



UNIVERSIDADE DA BEIRA INTERIOR  
Ciências da Saúde

# **Pilot-Model for oxidative post-competition recovery in swimmers**

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“Para ser grande, sê inteiro: nada  
Teu exagera ou exclui.  
Sê todo em cada coisa. Põe quanto és  
No mínimo que fazes.  
Assim em cada lago a lua toda  
Brilha, porque alta vive.”

Ricardo Reis, Odes



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# Resumo alargado

O treino desportivo com o objetivo de performance competitiva coloca os atletas sob um forte risco de desequilíbrio oxidativo, conhecido por stress oxidativo. A produção de radicais livres e espécies electrofílicas, como as Espécies Reativas de Oxigénio (ROS), são uma constante no metabolismo normal do organismo, no entanto, a maior taxa metabólica exigida pela demanda energética do exercício físico intenso, provocam uma produção de tais espécies a um nível superior às defesas antioxidantes disponíveis. Nesta situação de stress oxidativo, os radicais livres e ROS provocam danos a fulcrais estruturas e macromoléculas celulares, reagindo forte e rapidamente com estas, ameaçando a homeostasia celular.

Para controlar a ação nefasta dessas agressões oxidativas, os organismos possuem mecanismos de defesas antioxidantes, podendo estas ser de origem endógena ou exógena. Entre as defesas antioxidantes endógenas encontram-se proteínas expressas pelas células, e cuja expressão pode ser influenciada pelo ambiente oxidativo celular, como é o caso das Glutathione S-Transferases (GST). Desta forma, situações que criem stress oxidativo, como no treino desportivo, ativam a expressão das defesas antioxidantes.

Assim sendo, o treino desportivo regular e bem planeado, de forma a evitar danos constantes ao organismo, deve ativar uma resposta deste de forma a protegê-lo dessa agressão, preparando-o previamente para essa agressão. Essa preparação pode ser verificada através da expressão génica de fatores antioxidantes endógenos. Além disso, certos genótipos podem revelar-se vantajosos nesta proteção, nomeadamente os genótipos associados às várias isoformas das GSTs. Nestes, constam vários e frequentes genótipos Null (ausência do gene), o que permite uma grande variabilidade entre indivíduos para a disponibilidade de isoformas de GSTs.

O objetivo deste trabalho foi precisamente verificar a distribuição de genótipos Null/Present para duas isoformas de GSTs, a GSTM1 e a GSTT1, numa amostra de 20 nadadores portugueses de nível nacional. Para comparação de genótipos, foi recolhida semelhante informação a partir de um grupo de controlo constituído por 52 indivíduos aleatórios. Além disso, observou-se a expressão relativa de GSTT1 ao longo de 5 momentos distintos ao longo da época de Inverno (preparação geral, preparação específica, fase *taper* e dois momentos pós-competição) em 3 desses atletas, e a expressão relativa, também de GSTT1, 48h e 72h após uma competição, para 8 desses atletas.

Para conseguir alcançar isto, foi necessário montar uma técnica totalmente nova para recolher as amostras de forma rápida, fiável e praticável nas condições de treino, e otimizar todos os procedimentos laboratoriais para conseguir processar essas amostras de forma eficiente e rigorosa. As amostras foram recolhidas em papel de filtro de análises clínica, através de uma picada no dedo dos nadadores, antes do início do treino do dia definido previamente para recolha de amostras. As amostras foram ainda conservadas em invólucros

individuais para cada recolha a cada momento e de cada atleta, numa câmara-fria 4°C, no Centro de Investigação em Ciências da Saúde (CICS) da Faculdade de Ciências da Saúde (FCS) da Universidade da Beira Interior (UBI).

Para genotipagem dos nadadores em amostra, DNA foi extraído da amostra de sangue em papel utilizando o método do Chelex 100. Após extração, o DNA foi usado para amplificação enzimática da sequência específica dos genes da GSTM1 e GSTT1, pela técnica de PCR. Por fim, os resultados foram corridos por electroforese em gel de agarose, usando Green-safe como fator de marcação de DNA, e os resultados foram visualizados à luz ultravioleta num transiluminador. A presença de GSTM1 foi identificada pela presença de uma banda com cerca de 215bp, enquanto a presença de GSTT1 foi identificada pela presença de banda aos 473bp.

Para análise da expressão génica, RNA foi isolado a partir das amostras de sangue em papel, pelo método do Trizol. O RNA era correspondente a cada um dos momentos de recolha. De seguida o RNA foi convertido a cDNA através da técnica de transcriptase reversa, utilizando a enzima M-MLV. Por fim, o cDNA foi amplificado pela técnica de RT-PCR, para o gene GSTT1, tendo ainda como controlo a amplificação da  $\beta$ -Actin, também para cada um dos momentos de recolha e fazendo duplicados por uma questão de rigor. A expressão foi calculada através das curvas de amplificação de RT-PCR e utilizando o método  $\Delta\Delta C_T$ .

Não foram encontradas distribuições de genótipos GSTM1 e GSTT1 Null/Present estatisticamente significativas entre a nossa amostra de teste e o grupo de controlo. No contexto da expressão relativa de GSTT1, verificou-se que variações muito acentuadas ao longo da época desportiva ou após um exercício foram prejudiciais à performance física dos nadadores. Encontramos também algumas diferenças na recuperação das nadadoras, mantendo uma expressão mais alta e por um maior período de tempo após o exercício físico intenso que os homens. Além disso, verificou-se uma tendência para os indivíduos GSTM1 Null manterem os níveis de expressão relativa de GSTT1, ao longo da época e após um exercício intenso, mais estáveis, o que parece favorecer o seu rendimento. Conclui-se ainda que a análise da evolução da expressão relativa de GSTT1 em vários treinos, após uma competição ou outro exercício de elevada intensidade, pode ajudar a perceber qual a forma atual de um nadador.

## Palavras-chave

Glutathione S-Transferases, Espécies Reactivas de Oxigénio, treino desportivo, expressão génica.



# Abstract

Physical exercise have several health benefits, but it can also be a source of cellular damage. The energetic demands of physical exercise and training promote an increase on metabolic rate, and its pathways may produce secondary harmful compounds that will cause cellular damage. Some of those compounds are the free radicals and Reactive Oxygen species, which are highly instable molecules that react quickly, oxidizing important functional molecules such as proteins, membrane lipids and DNA, in a condition known as oxidative stress. To dampen the action of these molecules, the cells express antioxidant defence proteins. One of the most ubiquitous and polymorphic of those is the family of Glutathione S-Transferases (GSTs). The great physical load of competitive training creates serious oxidative stress on athletes so, it is expected that their expression of GSTs will vary throughout the season to overcome such aggression, quickly recovering from one training session and preparing the antioxidant defence for the next one.

Our main objective was to verify if the expression of a GST (GSTT1) varies throughout the season, as expected theoretically, and how it fluctuates after a competition. We also check if the distribution of the GSTM1 and GSTT1 Null/Present genotypes had some influence in the preparation and performance of our sample, consisting in 20 national level swimmers. A control group of 52 random individuals was also used to compare genotype distribution.

We collected blood samples in analytic filter paper, at 5 different moments throughout the winter season. DNA was isolated from a sample of each individual, amplified by PCR for our interest genes, and ran in agarose gel by electrophoresis to genotype our 20 swimmers. RNA was isolated from all the samples of a swimmer and converted in cDNA by reverse transcriptase. The relative expression of GSTT1 was done using  $\beta$ -actin (a housekeeping gene) as a control gene and the first collected sample of the swimmer as control condition, by the RT-PCR technic. Three swimmers were accessed for the whole 5 moments, while eight were only evaluated their expression at 48h and 72h after competition.

The results showed little influence in the distribution of genotype from swimmers to controls. The expression results show influence of the GSTT1 expression profile throughout the season and after an intense exercise with sport performance and as a fitness check tool.

# Keywords

Glutathione S-Transferases, Reactive Oxygen Species, sport training, gene expression.



# Index

1. Introduction	1
1.1. GSTs	1
1.1.1. Physiologic function and distribution	1
1.1.2. Acting mechanisms	2
1.1.3. GST classification	9
1.1.4. Structure and dimerization	11
1.1.5. Chromosomic location	12
1.1.6. Nrf2 and GST	14
1.2. Oxidative stress and physical exercise	16
1.2.1. Oxidative stress	16
1.2.2. Oxidative damage linked to physical exercise	18
1.2.3. Oxidative stress and GST	20
2. Objectives	21
2.1. Main objective	21
2.2. Secondary objectives	21
3. Materials and Methods	22
3.1. Subjects	22
3.2. Study design	22
3.3. Data collection and storage	24
3.4. Sample genotyping	24
3.4.1. DNA extraction	25
3.4.2. Polymerase Chain Reaction (PCR)	25
3.4.3. Electrophoresis	27
3.5. Gene expression	28
3.5.1. RNA extraction	28
3.5.2. cDNA synthesis	29
3.5.3. Real Time PCR (RT-PCR)	30

3.5.4. Relative expression calculus	33
3.6. Statistical analysis	34
3.6.1. Genotyping	34
3.6.2. Relative Expression	35
4. Results	36
4.1. Genotyping	36
4.2. Relative expression	38
5. Discussion	44
5.1. Genotype determination	44
5.2. Gene Expression	44
6. Conclusion	47
7. Bibliography	48
8. Annexes	56
8.1. Authorization Chart	56



## Figure list

- Figure 1: Pathways of GSH metabolism. Y represents a GSH substrate, while X represents a  $\gamma$ -Glutamyl moiety acceptor. Enzymes involved: 1)  $\gamma$ -Glutamylcysteine Synthetase; 2) Glutathione Synthetase; 3) Glutathione Peroxidases; 4) Glutathione Reductase; 5) Glutathione S-Transferases; 6)  $\gamma$ -Glutamyl Transferase; 7) Ectopeptidases. Adapted from (8). 3
- Figure 2: Simplified Mercapturic Acid Pathway. The GSH conjugation with a xenobiotic is catalyzed by a GST, resulting in a Glutathione S-Conjugate. Adapted from (27). 4
- Figure 3: Aromatic Amino Acids Catabolism. GSTZ1-1 has Maleylacetoacetate Isomerase activity, catalyst of the penultimate reaction in this catabolic pathway. 6
- Figure 4: Synthesis of Steroid Hormones. GSTA3-3 acts as a highly efficient keto-steroid isomerase and only exists in steroid producing tissues. 7
- Figure 5: Regulation of Prostaglandins signalling by action of GSTs, and the transcription factors that may be activated or inhibited by them. Adapted from article (4). 9
- Figure 6: Ball-and-socket Interactions between GST monomers. Red and blue regions are the ball and the socket, respectively. Adapted from article (2). 12
- Figure 7: Schematic representation of the Nrf2-Keap1 gene expression regulation. The Keap1 complex, due to its E3 ubiquitin ligase activity, constantly binds and marks Nrf2 for degradation. When electrophilic substances are present, due to oxidative stress, Keap1 cysteine residues get alkylated, releasing Nrf2 that can migrate to the nucleus, where it recognizes the ARE element of cytoprotective genes and promotes their expression. Adapted from article (40). 15
- Figure 8: The electron transport chain, in the inner membrane of mitochondrion. Electrons are transferred through proteins by a series of oxidoreductions, with reduction potential progressively higher. The electron energy differential through the chain is used to pump electrons to the inner mitochondrial membrane, by complexes I, III and IV, creating a chemiosmotic gradient. After complex IV, electrons are captured by oxygen, which reacts with hydrogen to form water. This whole transport, very well balanced can, nevertheless, let 0.4% to 4% of electrons escape and form ROS. Adapted from (47). 17
- Figure 9: Catabolism of purines. This metabolic pathway creates ROS by the Xanthine oxidase activity (Bold compounds have recognized electrophilic activity). Damage caused to DNA will

trigger this catabolism, in order to eliminate the damaged purine bases, but the action of xanthine oxidase results in peroxide, contributing to oxidative stress. 19

Figure 10: Well displacement on the RT-PCR plate. The wells represented as green squares were relative to the target gene, GSTT1, while the blue-coloured squares were relative to the housekeeping gene,  $\beta$ -actin. The wells with an inner rotated square were the Negative Controls, in which cDNA had been replaced by water. The numbering represents the sample collection order (1 for the first sample moment, and so on). This plate was repeated for each individual and for each target gene. 31

Figure 11: Optimization of annealing temperature and amplification cycles for GSTT1. Sample 1 and sample 2 correspond to two different individuals whose RNA was previously extracted and converted in cDNA. Annealing temperature was set to 58.0° C and were done 60 amplification cycles. NTC-1 is the Non-template control. In spite of its amplification, it occurs later than our samples. 32

Figure 12: Melting curves for GSTT1 and  $\beta$ -Actin genes. Each line correspond to a sample well, and all graphics are expressed as fluorescence units by °C. Correct amplification at a sample was considered when its melt peak was between the 80°C and 90°C. a) Dissociation lines for GSTT1 wells; b) Melting curve peaks for GSTT1; c) Dissociation lines for  $\beta$ -Actin wells; d) Melting curve peaks for  $\beta$ -Actin. 33

Figure 13: Gel images captured with UV light, for genotype analysis. On left side, an example of a null and a present genotype for GSTM1. On the right side, two examples for present GSTT1 genotype. In this case, bands migrated further than expect, so their length doesn't match with the DNA ladder. That situation is likely due to partial DNA degradation by haemoglobin contamination. 36

Figure 14: GSTT1 expression throughout the Winter season, for three different swimmers. Units are expressed in expression fold, comparing to the housekeeping-gene,  $\beta$ -actin. Baseline is the moment 1, Specific is the moment 2, Taper is the moment 3, and 48h and 72h are the fourth and fifth moments, relative to the post-competition recovery. 39

