTT1 positiivne fenotüüp omasid iseseisvalt vaadatuna küll vaid nõrka kataraktogeneesi soodustavat efekti, kuid potentseerisid GSTM1 positiivsest staatusest tulenevat haigestumisriski tõusu.
6. GSTM1 positiivne staatus on riskifaktoriks ka primaarse avatud nurgaga glaukoomi korral, kusjuures risk on tõusnud suitsetavatel isikutel.

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CURRICULUM VITAE

Gunnar Tasa

Citizenship:	Estonian citizen
Born:	February 10, 1965 in Tartu, Estonia
Address:	Department of Human Biology and Genetics, University of Tartu Ravila 19, 50411 Tartu, Estonia
Phone:	+372 7 374 217
Fax:	+372 7 374 212
E-mail:	Gunnar.Tasa@ut.ee

Education

1972–1975	Tartu Elementary School N 11
1975–1983	Türi Secondary School
1983–1991	Medical Faculty, University of Tartu
1991–1996	Postgraduate studies at the Department of Human Biology and Genetics, University of Tartu.

Employment history

1991	Scientist and assistant at the Department of Human Biology and Genetics, University of Tartu
2000	DNA specialist at the Estonian Bureau of Forensic Medicine.

Scientific work

The scientific work has been focused on the following areas:

1) polymorphic glutathione S-transferases in Estonian population, biology and possible connection with susceptibility to diseases;
2) molecular mechanisms of the gelatinous drop-like corneal dystrophy;
3) relation of depression and suicide to serotonin transporter gene polymorphism;
4) association of personality traits with genetic markers;
5) molecular arrangement of deleted regions in human genome.

Publications

Nineteen scientific publications.

CURRICULUM VITAE

Gunnar Tasa

Kodakondsus: Eesti
Sündinud: 10.02.1965 Tartu, Eesti
Address: Inimese bioloogia ja geneetika õppetool, Tartu Ülikool
Ravila 19, 50411 Tartu
Phone: +372 7 374 217
Fax: +372 7 374 212
E-mail: Gunnar.Tasa@ut.ee

Haridus

1972–1975	Tartu 11.-nes kaheksaklassiline kool
1975–1983	Türi Keskkool
1983–1991	Tartu Ülikooli arstiteaduskonna ravi eriala
1991–1996	Doktorantuur Tartu Ülikooli Inimese Bioloogia ja Geneetika õppetoolis.

Teenistuskäik

1991	Teadur ja assistent Tartu Ülikooli Inimese Bioloogia ja Geneetika õppetoolis
2000	DNA ekspert Eesti Kohtuarstlikus Ekspertiisibüroos.

Teadustegevus

Teadustöö on olnud seotud järgmiste valdkondadega:
1) polümorfsed glutatioon S-transferaasid Eesti populatsioonis, bioloogia ja võimalik seos haigusvastuvõtlikkusega;
2) päriliku silma sarvkesta tilkdüstroofia molekulaarsed mehhanismid;
3) depressiooni ja suitsidaalse käitumise seos geneetiliste polümorfismidega serotoniinergilises süsteemis;
4) isiksuseomaduste seos geneetiliste markeritega;
5) inimenoomis esinevate deletsioonide molekulaarne organisatsioon.

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