Figure 15: GSTT1 expression tendency after a competition, for eight different swimmers. Units are expressed in expression fold, comparing to the housekeeping-gene,  $\beta$ -actin. Our baseline is the first collected sample, then 48h and 72h are the samples collected at that time after the competition. 40

Figure 16: GSTT1 expression lines after the division by 2 groups, according to their evolution at the first 48h. Lines in blue correspond to male swimmers, and those in rose to female swimmers. a) swimmers who dropped their GSTT1 expression after the first episode; b) swimmers who had increased expression after the same episode. 40

Figure 17: GSTT1 expression lines after the division by 3 groups, according to their expression evolution at the 48h-72h period. Lines in blue correspond to male swimmers, and those in rose to female swimmers. a) Swimmers who greatly increased GSTT1 expression after the second episode; b) Swimmers with moderate expression increase; c) Swimmers who dropped or maintained their GSTT1 relative expression at this interval. 41

Figure 18: Expression tendency lines for our sample's swimmers, evidencing the groups divided by number of physical episodes. The orange lines are for swimmers with only one episode (the training), while black lines stand for swimmers who had two episodes (the competition 48h before the training). 42

Figure 19: Gene expression comparison between the one episode group and the two episodes group, in matter of gender influence. The rose lines mark female swimmers, while the blue lines correspond to male swimmers. a) Swimmers submitted to 2 episodes (competition + training); b) Swimmers submitted to only one episode (training). 42

Figure 20: Comparison of the GSTT1 expression in the two episode group versus the single episode group, for the influence of the GSTM1 genotype. GSTT1 genotype has not included because it not a differentiation factor, as all our swimmers were GSTT1 Present. Blue lines represent GSTM1 Null individuals and magenta lines are GSTM1 Present. a) Swimmers submitted to 2 episodes (competition + training); b) Swimmers submitted to only one episode (training). 43



## Table list

Table 1: Families and classes of GSTs, their members and substrate.	10
Table 2: Cytosolic GSTs' genes, chromosomic location, alleles, mutations and their effects.	13
Table 3: Training characteristics. WV (weekly volume); A0 (slow/regenerative swimming speed); A1 (aerobic, low intensity swim); A2 (swim speed at lactic anaerobic threshold); A3 (pace above the lactic anaerobic threshold); A4 (maximal aerobic potency pace); LT (Lactic Tolerance); LP (Lactic Potency); PR (Potency Resistance).	23
Table 4: Reagents <i>per</i> PCR reaction tube, for accessing GSTM1 and GSTT1 null/present genotypes in swimmers.	26
Table 5: GSTM1 null/present genotype distribution comparison between swimmers and controls.	37
Table 6: GSTT1 null/present genotype distribution comparison between swimmers and controls. † P-value calculated by Fisher's Exact Test, since one class count less than 5 cases.	37
Table 7: Combination of GSTM1 and GSTT1 null/present genotypes distribution between swimmers and controls.	37
Table 8: Combination of GSTM1 and GSTT1 null/present genotypes distribution between within swimmers, according to their distance speciality. <sup>(1)</sup> SDS vs MDS; <sup>(2)</sup> SDS vs Controls; <sup>(3)</sup> MDS vs Controls	37
Table 9: Combination of GSTM1 and GSTT1 null/present genotypes distribution between swimmers and controls according to their gender. <sup>(1)</sup> Male vs Male; <sup>(2)</sup> Female vs Female; <sup>(3)</sup> Male vs Controls; <sup>(4)</sup> Female vs Controls.	38
Table 10: Individual characteristics for the 8 swimmers eligible for GSTT1 relative expression analysis. All of them compared on the 5 sample collect moments. The nr of races and best position are related to the winter short course National championships. *Number of events (number of eliminatories); #Classifications achieved in all events, from the best to the worst.	38



## Abbreviation list

15d-PGJ <sub>2</sub>	15-deoxy- $\Delta^{12, 14}$ -prostaglandin J <sub>2</sub>
AA	Amino Acid
Ala	Alanine
AMV	Avian Myeloblastosis Virus
ARE	Antioxidant Response Element
ATP	Adenosine Triphosphate
bp	Base Pair(s)
cDNA	Complementary DNA
CICS	Health Sciences Research Centre
CnC	Cap'n'Collar
C <sub>T</sub>	Cycle threshold
Cul3	Cullin 3
Cys	Cysteine
DDCT	d-Dopachrome Tautomerase
DHA	Dehydroascorbic Acid
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
FCS	Faculty of Health Sciences
Glu	Glutamate
Gly	Glycine
GSH	Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-Transferase
HWE	Hardy-Weinberg Equilibrium
Ile	Isoleucine
JNK	c-Jun NH2-terminal kinase
kb	Kilobase
Keap1	Kelch-like ECH Associated Protein 1
LP	Lactic Potency
LT	Lactic Tolerance
LTC4	Leukotriene C4
MAPEG	Membrane-Associated Proteins of Eicosanoids and Glutathione metabolism
MDS	Middle-Distance Swimmer
M-MLV	Moloney-Murine Leukaemia Virus
MRP	Multidrug Resistance Protein
NFE2L2	Nuclear factor erythroid-2-related-factor 2 gene
Nrf2	Nuclear factor erythroid-2-related-factor 2
PCR	Polymerase Chain Reaction
PGX <sub>y</sub>	Prostaglandin X <sub>y</sub> (X <sub>y</sub> represent the different isoforms)
PR	Potency Resistance
PR	Potency Resistance
Prx VI	1-cys Peroxiredoxin
Rbx1	Ring-box 1
RNA	Ribonucleic Acid

ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
RT-PCR	Real Time Polymerase Chain Reaction
SDS	Short-Distance Swimmer
SNP	Single Nucleotide Polymorphism
UBI	University of Beira Interior
Val	Valine
γGT	γ-Glutamiltransferase





# 1. Introduction

The physical exercise practice naturally obliges to an oxygen consumption rate increase, comparing to the basal state, so the body can obtain the energy required to perform the necessary motor and sensorial actions. However, energy attainment, by complete lipid and carbohydrate metabolization at mitochondrial cristae, involves the electron chain transporter, a process that may cause Reactive Oxygen Species (ROS), since oxygen is the final electron receptor. ROS are very reactive chemical species that strongly and quickly react, for instance, with lipids and nucleic acids, oxidizing them. Thus, physical exercise potentiates ROS production, arising oxidative stress at cells. In order to protect cells from such menace, there are detoxifying enzymes, such as Glutathione S-Transferases (GSTs), whose expression is mediated by several transcription factors, amongst them Nrf2. Thus, to keep organism's homeostasis, it is expected that a competitive athlete has changes at gene expression for the transcription factors which regulate antioxidative protection, so he can be prepared against the extraordinary oxidative stress he is subjected to.

In this work, we investigate the Null/Present genotype distribution for two GSTs (GSTM1 and GSTT1) for a swimmer group and a control group, and evaluate the fluctuations of the GSTT1 relative expression along different training phases on a sports season and after a competition.

## 1.1. GSTs

Glutathione S-Transferases are a multifunctional and ubiquitous dimeric phase II detoxifying enzyme superfamily (1-3). They are usually divided into 3 main families, according to their intracellular location: Cytosolic, Mitochondrial and Membrane-Associated Proteins of Eicosanoids and Glutathione metabolism (MAPEG family) (2, 4).

### 1.1.1. Physiologic function and distribution

The main function of GSTs is to metabolize and detoxify chemical electrophiles, carcinogens, pollutants and products of oxidative stress (2, 4), that are responsible to damage at membrane lipids and DNA (4). However, besides cellular detoxification and antioxidant protection, GSTs take also an important role at eicosanoids, prostaglandins and steroids metabolism, tyrosine catabolism and apoptosis (2-5). Theta class enzymes have a highly

specific sulphatase activity (2), while Zeta class has maleyloacetoacetate isomerase activity (the penultimate step at phenylalanine and tyrosine catabolism) (2, 4). The role at prostaglandins metabolism is ensured by Sigma class, which acts as a Prostaglandin D synthase, a mediator of allergic and inflammatory responses (2, 4), and by the MAPEG family, that are involved at the synthesis of leukotrienes, too (2, 4). This role at Eucasanoids and Prostaglandins has a major impact at gene expression, due the activation of divergent regulatory pathways (figure 6) (2, 4). Omega class enzymes are crucial at ascorbic acid maintenance at brain, since they show dehydroascorbate reductase activity (2, 4, 6). All classes have also a peculiar ability, called ligandin function (2). This property is the ability to bind several non-substrates, such as bilirubin, steroids and xenobiotics, often but not always at the dimer interface. Nevertheless, when a non-substrate is bound, even at non-active site, the active site is affected, and renders the enzyme either inhibited or inactive (2). At last, GSTP1 conjugates with chemotherapeutic drugs, being highly expressed at cancer cells that resist medication, but also regulate a c-Jun NH<sub>2</sub>-terminal kinase (JNK) pathway, that is involved at apoptosis mechanisms (5). Thus, GSTP1 has both importance at preventing cancer evolution, and on cancer spreading (2, 5).

Although GSTs are ubiquitously expressed by cells, not all subclasses are expressed evenly throughout all tissues. A good example is the hepatic tissue, which expresses GSTM1 but does not GSTP1 (7).

### 1.1.2. Acting mechanisms

GSTs are known to typically catalyse the conjugation of Glutathione (GSH) and toxic compounds, such as xenobiotics, electrophile substances and oxidative stress products, but they can also take part in isomerization and reduction reactions of those compounds (2). Glutathione is the most abundant tripeptide in cells, and it is constituted by glutamate (Glu), cysteine (Cys) and glycine (Gly). It is synthesized at the cytosol by action of the  $\gamma$ GluCys ligase, resulting the dipeptide  $\gamma$ GluCys, after combined with Gly by the glutathione synthase (figure 2) (4, 8); both enzymes require ATP (Adenosine-triphosphate) and are substrate-inhibited by high GSH concentrations (8).

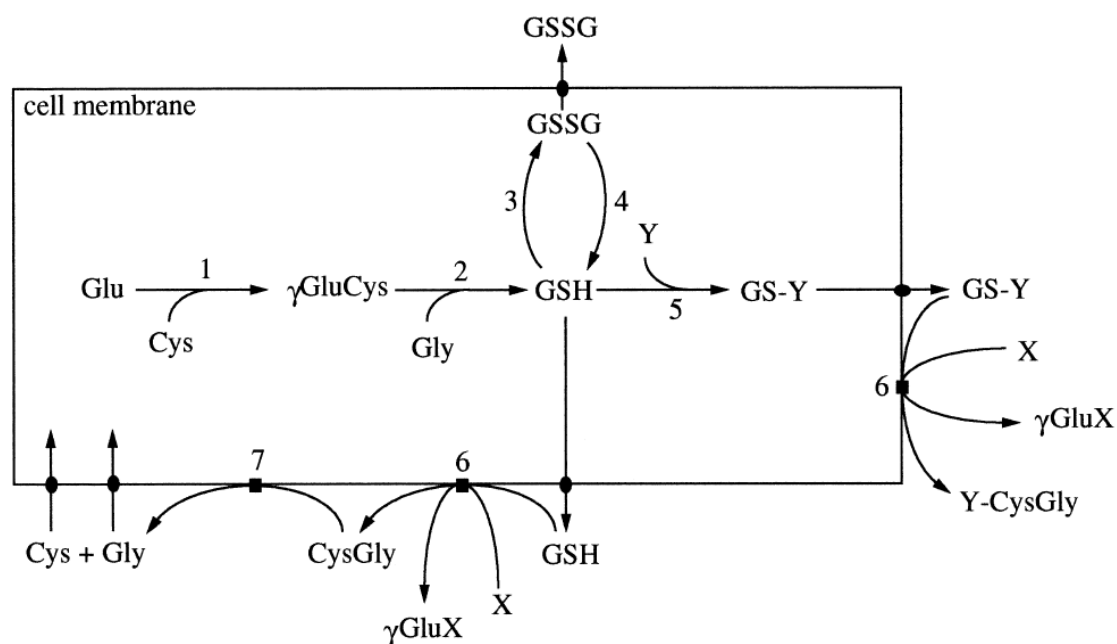


Figure 1: Pathways of GSH metabolism. Y represents a GSH substrate, while X represents a  $\gamma$ -Glutamyl moiety acceptor. Enzymes involved: 1)  $\gamma$ -Glutamylcysteine Synthetase; 2) Glutathione Synthetase; 3) Glutathione Peroxidases; 4) Glutathione Reductase; 5) Glutathione S-Transferases; 6)  $\gamma$ -Glutamyl Transferase; 7) Ectopeptidases. Adapted from (8).

The conjugation reaction attenuates the oxidative effects that free radicals cause to cellular structures, reducing them to more stable and less reactive chemical species, more soluble and easier to excrete (2-4, 9). The typical conjugation reaction (figure 3) involves the activation of the thiol group (-SH) of cysteine to react with a xenobiotic or other compound for excretion (2). This chemical group is also known by mercaptan (from Latin, *mercurium captans*, meaning capturing mercury), so this reaction may be referred as Mercapturic Acid Pathway too (4). After conjugation, it follows several steps to complete substrate's excretion (Figure 2). First, the  $\gamma$ -glutamyl moiety or glutathione conjugate is removed by the  $\gamma$ -Glutamyltransferase ( $\gamma$ GT), remaining CysGly or CysGly conjugate (8). The conjugates are N-acetylated to further elimination by the transmembranar MRP (Multi Resistance-associated Proteins), which are members of the C family of ABC transporters (4).

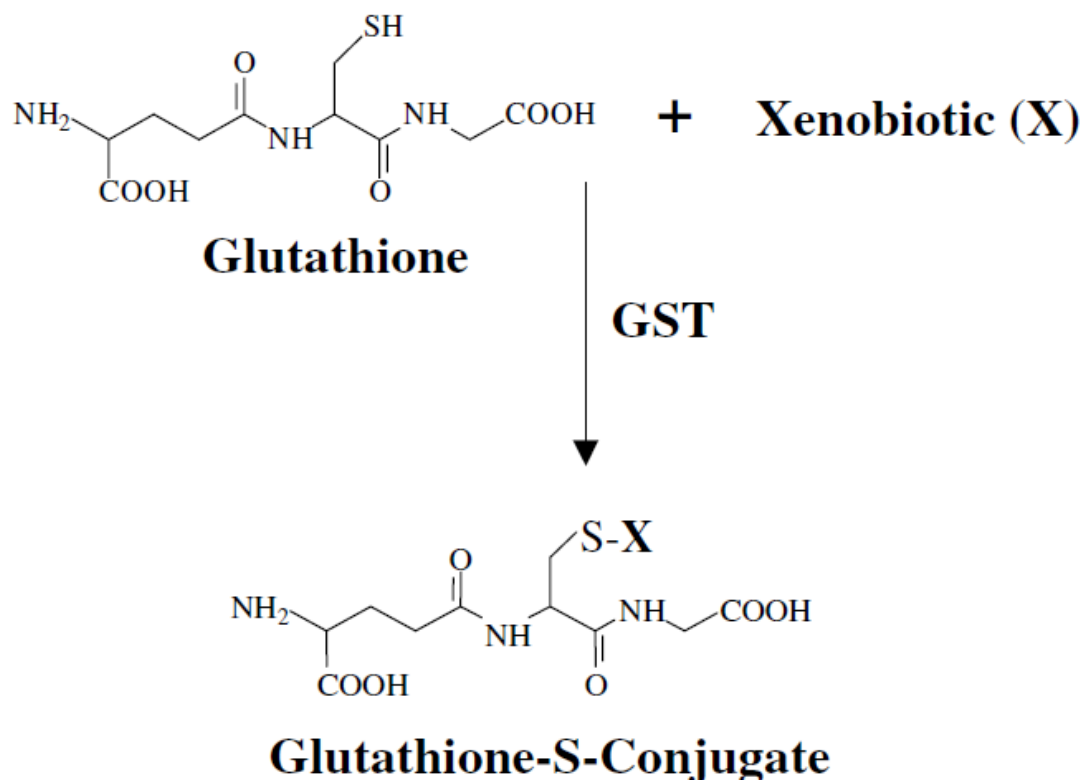


Figure 2: Simplified Mercapturic Acid Pathway. The GSH conjugation with a xenobiotic is catalyzed by a GST, resulting in a Glutathione S-Conjugate. Adapted from (27).

However, at some circumstances, the resulting products from this pathway are even more reactive than the substrate (4). Amongst the chemical functional groups that may lead to this effect are the short-chain alkyl halides that contain two functional groups (4, 10) and the 1,2-dihaloethanes (4, 11). Both chemical species result in unstable glutathione conjugates which possess an electrophilic centre capable of modifying DNA (4, 10, 11). Some compounds formed from plant glucosinolates are conjugated with GSH by GST to yield thiocarbamates, which are exported out of the cell by MRP (4). Outside the cell, they may spontaneously degrade into isothiocyanates, releasing GSH, and then be reimported to be once more conjugated with GSH (4). This creates a cycle that leads to a fatal depletion of intracellular GSH, occurring protein thiocarbonylation, a condition that leads to cell death (4). There are also cases of tissue-specific toxicity for some chemicals. For instance, haloalkanes and haloalkenes conjugation, which occurs mainly at liver, can generate reactive thiols at kidney, through the action of renal cysteine conjugate  $\beta$ -lyase (2, 4, 12).

The products of oxidative stress are subproducts of the partially reduced  $O_2$ , such as the superoxide anion ( $O_2^-$ ), the hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $HO\cdot$ ), and they arise not only through the oxidative phosphorylation of aerobic respiration, but also through 5-lipoxygenase, cyclooxygenase, cytochrome P450 and xanthine oxidase catalysed

reactions (4). Even when combined with antioxidant defence proteins, the degradation of those species can result into products that are also cytotoxic and mutagenic (4, 13). GST isoenzymes are one of the enzymes that protect cells against the subproducts of the oxidative stress (4). GST's are particularly involved in protection against the electrophiles derived from membrane lipids' oxidation, because they conjugate GSH to several end-products of lipid peroxidation, such as 4-hydroxy-2-alkenals of between 6 and 15 carbon atoms in length, 2-alkenals acrolein and crotonaldehyde, phosphatidylcholine hydroperoxide, cholesteryl hydroperoxides (14) and fatty acid hydroperoxides (4). Besides, GST's can indirectly combat those damages through regeneration of 1-cys Peroxiredoxin (Prx VI), an enzyme that reduce phospholipid hydroperoxides to their respective alcohols (4). Prx VI acts by oxidizing his Cys residue at position 47 to sulfenic acid, and later GST reactivates it through glutathionylation followed by spontaneous reduction of the mixed disulphide by GSH (4, 15). The base propenals that result from nucleotides oxidation are also detoxified by GSTs, as well as the products of catecholamine oxidation, which are harmful due to  $O_2^-$  production by redox cycling (4, 16, 17).

GSTs intervene in the degradation of aromatic amino acids (figure 4), namely the Zeta Class, which is a maleylacetoacetate isomerase (2, 4, 18, 19), as previously mentioned. Phenylalanine is degraded in a six-step reaction to fumarate and acetoacetate, that is an intermediate compound of Krebs Cycle, also known as Citric Acid Cycle. The intermediates of this reaction are Tyrosine, 4-Hydroxyphenylpyruvate, Homogentisate, Maleylacetoacetate, and Fumarylacetoacetate, thus GST class Zeta catalyses the penultimate step of this reaction (Maleylacetoacetate to Fumarylacetoacetate) (2, 4). GSTO1 and GSTO2 are also important for cells metabolism, since they help maintain the ascorbate homeostasis, due to its Dehydroascorbate reductase activity (2, 6). This discovery was surprising, because it is the only known GST-dependent reaction that GSH does not bind the G site; instead, Dehydroascorbate (DHA) binds to the G site, and GSH forms a disulfide bond with a cysteine in the active site (2, 6). This cysteine residue donates an electron to DHA that will trigger the capture of hydrogen from GSH, resulting Ascorbate (2, 6). However, class-omega isoenzymes doesn't have the same substrate affinity, and so GSTO2 shows more affinity to DHA than GSTO1, maybe due to the size of the active-site cavity (2, 6).

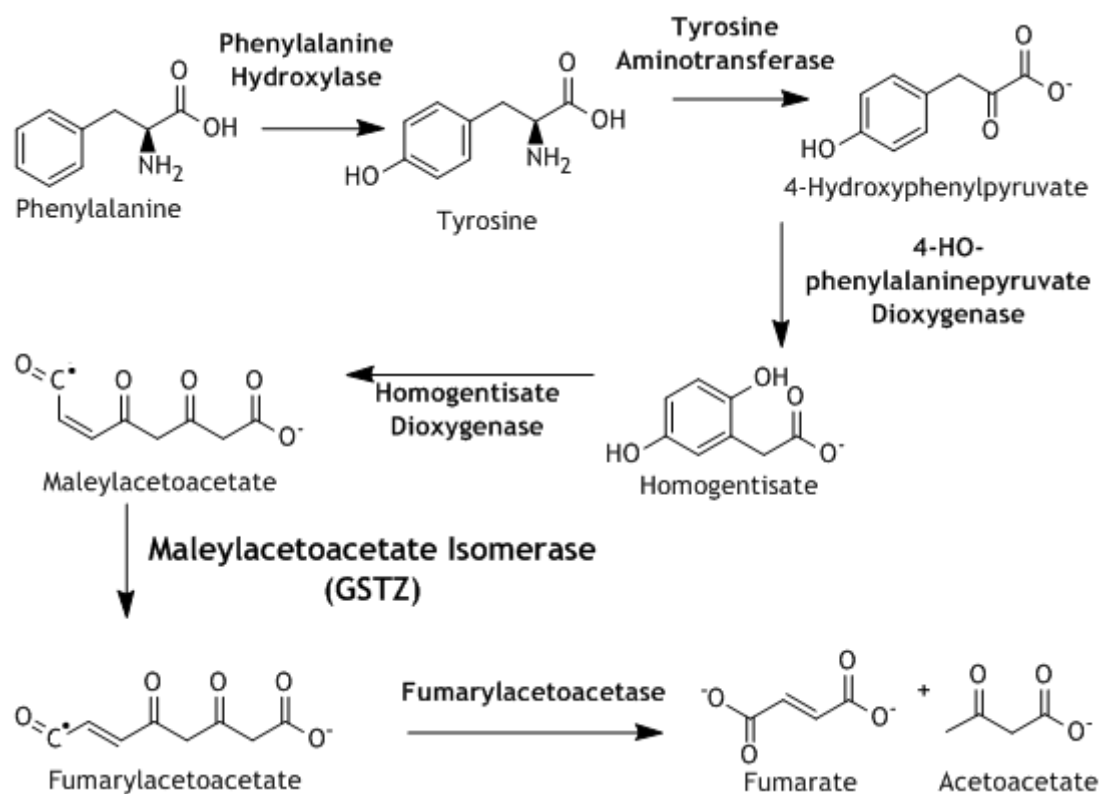


Figure 3: Aromatic Amino Acids Catabolism. GSTZ1-1 has Maleylacetoacetate Isomerase activity, catalyst of the penultimate reaction in this catabolic pathway.

Alpha GSTs isoenzymes participate in Synthesis of Steroid Hormones. Both testosterone and progesterone are synthesized from 3 $\beta$ -hydroxi-5-pregnene-20-one, a cholesterol metabolite (2, 4). This compound can be converted to  $\Delta^5$ -androstene-3,17-dione, an intermediate of the testosterone pathway, or  $\Delta^5$ -pregnene-3,20-dione, an intermediate of the progesterone pathway, by action of 3 $\beta$ -hydroxysteroid dehydrogenase (2, 4, 20). Then, both these 3-keto- $\Delta^5$ -steroids are converted to their 3-keto- $\Delta^4$ -steroid isomers, also by action of 3 $\beta$ -hydroxysteroid dehydrogenase, which has found to exhibit keto-steroid isomerase activity (2, 4). However, in steroidogenic tissues, GSTA3-3 has found to have a 230-fold higher catalytic efficiency for isomerization of 3-keto-  $\Delta^5$ -steroids than 3 $\beta$ -hydroxysteroid dehydrogenase, therefore GST is more likely to catalyse this reaction *in vivo* (2, 4, 20).

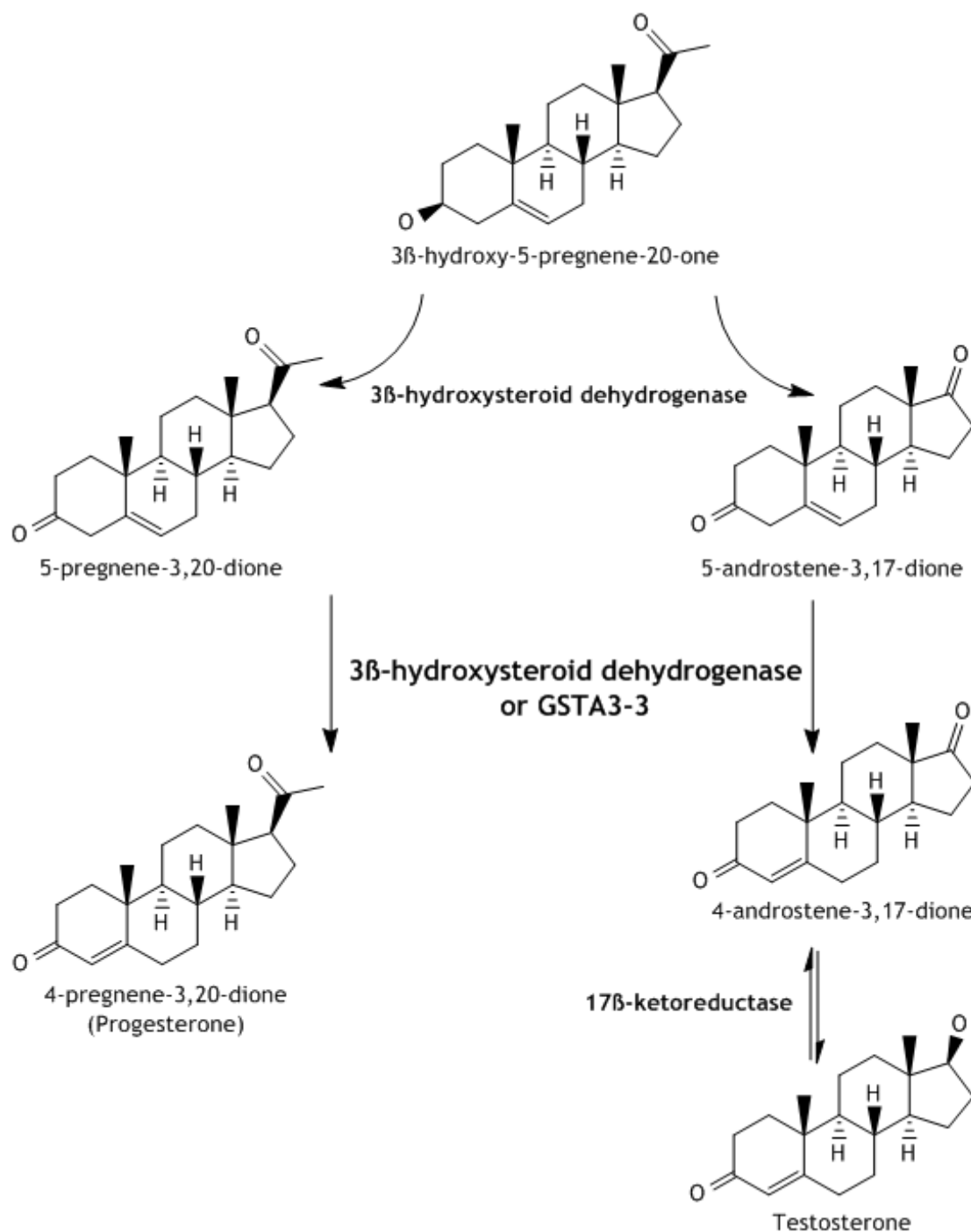


Figure 4: Synthesis of Steroid Hormones. GSTA3-3 acts as a highly efficient keto-steroid isomerase and only exists at steroid producing tissues.

GSTs also play an important role at the synthesis and inactivation of eicosanoids, a group of metabolites derived from arachidonic acid. Many GSTs were suggested to be responsible for the isomerization of the Prostaglandin  $H_2$  ( $PGH_2$ ) to Prostaglandins  $D_2$  ( $PGD_2$ ) and  $E_2$  ( $PGE_2$ ), or responsible for its reduction to  $PGH_{2\alpha}$ , but now is clear that certain GSTs are very specific for some of those reactions (4). For instance, sigma-class GST has found to have GSH-dependent Prostaglandin  $D_2$  Synthase activity in humans (2, 4, 21), while two human

brain's cytosolic GSTM homodimers (GSTM2-2 and GSTM3-3) present PGE<sub>2</sub> synthase activity (4, 22) and the MAPEG family enzymes show PGF<sub>2α</sub> synthase activity (4, 23). The influence of GSTS1 is the most interesting of the above-mentioned, since it also participates in the formation of the downstream cyclopentone prostaglandin 15-deoxy-Δ<sup>12, 14</sup>-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), which acts as a signalling molecule (figure 6), rather than the typical pro-inflammatory role of most prostaglandins (4). Cyclopentone prostaglandins, like 15d-PGJ<sub>2</sub>, are also substrates for GSH conjugation by several GSTs (GSTA1-1, GSTA2-2, GSTM1-1, GSTP1-1) (24), and thus their elimination is facilitated through Multidrug Resistance Protein (MRP) 1 and MRP3 transporters (25). Leukotrienes, another eicosanoid class, are critically influenced by GSTs, namely by the MAPEG family, in which several members intervene in the Leukotriene C<sub>4</sub> (LTC<sub>4</sub>) synthesis (group I and group II enzymes) (4), and in the 5-lipoxygenase activation, uniquely done by FLAP and MGTS2 (4, 26).

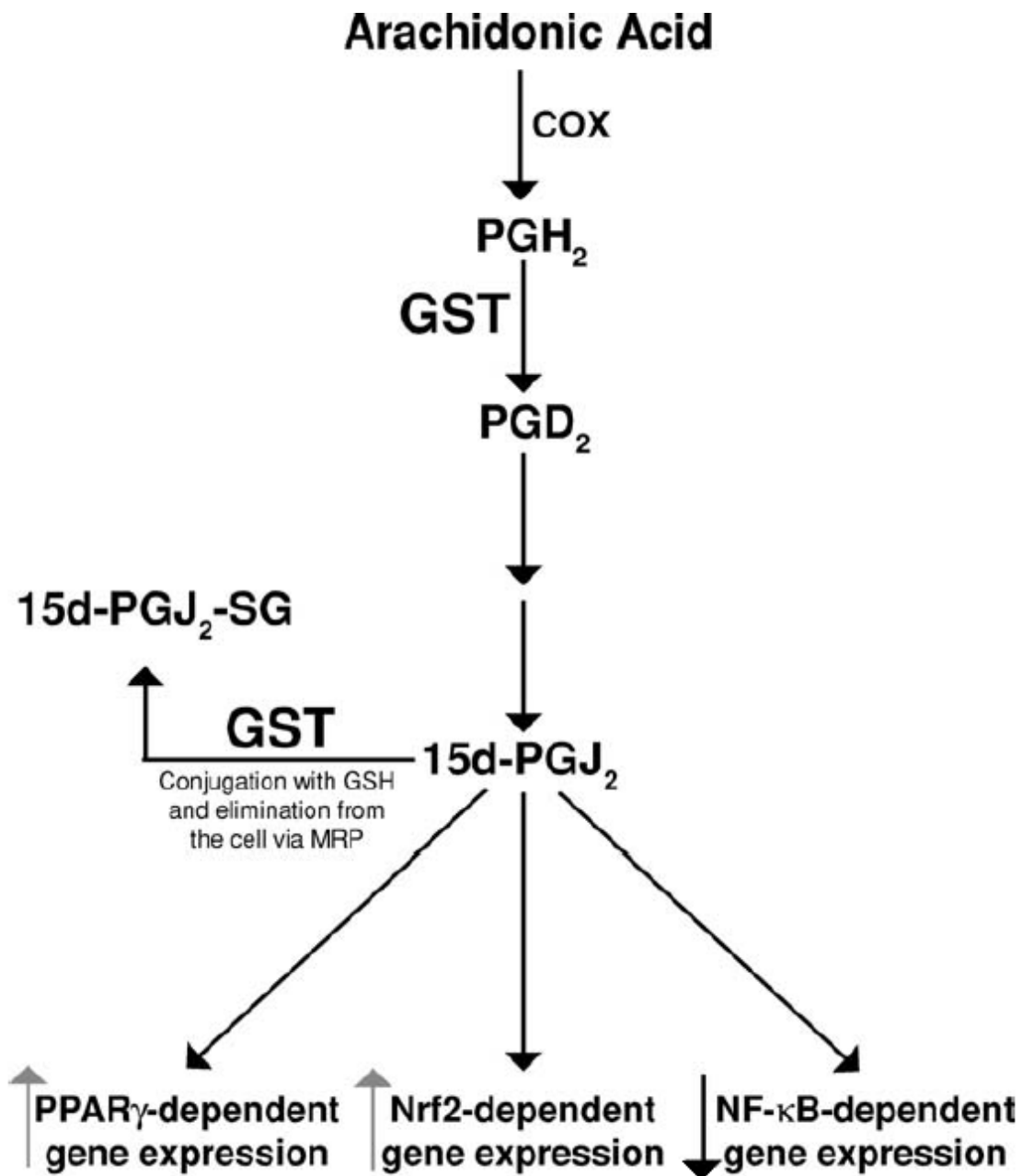


Figure 5: Regulation of Prostaglandins signalling by action of GSTs, and the transcription factors that may be activated or inhibited by them. Adapted from article (4).

### 1.1.3. GST classification

GSTs are classified, as above mentioned, according to their cell location. There are 3 major families: cytosolic or soluble, mitochondrial and peroxisomal, and microsomal or MAPEG (2-4).

Cytosolic GSTs are especially polymorphic, being further divided into 7 different classes, according to their amino acid sequence, identified by Greek letters:  $\alpha$  (alpha, five members),  $\zeta$  (zeta, one member),  $\theta$  (theta, two members),  $\mu$  (mu, five members),  $\pi$  (pi, one member),  $\sigma$  (sigma, one member) and  $\omega$  (omega, 2 members) (2-4, 27, 28). Some authors include also the class  $\kappa$  (kappa, one member), in spite of being a mitochondrial GST, because it is also soluble, have other similar structure and may appear on cytosol (3, 4, 29). Those classes are the only yet discovered in mammals, however there are plenty more GSTs classes for plants and even for other animal classes, such as insects (30). Classes should be designated by the name of the Greek letter (Alpha, Mu, etc.), abbreviated as Latin capital letters (A, M, and so on) (28). Class members are numbered by Arabic numerals and a GST protein dimer is distinguished by their monomers (e.g. GSTM1-M1 is a dimer of two subunits 1 in the Mu class) (28).

In spite of this huge variability, those enzymes have roughly 30% sequence homology (27); more than 40% sequence homology amongst the same class, but less than 25% between proteins from different classes (2). These similarities point that all cytosolic GSTs share a mutual precursor, from which had evolved divergently (29). Since GSTK1 share an N-terminal amino acid sequence motif solely with GST class Theta, but share the motif II with all other cytosolic GST classes, it suggests that GSTK1 is the precursor of GST class Theta, and class Theta is the precursor of classes Alpha, Mu, Pi and Sigma (29).

Table 1: Families and classes of GSTs, their members and substrate.

Cytosolic GSTs		
GST class (2, 4)	Members (2, 4)	Substrate (4, 6, 19, 27, 29, 30)
Alpha ( $\alpha$ )	GSTA1, GSTA2, GSTA3, GSTA4, GSTA5	<b>Endogenous:</b> Prostaglandins, lipid peroxidation products generated by reactive oxygen species <b>Exogenous:</b> Chemotherapeutic agents
Zeta ( $\zeta$ )	GSTZ1	<b>Exogenous:</b> Dihaloacetic acids <b>Endogenous:</b> Tyrosine degradation pathway
Theta ( $\theta$ )	GSTT1, GSTT2	<b>Exogenous:</b> Molecules with epoxide groups
Mu ( $\mu$ )	GSTM1, GSTM2, GSTM3, GSTM4, GSTM5	<b>Endogenous:</b> Prostaglandins, Lipid peroxidation products generated by reactive oxygen species, O-Quinones of catecholamines and dopamine
Pi ( $\pi$ )	GSTP1	<b>Exogenous:</b> Polycyclic aromatic hydrocarbons, Chemotherapeutic agents, $\alpha$ , $\beta$ unsaturated aldehydes

Sigma ( $\sigma$ )	GSTS1	<b>Endogenous:</b> Prostaglandins, Lipid peroxidation products generated by reactive oxygen species
Omega ( $\omega$ )	GSTO1, GSTO2	<b>Endogenous:</b> Dehydroascorbate
<b>Mitochondrial GSTs</b>		
Kappa ( $\kappa$ )	GSTK1	<b>Endogenous:</b> Lipid peroxidation products generated by reactive oxygen species
<b>MAPEG GSTs</b>		
gp I	MGST2, FLAP, LTC <sub>4</sub> S	<b>Endogenous:</b> Leukotriene, non-enzymatic binding of arachidonic acid
gp II	MGST3	<b>Endogenous:</b> Leukotriene
gp IV	MGTS1, PGES1	<b>Endogenous:</b> Prostaglandins

### 1.1.4. Structure and dimerization

Cytosolic GSTs have a well-conserved secondary and tertiary structure (2). A single unit is composed by an N-terminal  $\alpha/\beta$  domain (the G site, for binding GSH), and an all- $\alpha$ -helical domain (the H site, for binding hydrophobic substrates) (2, 3, 31). The G site is a thioredoxin-like domain that contains four mixed  $\beta$ -sheet strands, whose strand 3 is antiparallel to the others; the H site consists of five to six alpha helices (2). However, the G site only achieve its full catalytic activity once GSTs dimerize, because it locates on a cleft between both subunits (31). Some classes differ only by the number of such elements (like the extra  $\alpha_9$  helix of classes alpha, theta and omega), others have unique features, like the C-terminal mu-loop, the N-terminal extension of classes mu and omega, and the long loop between helices  $\alpha_4$  and  $\alpha_5$  of theta enzymes (2). These variations lead to a wide range of available substrates for GSTs (3).

GSTs always form dimers, both homodimers or heterodimers, i.e., GST dimers may be an association between two equal GSTs subunits (GSTM1-M1, for instance), of the same class (GSTM1-M2) or from different classes (GSTM1-P1) (7), which increases complex's protein stability and increases the range of available substrates (2, 4, 7). Recent studies point that heterodimers always are formed by same class GSTs, though. The dimer is formed by ball-and-socket or lock-and-key interactions, organized around a twofold axis, and through an extensive interface (2). These interactions occur between the domain I and the domain II of each subunit, particularly residues F52 and M51 from a loop between an  $\alpha$ -helix and a  $\beta$ -sheet from a subunit interacts with an hydrophobic pocket between two  $\alpha$ -helices of the other

subunit, as a typical ball-and-socket interaction (Figure 2) (2). There is also a region of hydrophilic interaction between subunits at near base of the twofold axis, involving residues of two  $\alpha$ -helices (2).

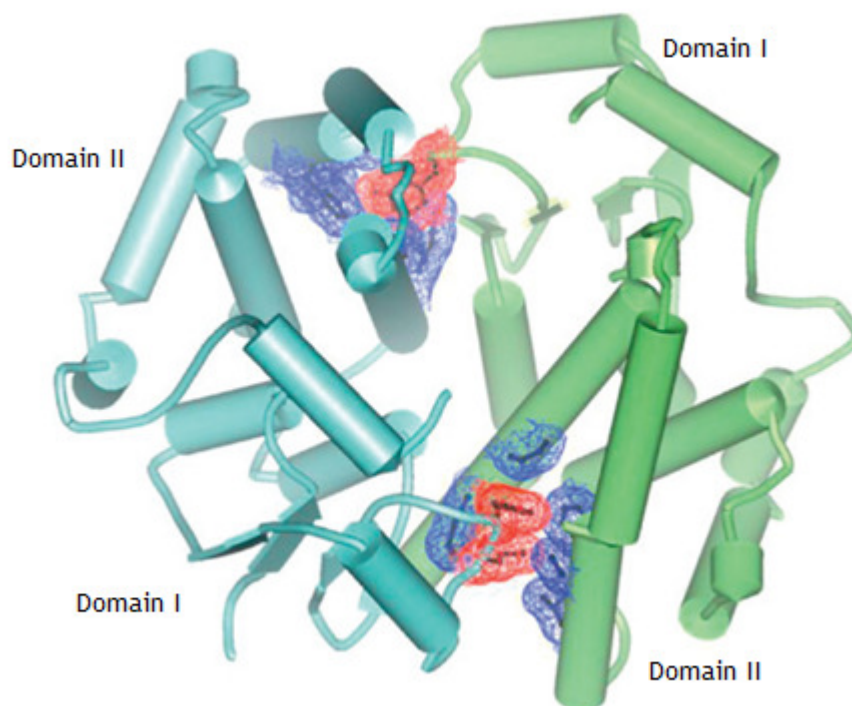


Figure 6: Ball-and-socket Interactions between GST monomers. Red and blue regions are the ball and the socket, respectively. Adapted from article (2).

It is not known yet how occurs the formation of GST heterodimers, but it seems to happen *in vitro*, under non-denaturing conditions, between GSTs classes Mu and Pi (7). Moreover, any GST class may have affinity to form heterodimers with subunits from different classes, since the amino acids residues at dimer interface are well conserved (7). *In vivo*, heterodimer association within different GST classes may be limited due to biological activity; for example, GSTM1-P1 heterodimers should not be possible at liver because it does express class Mu enzymes but not class Pi, but may be possible at other tissues that express both classes (7). In the breast, the predominant GST is the Pi class (32).

### 1.1.5. Chromosomic location

GSTs genes can be divided into two gene superfamilies: the cytosolic or soluble enzymes genes (including the mitochondrial Kappa class) and the microsomal or MAPEG enzymes genes (1). Each class has its own gene family, and each one is located at a different chromosome (1). For soluble GSTs, alpha on chromosome 6, mu on chromosome 1, theta on

chromosome 22, pi on chromosome 11, zeta on chromosome 14, sigma on chromosome 4, kappa (chromosomal location not known, probably mitochondrial) and omega on chromosome 10 (1). There are polymorphisms in several of those genes, but the focus is on those which present allelism, namely the mu, theta and pi families.

The mu class gene family are on a 100kb cluster at chromosome 1p13.3, in tandem (5'-GSTM4-GSTM2-GSTM1-GSTM5-GSTM3-3') (1, 32). There are three known polymorphisms at GSTM1, resulting into three possible alleles: GSTM1\*0, GSTM1\*A and GSTM1\*B (1, 4). GSTM1\*0 represents the deletion of the gene, and homozygotes for this allele can't express the protein; GSTM1\*A and GSTM1\*B differ only by a base pair (bp) at exon 7, their monomers can form dimers it one another, and show equal catalytic efficiency (1). On the other hand, null genotype is associated with increased breast cancer risk (3, 4). GSTM3 has two different alleles, GSTM3\*A and GSTM3\*B, the latter having a 3 bp deletion at exon 6. This deletion creates a recognition motif for YY1 transcription factor, so the regulation of those two alleles may be different (1, 32).

GSTT1 and GSTT2 are located on chromosome 22q11.2, 50kb apart each other, and have similar structures (5 exons and same intron/exon boundaries) (1, 32). GSTT1 may be deleted, the GSTT1\*0 allele, and 1 at each 5 individual amongst Caucasian population is homozygote for it, so can't produce the encoded enzyme (1). GSTT2 gene is side-to-side to d-dopachrome tautomerase gene (DDCT), sharing a common bidirectional promoter on the sequence between the two genes and are both duplicated in an inverted repeat (1, 32). Several polymorphisms are described for GSTT2, including an amino acid substitution and a nonsense mutation, but it was not found any functional implications (1).

The single gene for class Pi (GSTP1) is at 11q13, is 2,8kb long and has 7 exons, encoding a protein of 209 amino acids (32). This gene is regulated by a CpG island at the promoter, which appear to be hypermethylated in one third of the primary breast cancer, inactivating the gene (32). There are two SNP's (Single Nucleotide Polymorphism) at this gene that cause amino acid substitution and shift substrate specificity: one at codon 105, exon 5 (Ile/Val) and another at codon 113, exon 6 (Ala/Val) (32).

However, there are much more information about mutations and SNPs present at the several GST's genes. Table 2 depicts a brief compilation of most information available in the scientific bibliography about these genes, their mutations, alleles and effects on proteins.

Table 2: Cytosolic GSTs' genes, chromosomic location, alleles, mutations and their effects.

GST class	Genes (1, 2, 28)	Chromosomic location (1, 27, 28, 33)	Alleles (1, 27)	Mutation nature or effect (4)
Alpha ( $\alpha$ )	GSTA1	6p12.1	GSTA1*A	"Wild type"
	GSTA2		GSTA1*B	Base substitution, low protein levels
	GSTA3			
	GSTA4		GSTA2*A	"Wild type"
	GSTA5		GSTA2*B-E	

				Base and AA substitution
Zeta ( $\zeta$ )	GSTZ1	14q24.3	GSTZ1*A-D	Four different base and AA substitutions
Theta ( $\theta$ )	GSTT1 GSTT2	22q11.2	GSTT1*0 GSTT1*A GSTT2*A-B	Gene deletion “Wild type” Base and AA substitution
Mu ( $\mu$ )	GSTM1 GSTM2 GSTM3 GSTM4 GSTM5	1p13.3	GSTM1*0 GSTM1*1x2 GSTM1*A-B GSTM3*A GSTM3*B GSTM4*A GSTM4*B	Gene deletion Gene duplication, protein overexpression Base/AA substitution “Wild type” 3bp deletion, Protein unchanged “Wild type” Base substitution on intron, protein unchanged
Pi ( $\pi$ )	GSTP1	11q13	GSTP1*A-D	Four different base and AA substitutions
Sigma ( $\sigma$ )	GSTS1	4q22.3	GSTS1*A GSTS1*B	Inversion, “reference” protein levels Inversion, protein unchanged
Omega ( $\omega$ )	GSTO1 GSTO2	10q25.1	GSTO1*A/C GSTO1*B/D GSTO2*A-B	Base/AA substitution Base/AA deletion and Base/AA substitution Base/AA substitution

### 1.1.6. Nrf2 and GST

There is a positive feedback between Nrf2 and GSTs. Nrf2 recognizes genes with the ARE element in its promoter region, increasing their expression (34-36). GSTs do have such element, and so their expression may be influenced by the activation of the transcription factor Nrf2 (36). On the other hand, some cytosolic GSTs intervene in the metabolism of prostaglandins, particularly 15d-PGJ<sub>2</sub>, a group of pro-inflammatory biomolecules that can activate Nrf2, and thus the induction of gene expression through the antioxidant response element (ARE) (2, 4, 34).

Nrf2 is widely expressed by all cells, however, its function is suppressed by the conjugation with Keap1 (Kelch-like ECH Associated Protein 1), with whom forms cytosolic protein complexes (34, 35, 37, 38). Keap1 is also associated to two other proteins, Cullin 3 (Cul3) and Ring-box 1 (Rbx1), that form together a complex E3 ubiquitin ligase which ubiquitinates Nrf2, to further proteasome degradation, keeping it at low concentration at cytosol (34, 38, 39). When cells are exposed to oxidative stress or electrophile substances, cysteine residues at Keap1 are alkylated, acting as sensors (38, 39). This alkylation is thought to promote structural changes on E3 ubiquitin ligase complex, inactivating it, and thus interrupting Nrf2 ubiquitination, which accumulates freely on cytosol (38-40). At last, Nrf2 migrates to the nucleus, where it dimerizes with a small Maf protein, to recognize and bind to the Antioxidant Response Element (ARE), a cis element that consists on a nucleotide sequence present at the promoter of most cytoprotective genes (41).

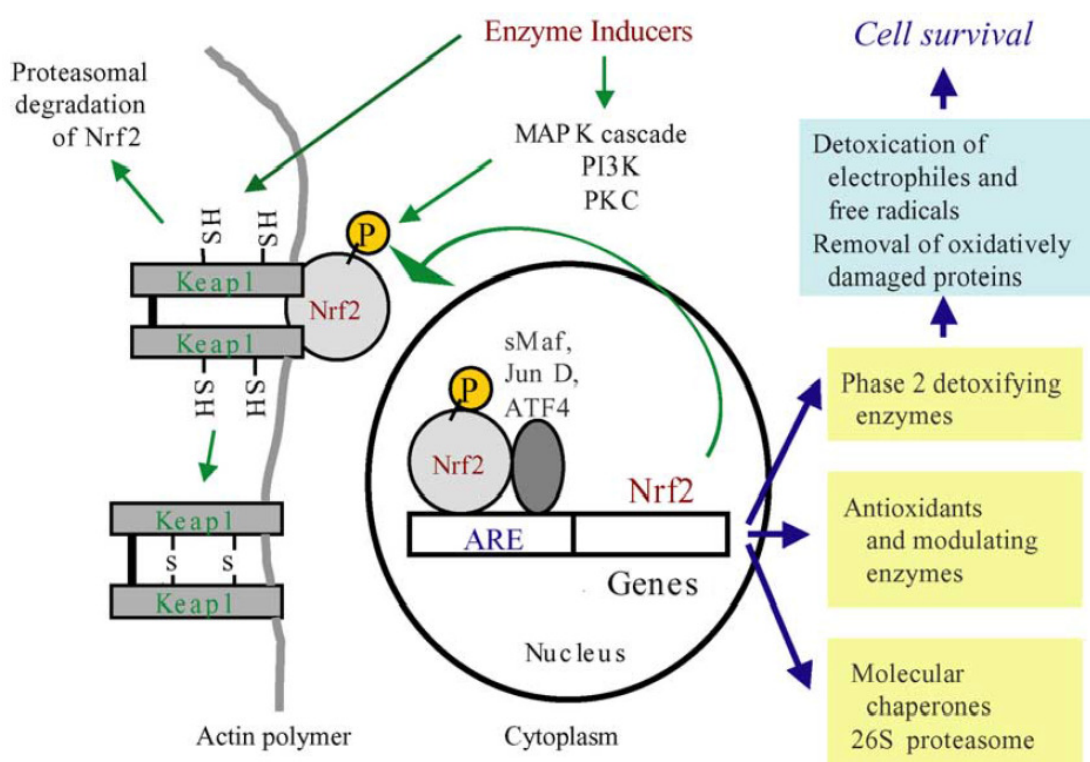


Figure 7: Schematic representation of the Nrf2-Keap1 gene expression regulation. The Keap1 complex, due to its E3 ubiquitin ligase activity, constantly binds and marks Nrf2 for degradation. When electrophile substances are present, due to oxidative stress, Keap1 cysteine residues get alkylated, releasing Nrf2 that can migrate to the nucleus, where it recognizes the ARE element of cytoprotective genes and promotes their expression. Adapted from article (40).

The gene that encodes Nrf2 transcription factor is known as NFE2L2. It is a ubiquitously expressed gene, but its highest expression occurs, in descending order, in the kidney, muscle, lung, heart, liver and brain (42). As previously referred, Nrf2 is a transcription factor whose function is to recognize and bind the ARE element, a regulatory cis-element which exists at the promoter region of most cytotoxicity-protective and phase II detoxification enzymes

genes. This element core sequence is 5'-TGACNNNGC-3' (36). As for its active-form structure, Nrf2 forms protein dimers with small Maf proteins, resulting in a Cap'n'colar (CnC) leucine-zipper transcription factor (36, 41, 42).

## 1.2. Oxidative stress and physical exercise

### 1.2.1. Oxidative stress

Oxidative stress is a condition that results from an unbalanced production of free radicals when compared to the body's antioxidative capacity to dampen their harmful action (43-45). The production of free radicals is impossible to avoid, since the main metabolic pathways in humans produce such reactive compounds. The electron transport chain at the mitochondrion's inner membrane (figure 8), a common step for both aerobic respiration and lipid oxidation, is one of the principal sources for ROS, a family of chemical compounds that cause oxidative stress. In this step, electrons are transferred throughout 4 protein complexes, which use the electromotive energy from them to pump hydrogen ions ( $H^+$ ) to the mitochondrion's intermembrane space, creating an acidic mean and a chemiosmotic gradient (46-48). Electrons can't be left loose into cytoplasm, because they would cause damage to cellular structures, and therefore are received by oxygen atoms, the final electron acceptor, after chain's protein Complex IV. A fifth protein complex, a transmembrane complex denominated by ATP-synthetase A, uses that chemiosmotic gradient to create ATP, using a proton-motive force to combine ADP and inorganic phosphate (47-49).

The electron transfer in this chain is only possible because each electron acceptor as a higher reduction potential than the one before (47, 48). However, some electrons can escape the chain before reaching the final acceptor, and may be conjugated by oxygen and oxygenated compounds into Reactive Oxygen Species such as hydroxide ( $HO\bullet$ ), peroxide ( $H_2O_2$ ) and superoxide ( $O_2^{\bullet-}$ ) ions (50, 51). The complex II, ubiquinone, and cytochrome C are the main sources for electron leakage that promotes ROS, and about 0.4% to 4% of all  $O_2$  used by the cell forms superoxide ions (47, 51, 52).

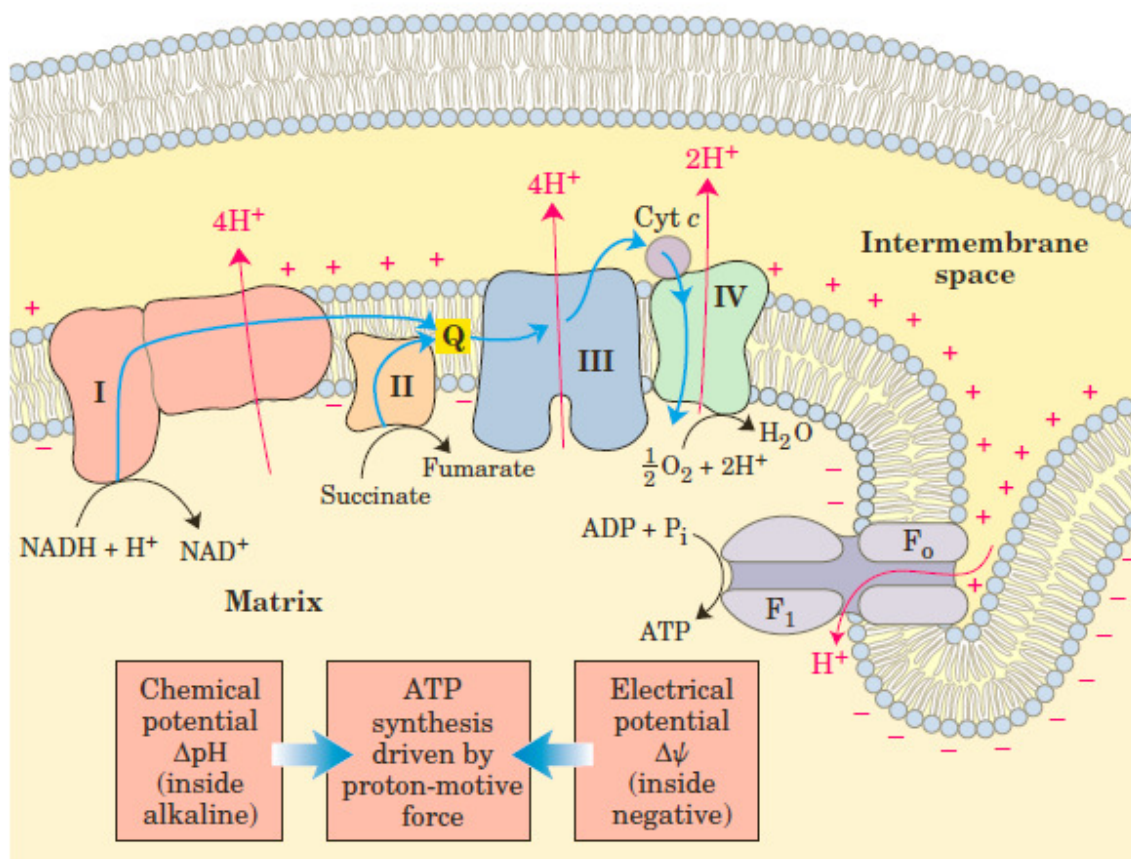


Figure 8: The electron transport chain, in the inner membrane of mitochondrion. Electrons are transferred through proteins by a series of oxidations, with reduction potential progressively higher. The electron energy differential through the chain is used to pump electrons to the inner mitochondrial membrane, by complexes I, III and IV, creating a chemiosmotic gradient. After complex IV, electrons are captured by oxygen, which reacts with hydrogen to form water. This whole transport, very well balanced can, nevertheless, let 0.4% to 4% of electrons escape and form ROS. Adapted from (47).

ROS are highly unstable and reactive chemical species, and often are responsible for the induction of other free-radicals, through reactions triggered by their action (51). The reactivity of the free radical is due to the unpaired electron in their electronic orbit, a condition that causes instability due to charge unbalance between positive charges (protons) and negative charges (electrons). To balance this condition, radicals react quickly with other molecules so their electronic orbit become fully filled, “stealing” electrons from other molecules and causing Oxidative Damage. Rather than inducing more free radicals, these compounds several damages to cells, such as membrane lipid peroxidation, damage to nucleic acids bases and proteins, inflammatory process, which can compromise its viability (50, 51).

Detoxification enzymes are able to attenuate those damages by reacting with free radicals before they react with macromolecules, or by catalysing reactions between free radicals and antioxidative compounds (45). Drug metabolism is commonly divided into 3 phases: the phase I, modification of drugs to react with furthers cell defensive agents; phase II, conjugation of the modified drugs with cell protective molecules to dampen its action; and phase III, excretion of the drugs due to more modifications to facilitate its elimination from

the organism. ROS and free radical are reactive for themselves, so phase II detoxification enzymes are the most important agents to dampen its effects. Therefore, GSH and GST are very important to maintain oxidative balance. GST is an enzymatic catalyst that uses GSH and a reactive compound as substrates (reactive compounds depend on GST isoform, see table 1), and the result of the reaction is oxidized glutathione (GSSG) and the reduced, now less reactive molecule. Once the reserves of GSH are depleted due to an excessive production of ROS, oxidative stress will arise (45). Hopefully, GSH is swiftly regenerated by a flavoprotein GSSG reductase, using NADPH to donate an electron to GSSG, resulting NADP<sup>+</sup> and GSH (45).

### 1.2.2. Oxidative damage linked to physical exercise

Physical exercise requires an increase in energy production by the body, through ATP synthesis and utilization. For mammals, and particularly for humans, the principal energetic pathway is the aerobic respiration, which is the main endogenous ROS source (44, 53). To face such energetic demand, the amount of respiration cycles and oxygen intake increase, thus augmenting the quantity of ROS produced (44, 50, 54), to a level that our body's antioxidant defences can't handle (43, 50).

Furthermore, ROS produced by the electron transport chain cause cellular damages that have cumulative effects (50), inducing further production of ROS, throughout other metabolic pathways such as the respiratory burst of lymphocytes or xanthine oxidase activity (figure 9) (44, 53, 54). Exhaustive exercise may impair the immune system (50, 55), rapidly increasing the number of circulating neutrophils but reducing the number of lymphocytes, which might be reflected in the increased risk for upper respiratory tract infections (44). Erythrocytes also contribute to the increased ROS production during exercise, because superoxide anion is produced as result of the first step of heme oxidation (44, 54). Besides, in spite of every cell is susceptible to severe impairment due to ROS (particularly due to peroxidation), erythrocytes are especially susceptible due to the lack of repair mechanisms, causing haemolysis (44). This oxidative effect may be significantly greater in runners due to the trauma-induced haemolysis at their feet, which releases free haemoglobin, highly oxidative due to the iron-containing heme group, to the blood stream (43). Oxidative stress tend to lead, ultimately, to activation of apoptosis (44), and this phenomenon is associated to the inflammatory acute phase response, which is, in its turn, linked to neutrophil increasing number (55). It is interesting to notice that, this neutrophil exercise-induced activation is coupled with a decrease in the antioxidant activity (55). Thus, we can consider that there is a positive feedback in exercise-induced oxidative stress.

Exercise, increasing metabolic rate and thus body temperature, also increases the rate of ROS produced in the mitochondrion of myocytes, as they undergo increased electron uncoupling (54). Shorter efforts, such as for short distances and anaerobic training, due to lactate production, cause blood and muscle acidosis and this is associated to higher

catecholamines' levels (56, 57). In its turn, catecholamines are easily oxidized, and that reaction produces ROS (54, 56). Besides this effect, anaerobic exercise contributes to oxidative stress by purine degradation, stimulating xanthine oxidase activity (figure 9) (54, 56). The high intensity this training provoke periods of blood shunt due to the intense muscle contraction, causing brief moments of ischemia, followed by reperfusion, along with mechanical muscle damage (57, 58). Proteins are then prone to get carbonylated due to free radicals, causing structural and functional changes (58, 59). Local radical insult will cause death to some myocytes, releasing calcium to the extracellular mean, interfering with the homeostasis of this apoptotic metal (57, 58). Once more, to “clean” cell fragments is activated the inflammatory response and neutrophils will be recruited, contributing with more oxidative species due to their respiratory burst (57, 58).

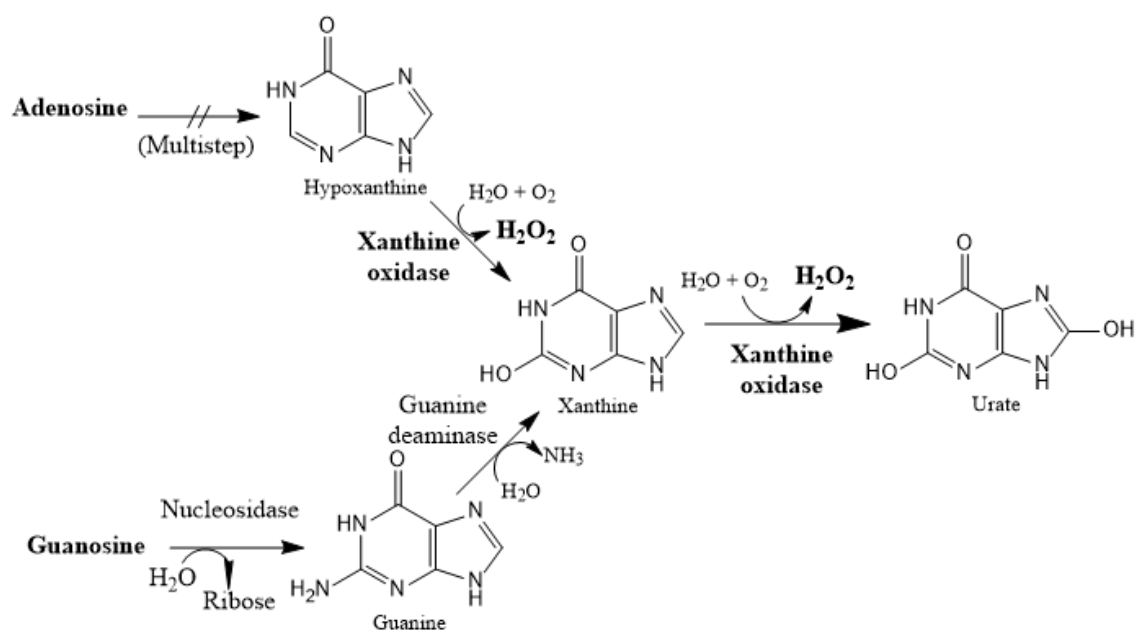


Figure 9: Catabolism of purines. This metabolic pathway creates ROS by the Xanthine oxidase activity (Bold compounds have recognized electrophilic activity). Damage caused to DNA will trigger this catabolism, in order to eliminate the damaged purine bases, but the action of xanthine oxidase results in peroxide, contributing to oxidative stress.

All those damages have a functional effect. Inflammation and apoptosis are correlated to overtraining in athletes (50). Lipid peroxidation and protein carbonylation cause impairment of muscle cells and is reflected in strength and endurance decrease (58). Hopefully, physical exercise, creating this situation of oxidative stress repetitively, may promote physiological adaptations according to duration and intensity of those electrophilic assaults (43, 50). Despite all damage linked to exercise that ultimately increases oxidative stress, there are evidences that their continued assault upregulates endogenous antioxidant defences (58). Total antioxidant capacity is higher in long-term trained individuals than in a sedentary group (60), and a group of untrained individuals show less lipid oxidation after intense exercise, after entering a regular physical activity training plan (61). It seems that training promotes antioxidant adaptation in a similar fashion to other principles of Sport

training (58, 62), such as the supercompensation principle. At each training session, an athlete must be under a certain oxidative threshold, above his antioxidant capacity, to permit his body to recuperate and achieve a higher antioxidant level at the beginning of the next session (58).

### **1.2.3. Oxidative stress and GST**

As previously stated, in spite of all damage caused by exercise and exercise-induced ROS production, the body's antioxidant defences are able to adapt against continued free-radical assault. GSTs are phase II detoxification, and are amongst the endogenous antioxidant defences of our organism. These isoenzymes have ROS as one of their substrates (4), which are also one of the reactive chemical species that contribute to oxidative stress, due to their high oxidative potential. So, the redox equilibrium within the cell depends, in a certain extent, on a balance between ROS production and GST activity (45). Besides, the carbonyls resulting from protein damage due to oxidative stress are another GST substrate (4). So, whatever is the source of the oxidative stress, either from aerobic or anaerobic exercise, GSTs may play a central role in the defence and recovery of their damage. Thus, the different functional genotypes for GSTs may be responsible for different responses to the oxidative stress (63).

## 2. Objectives

### 2.1. Main objective

The main purpose of this thesis was to verify the effect of competitive training over the expression of endogenous antioxidant genes, namely over two GST isoforms, GSTM1 and GSTT1. The results of this investigation may lead to new training approaches, helping coaches to plan the training throughout the season in a fashion that will protect their athletes from the damage suffered from the exhausting exercise, and better managing their performance peak.

This objective may be summarize as a pilot-model to evaluate consistently how the recovery of swimmers evolves, which can promote the individualization of the recovery times, according to the applied exercise charge.

### 2.2. Secondary objectives

To achieve our main goal, there are several protocols that need optimization. This investigation aims to facilitate new training approaches that are reliable and practicable. Therefore, the developing and optimization of new techniques to collect samples in a minimal invasive manner, to store them without contamination nor degradation, and to extract RNA in sufficient concentration to permit further expression analysis, is probably one of the most innovative points of this work.

Additionally, data collected may be used to verify the differential genotype distribution for different groups within our sample. In spite of all our subjects are competitive swimmers, there is room for genotype distribution from male to female athletes, from short-distance swimmers (SDS) to middle-distance swimmers (MDS).

The same kind of comparison will be possible for the expression. Besides the global effect of training in the expression of GSTM1 and GSTT1, we are interested in knowing if different groups are differentially influenced by this stimuli.

## 3. Materials and Methods

### 3.1. Subjects

30 Portuguese swimmers of national level volunteered to serve as subjects (13 males and 7 females). It was defined that national level swimmers are ( $20 \pm 3.25$  years old;  $1.77 \pm 0.05$  m of height;  $72.93 \pm 6.34$  kg of body mass;  $23.19 \pm 1.80$  kg.m<sup>-2</sup> of body mass index) the ones with regular presence in the national championships.

For statistical analysis, we further divided swimmers into smaller groups. We divided the swimmers their distance speciality, not considering their swimming style. We considered that all swimmers specialized in distances of 200m and under were Short-Distance Swimmers (SDS), and all swimmers specialized on distances ranging from 400m to 1500m were Middle-Distance Swimmers (MDS). This division was done because the ROS produced by short-distance training are not exactly equal to those arising from Middle-distance training. The higher intensity, muscular potency and anaerobic demand required by short efforts are linked to a prevalence from ROS derived from purine catabolism, catecholamine oxidation and mechanical muscle damage. On the other hand, the less intense, more oxidative and aerobic demand that is required for middle distances is associated mainly to ROS production from electron leakage, inflammation and haemolysis. However, all those exercise-induced ROS production occurs in both.

A control group was designated in order to evaluate the genetic drift in national level swimmers, for GSTM1 and GSTT1 genotypes. This group aimed to represent the general population, and consisted in 52 individuals, from whom 38 were men and 14 were women, from all ages and ethnies.

Samples were collected at 5 different moments, at different training phases throughout a sport season. Every sample was collected before the training session of that day. More detailed information about sample collection and the exact dates and training phases is provided in section 3.2.

### 3.2. Study design

Samples were collected at 5 different occasions during the 2012-2013 calendar, throughout the winter season (short pool). The first 3 samples are correspondent to 3 different training macrocycles, and the last two samples were collected in the week after the most important competition of this season. At every sample collection moment, the blood

was collected right before the start of the afternoon training session, to prevent the interference of that session's exercises on our data.

The first sample for each swimmer was taken at the second week of September 2013, correspondent to the first week of training after the summer break, and to the general preparation phase. The exercises of this phase aim to prepare the swimmers for the upcoming, more exhausting training sessions. For these reasons, this sample was defined as the control condition for gene expression calculations.

The second sample was collected at the pre-competitive phase, on 22<sup>nd</sup> November 2012, the Thursday before junior/senior Regional Winter Championships. This training period followed a macrocycle of general preparation, which focus in further specialization to prepare the swimmers for their speciality.

The third sample was collected at the competitive phase, on 6<sup>th</sup> December 2012, towards the end of the small taper phase (two weeks) and just before the most important competition of winter season (the winter short course national championship).

The last two moments were collected to verify the recovery of swimmers after an exhaustive exercise: the forth sample was collected on 11<sup>th</sup> December 2012, 48h after competition, and the fifth sample was collected on 12<sup>th</sup> December 2012, 72h after competition.

Every sample was collected before the training session of that day. In the time period between tests the swimmers completed a full training preparation. Swim training generally consisted of a mixture of low, moderate and intense training (see table 3 for further detail).

Table 3: Training characteristics. WV (weekly volume); A0 (slow/regenerative swimming speed); A1 (aerobic, low intensity swim); A2 (swim speed at lactic anaerobic threshold); A3 (pace above the lactic anaerobic threshold); A4 (maximal aerobic potency pace); LT (Lactic Tolerance); LP (Lactic Potency); PR (Potency Resistance).

Week	WV (Km)		A0	A1	A2	A3	A4	LT	LP	PR
10-16 Sept.	4.0	Aerobic	2.0	1.0	1.0	0	0	0	0	0
17-23 Sept.	16.0		8.0	5.5	2.5	0	0	0	0	0
24-30 Sept.	28.3		13.6	8.0	5.0	1.5	0	0	0.2	0
1-7 Oct.	44.0		19.4	11.0	9.0	3.0	1.2	0	0.4	0
8-14 Oct.	48.1		18.1	12.0	11.0	4.5	2.0	0	0.5	0
15-21 Oct.	48.1	Aero. Anaero.	15.0	12.8	9.0	6.0	4.0	0.8	0.5	0
22-28 Oct.	53.4		17.6	12.0	11.0	7.0	4.0	0.8	0.6	0.4

29-4 Nov.	53.4		20.3	13.1	8.0	6.0	4.0	1.0	0.5	0.5
5-11 Nov.	57.9	Specific	24.4	15.0	9.0	3.5	3.0	1.5	0.5	1.0
12-18 Nov.	53.5		21.9	13.0	10.0	2.5	3.0	1.6	0.5	1.0
19-25 Nov.	48.1		21.8	10.0	9.0	1.6	3.0	1.2	0.5	1.0
26-2 Dec.	40.0		20.2	9.0	6.0	1.2	1.5	0.8	0.5	0.8
3-9 Dec.	22.0	Taper	13.3	4.7	1.5	0	0.8	0.6	0.3	0.8
10-16 Dec.	22.0		16.1	4.9	1.0	0	0	0	0	0
17-23 Dec.	19.1		16.1	2.0	1.0	0	0	0	0	0

### 3.3. Data collection and storage

Samples were obtained by a sting on a finger by a lancing device (Accu-Chek® Safe-T Pro Uno, F. Hoffmann-La Roche Ltd, Switzerland) and consisted on a blood drop of approximately 1cm<sup>2</sup> from each individual, at each moment, collected in a filter paper (refer brand). They were kept at 4°C to avoid water crystallization that would cause damage to nucleic acids. Filter paper was used because it absorbed the blood drops faster than vegetal paper or even regular paper, which permitted a faster sample drying for proper storage. Besides, several studies refer that sample collection on filter paper and storage at 4°C maintains sample integrity, even for long time periods (64-66).

Before collecting the samples, all individuals, both swimmers and controls, were invited to sign an authorization chart, a free and elucidated consent term, where it was totally described the scope and aim of the research, its scientific meaning and the possible uses of the collected data. Their signature seal the authorization or non-authorization of sample treatment and storage. Samples were kept under the investigator's guard, at the cold chamber of the Health Sciences Research Centre (CICS), at the Faculty of Health Sciences (FCS), of the University of Beira Interior (UBI).

### 3.4. Sample genotyping

Before running into gene expression of our interest end-products (GSTM1 and GSTT1), we accessed all our individuals for their genotype for both genes, since there are null genotypes in both. This is a measure to save time and reagents that would otherwise be

wasted by accessing gene expression (by RT-PCR) on samples of individuals that don't have null genotype, and so they couldn't express the gene.

### 3.4.1. DNA extraction

DNA extraction was performed using the Chelex 100 (Bio-Rad®, Munich, Germany) method. The Chelex 100 is a divinylbenzene estirene copolymer, containing iminodiacetate ions that bind metallic ions, thus acting as a quelant. It has a high selectivity and affinity towards metallic ions, unlike other weak cationic exchange resins. The final result is the precipitation of all DNA contaminants, but it maintains the non-metallic ions at same concentration in solution with DNA, promoting its stability.

It was added 200µl of Chelex at 5% in sterile Milli Q water solution to each sample, which consisted of, roughly, half of a blood spot on paper. Each reaction took place in a 1,5ml Eppendorf, and it was incubated at 100°C for 20 minutes, followed by centrifugation at 13000RPM for 3 minutes. At last, the aqueous phase was isolated, since it contains the extracted DNA.

When the extract presented visible contamination by haemoglobin, a purification of the DNA extract was done, following the same protocol.

### 3.4.2. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was performed to access the presence or absence of our antioxidant genes of interest in this thesis (GSTM1 and GSTT1), amplifying those sequences for further analysis by electrophoresis. PCR mimics the natural DNA replication, and needs the same base reagents: a DNA polymerase enzyme, dNTPs, primers and the DNA template for semiconservative replication. This permits the amplification of specific sequences in a sample several times, *in vitro*, through a series of replication cycles (usually ranging from 30 to 40 cycles). Each replication cycle involves the same steps as the natural semiconservative replication of DNA at cells: the double strand has to be open (denaturation step), then are added primers for DNA polymerase recognition (annealing step), which extends the sequence by addition of dNTPs according to the template strand (extension step).

In this research technique, the reactions occur in a device that permits quick temperature changes on samples, named thermocycler, because each step has a specific ideal temperature to occur. In a typical PCR reaction, the denaturation step occurs at high temperature (above 90°C) to completely separate both DNA strands, since the reaction mix do not include the enzymes that are responsible for doing it *in vivo*. The annealing step is the most variable because each pair of primers has different optimal annealing temperatures that have to be optimized. Primers must be design according to the sequence (gene) of interest and whether it is going to be amplified genomic DNA or cDNA, and each sequence has to pair

with a different DNA strand, so it can be possible the bilateral elongation of the interest gene (a forward primer for the leading strand, and a reverse primer for the lagging strand). The optimal temperature for this step usually ranges from 50°C to 60°C. The last step is the extension or elongation, and its optimal temperature depends on the enzyme with DNA polymerase activity that is used. The most commonly used enzyme is the Taq DNA polymerase, obtained from a thermophile microorganism (*Thermus aquaticus*), because it is resistant to high temperatures and drastic temperature changes, to which samples are submitted during PCR. For better results with this enzyme, it is added a reaction buffer and a magnesium source, which is a co-factor, and the optimal temperature is 72°C.

In this thesis, the interest genes were GSTM1 and GSTT1, while  $\beta$ -Actin was used as control. The primers were design with attention to further application for expression assays, so the sequences could not be within introns. For GSTM1 the primer sequences used were 5'-GGTGGCATATAAACTGGGTTG-3' (reverse) and 5'-GAACTCCCTGAAAAGCTAAAG-3' (forward). Primer sequences for GSTT1 were 5'-ACGACCGGTACTAGGCCACT-3' (forward) and 5'-TTCCTTACTGGTCCTCACATCTC-3' (reverse). The resulting amplification fragments were 215bp long for GSTM1 and 473bp long for GSTT1. For  $\beta$ -Actin gene, only used later for RT-PCR, the primers used were 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' (forward) and 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3' (reverse). Each reaction tube had a total volume of 25 $\mu$ l, which consisted in a mix detailed in table 3. The reactions took place in a thermocycler TProfessional Basic (Biometra®, Göttingen, Germany), according to the following program: a starting DNA denaturation at 94°C for 5 minutes, followed by 30 amplification cycles (consisting in denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute and extension at 72°C for 1 minute), a final extension at 72°C for 5 minutes and stop and storage at 4°C.

Before using collected samples for this protocol, the process had to be optimized through pilot samples, obtained from random individuals. Program temperatures and step length were optimized in a previous study that used the same primers for GSTM1 and GSTT1 (3).

Table 4: Reagents per PCR reaction tube, for accessing GSTM1 and GSTT1 null/present genotypes in swimmers.

GSTM1	GSTT1	Manufacturer
7.8 $\mu$ l Water Mili Q	7.8 $\mu$ l Water Mili Q	
0.5 $\mu$ l GSTM1 forward Primer (10pmol)	0.5 $\mu$ l GSTT1 forward Primer (10 pmol)	Sigma-Aldrich®, St. Louis, USA
0.5 $\mu$ l GSTM1 reverse Primer (10 pmol)	0.5 $\mu$ l GSTT1 reverse Primer (10 pmol)	Sigma-Aldrich®, St. Louis, USA
0.5 $\mu$ l $\beta$ -Actin forward Primer	0.5 $\mu$ l $\beta$ -Actin forward Primer	Sigma-Aldrich®, St. Louis, USA

(10 pmol)	(10 pmol)	
0.5 µl B-Actin reverse Primer (10 pmol)	0.5 µl B-Actin reverse Primer (10 pmol)	Sigma-Aldrich®, St. Louis, USA
1 µl dNTP (100 nM)	1 µl dNTP (100 nM)	Sigma-Aldrich®, St. Louis, USA
2.5µl Taq polymerase Buffer	2.5 µl Taq polymerase Buffer	NZYTech®, Lisbon, Portugal
1.5 µl MgCl <sub>2</sub> (1.5 nM)	1.5 µl MgCl <sub>2</sub> (1.5 nM)	NZYTech®, Lisbon, Portugal
10 µl DNA extract	10 µl DNA extract	(Previously extracted with Chelex 100)
0.2 µl Taq polymerase (1 U)	0.2 µl Taq polymerase (1 U)	NZYTech®, Lisbon, Portugal

### 3.4.3. Electrophoresis

PCR fragments were visualised by electrophoresis, providing the insight needed for the following gene expression assessment of our individuals. This technic is based on the application of a constant electric field to move samples through a gel matrix. The movement direction of samples depends on their charge; for nucleic acids, the samples are negatively-charged and so migrate towards the anode (positive pole). The speed of migration depends on several variables, such as gel composition and density, electrical potential and intensity applied, and sample weight. The variations at speed migration, in samples under the same matrix, electrical and buffer conditions, and at same run-time, result into different endpoint migration distances, which are used to differentiate the composition of each sample. The samples are visible under UV light as luminous bands due to the application of fluorescent dyes into the gel that are intercalated in between base pairs. The image is usually capture by an equipment

PCR fragments were separated in a 2% SeaKem® LE Agarose gel (Lonza Walkersville Inc, Walkersville, USA) prepared in 50ml TAE buffer and 15µl Green-Safe fluorescent dye (NZYTech®, Lisbon, Portugal), for 45min at 80V, powered by PowerPac™ Universal Power Supply (Bio-Rad®, München, Germany). Images were acquired in a UV Transiluminator FireReader (UviTech®, Cambridge, UK).

## 3.5. Gene expression

Gene expression was accessed to understand how the training phase influences the expression of antioxidant protection genes. RNA has to be extracted from our samples on paper, then cDNA has to be synthesized from the mRNA template for further amplification by RT-PCR. The results from this technique are used to evaluate the relative gene expression, i.e., how many times a gene is expressed when compared to another. In this thesis, the target genes are GSTT1 and GSTM1, and their expression is compared relatively to a housekeeping gene,  $\beta$ -actin.

### 3.5.1. RNA extraction

RNA extraction was performed recurring to TRIzol<sup>®</sup> reagent (Ambion<sup>®</sup>, Life Technologies<sup>™</sup>, Paisley, UK). TRIzol<sup>®</sup> is a reagent created to isolate whole-high quality RNA even from small samples, based on the acid guanidinium thiocyanate-phenol-chloroform extraction method (67). Although the TRIzol<sup>®</sup> designation derives from the three reagents needed for this method (TRI means three), this reagent is a solution of only guanidinium thiocyanate and phenol, while chloroform is added later to permit phase separation.

The original protocol for RNA extraction by TRIzol<sup>®</sup> was slightly modified to meet this work's demands. First of all, the samples were stored in filter paper, while the original protocol was meant for tissue samples. Due to that, the early steps of the method had to be optimized to ensure the best extraction efficiency. It was tried the direct application of TRIzol<sup>®</sup> on the samples, and a blood-elution method used previously in other studies (64-66). On the direct application, blood spots were cut into pieces to a 1.5ml Eppendorf tube, and then it was added 1ml TRIzol<sup>®</sup> reagent to advance with the normal procedure. On the elution method, the blood was eluted from the paper for 1h in a 1.5ml Eppendorf tube with 500 $\mu$ l RNase-free water at room temperature and gentle rocking, and then RNA extraction proceeded from 140 $\mu$ l from the eluted sample (65). The final result led to the choice of the first method, once the extracted RNA was more concentrated. Those concentrations were accessed by spectrophotometry, by the absorbance at 260nm, based on Lambert-Beer's Law, by NanoPhotometer<sup>™</sup> (Implen GmbH, München, Germany).

The protocol adopted for RNA extraction consisted on the following steps:

- The blood spot was cut from the filter paper, cut into smaller pieces and collected in a 1.5ml Eppendorf tube.
- It was added 1ml TRIzol<sup>®</sup> reagent and incubated with the sample for 5 to 10 minutes at room temperature.
- A centrifugation for 10 minutes, at 4°C and 10,000 RPM, followed to help both paper degradation and sample elution.

- To create the aqueous phase and precipitate most contaminants, it was added 0.2µl chloroform, followed by a 15 second vortex homogenization and incubation for 3 minutes at room temperature.
- After incubation, the reaction solution was centrifuged for 15 minutes, at 4°C and 13,000 RPM, to promote phase separation.
- Aqueous phase was then recovered, since it contained the RNA extract.
- Next, it was added 0.5 ml isopropanol to the recovered supernatant, and it incubated for 10 minutes at room temperature, to promote RNA precipitation.
- Centrifugation for 10 minutes, at 4°C and 13000 RPM, followed to form a RNA pellet. This pellet is white and usually very small, which turns it often difficult to spot.
- After discarding the supernatant, the pellet was washed by the addition of 0.5ml DEPC Ethanol solution 75%, and centrifuged once more, for 5 minutes at 4°C e 11,000 RPM.
- The resulting supernatant is discarded again and the remaining pellet is dried for 5 to 10 minutes at room temperature.
- Finally, RNA is resuspended in 20µl DEPC-treated water and incubates for 10 minutes at 60°C.

The volume added of each reagent was also target for modification. It was tried to reduce the volume added to an half however, the results obtained in those tests were not as good as with recommended volume, especially as accounts for TRIzol®. This last step was also slightly modified in order to improve the final RNA concentration, so it was added as less DEPC-treated water, so extracted could be RNA were more concentrated.

### 3.5.2. cDNA synthesis

mRNA was converted to cDNA in order to permit amplification by Real Time Polymerase Chain Reaction (RT-PCR), and relative gene expression analysis. This synthesis is an in vitro adaptation of a naturally occurring phenomenon, through action of a group of enzymes, mostly found on retro-viruses, called reverse transcriptases. There are two major reverse transcriptase used in vitro, the Avian Myeloblastosis Virus (AMV) and the Moloney-Murine Leukemia Virus (M-MLV).

For this thesis, M-MLV was used to synthesize cDNA from the mRNA extract. The protocol used was the reverse transcriptase manufacturer's manual, but due to the extracted RNA's low concentration, the water volume to be added was replaced by RNA extract. This modification, nevertheless, is predicted by the original protocol, as the sterile water volume to be added is calculated in function of RNA extract volume, 9µl-X, where X is the RNA extract volume. The reactions, one for each sample collected from an athlete, were prepared in thermocycler Eppendorf tubes, and consisted in 9µl RNA extract, 2µl Randomprimer

(Promega®, Madison, USA), 1µl dNTPs (100 nM, Sigma-Aldrich®, St. Louis, USA), performing 12µl total volume. The solutions were then heated to 70°C for 5 minutes and briefly cooled to 4°C in a thermocycler.

Meanwhile, a mix was prepared to add to the previous solution. This mix was prepared in function of reaction's number ( $n+1$ , where  $n$  is the number of reactions), and consisted, *per* reaction, of 4µl 5x M-MLV Reverse Transcriptase (RT) Buffer (Promega®, Madison, USA), 2µl Dithiothreitol (DTT) (0.1M), 1µl RNase inhibitor (Promega®, Madison, USA) and 1µl M-MLV RT (Promega®, Madison, USA). The sum of these volumes is 8µl, and that amount is transferred to each reaction tube, after the cooling.

After mix addition to reaction tubes and solution homogenization, by quick-spin, cDNA synthesis is ready to start. The synthesis requires also a three-step process, each one at different temperature and duration, thus a thermocycler is used to perform it. First, samples were incubated at 25°C, followed by an elongation step at 37°C (ideal temperature for M-MLV RT activity) for 1 hour, and a final step at 70°C for 15 minutes, to inactivate the enzyme.

The synthesized cDNA is stored at 4°C for further usage.

### 3.5.3. Real Time PCR (RT-PCR)

RT-PCR is a very similar technique to conventional PCR, an improvement to permit simultaneously amplify and quantify DNA, cycle after cycle. Because this data acquisition, which permits continuous track of the DNA amount, it was called Real Time. However, several other designations were adopted in function of the technique's aim. It is referred as quantitative PCR (qPCR) when the objective is to know DNA's concentration on a sample, but also as Reverse Transcriptase PCR, when it is used to synthesize cDNA. Nevertheless, its main application is a fusion of the abovementioned uses; it is used to amplify cDNA in order to access relative expression of a target gene. This technique is sometimes denominated as Real Time Reverse Transcriptase PCR, since it aims to evaluate cDNA copies on a sample after each amplification cycle, yet it is dependent of a Reverse Transcriptase to previously synthesize that cDNA from the extracted RNA.

The technique by itself uses the same principle as the conventional PCR. DNA is amplified by the exact same steps and reagents. However, RT-PCR needs a probe that will signalize the amplification, and the equipment, the thermocycler, has to have an extra unit to identify that probe. At each amplification cycle, a probe is incorporated in the amplicon (the enzymatic complex that enable amplification) and suffer a change that permit signal emission. Usually, that signal is light emission by a fluorophore that is then received by a light-sensitive system, which calculates the copy number in function of the perceived light intensity. In order to simplify laboratorial work and to ensure reagent's compatibility, probes are sold in mixtures containing also the DNA polymerase, its buffer, dNTP's and  $MgCl_2$ . Each reaction occurs on a RT-PCR plate well, which is sealed by a plastic film.

For this thesis, it was used Sybr Green as light probe. This probe binds to the minor groove of the recently-synthesized DNA, and its fluorescence increases 100 to 200 fold when bound (68). Since all our athletes presented GSTT1 Present genotype, we choose this gene to compare its expression relatively to the housekeeping gene  $\beta$ -actin, for all samples of an individual at a time, and duplicates for every moment were done for better data reliability. First, it was prepared a mix for each gene. The mix for GSTT1 gene consisted in 10 $\mu$ l Maxima Sybr Green/ROX qPCR Master Mix (2x) (Fermentas - Thermo Fisher Scientific Inc., Vilnius, Lithuania), 0.5 $\mu$ l forward primer (Sigma-Aldrich®, St. Louis, USA), 0.5 $\mu$ l reverse primer (Sigma-Aldrich®, St. Louis, USA ) and 4 $\mu$ l DEPC-treated water for each reaction, following the n+1 rule. It was added 5 $\mu$ l synthesized cDNA and 15 $\mu$ l mix to each plate well. This amount of added cDNA volume is much higher than the recommended (1-2 $\mu$ l) the concentration of resulting cDNA was too low for reliable results when 1 $\mu$ l and 2 $\mu$ l were added.

It was added also a Negative control for each gene, in which cDNA was replaced by sterile water. The plate (Bio-Rad®, München, Germany) was sealed by a sticky plastic film, especially prepared for this use. Figure 10 depicts the samples' displacement on the plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C				1	2	3	4	5	1			
D				1	2	3	4	5				
E				1	2	3	4	5	1			
F				1	2	3	4	5				
G												
H												

Figure 10: Well displacement on the RT-PCR plate. The wells represented as green squares were relative to the target gene, GSTT1, while the blue-coloured squares were relative to the housekeeping gene,  $\beta$ -actin. The wells with an inner rotated square were the Negative Controls, in which cDNA had been replaced by water. The numbering represents the sample collection order (1 for the first sample moment, and so on). This plate was repeated for each individual and for each target gene.

The reaction ran in an iQ5 Multicolor Real-Time PCR Detection System thermocycler (Bio-Rad®, München, Germany), according to the following program: a starting DNA denaturation at 95°C for 3 minutes, followed by 60 amplification cycles (consisting in denaturation at 95°C for 10s, annealing at 58°C for 30s and extension at 72°C for 30s). Before

running this thesis' samples, the annealing step was optimized for the different gene primers, aiming the best amplification efficiency, which was found to be at 58° C (figure).

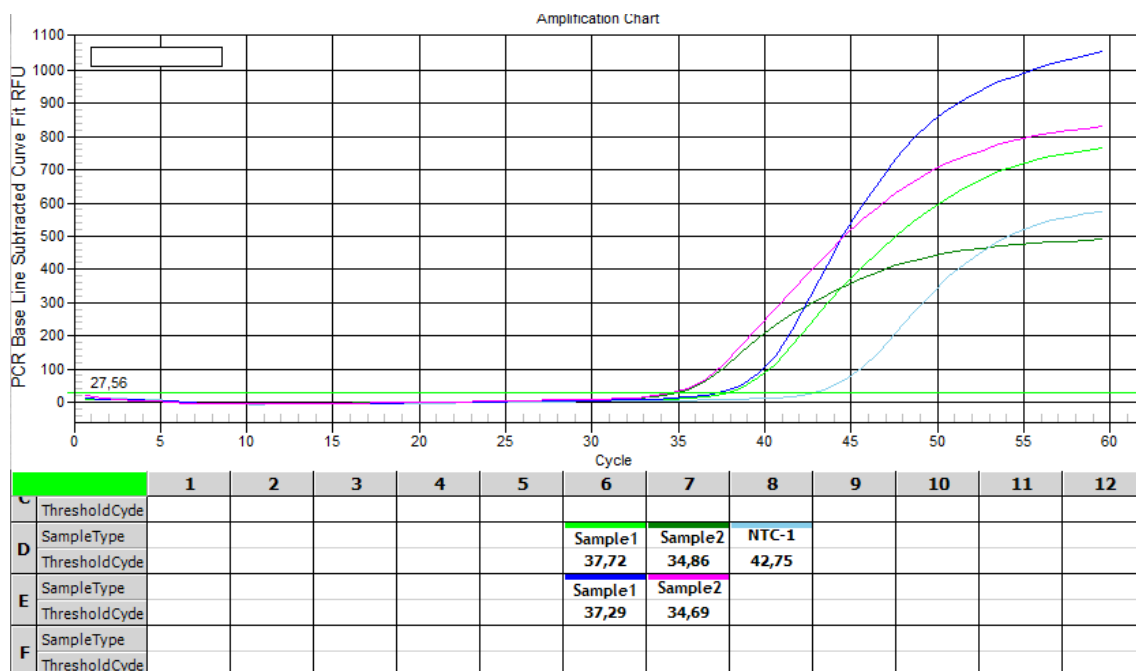


Figure 11: Optimization of annealing temperature and amplification cycles for GSTT1. Sample 1 and sample 2 correspond to two different individuals whose RNA was previously extracted and converted in cDNA. Annealing temperature was set to 58.0° C and were done 60 amplification cycles. NTC-1 is the Non-template control. In spite of its amplification, it occurs later than our samples.

This optimization was performed recurring to a pilot test using cDNA previously synthesized from samples from volunteers, used when optimizing upstream methods, such as RNA extraction. Real-Time data was acquired at the end of each step, and consisted in the fluorescence emitted by the Sybr Green probe. As a quality assay to found if there was primer amplification instead of gene fragment amplification, it was added a melting curve analysis, ranging from 55° C to 95° C, by 0.5° C increments lasting 10s, and fluorescence data was collected at the end of each increment to plot that curve (Figure 12). Data collected was analysed by iQ5 software v2.0 (Bio-Rad®, München, Germany) to plot the amplification graphs for further calculations.

el

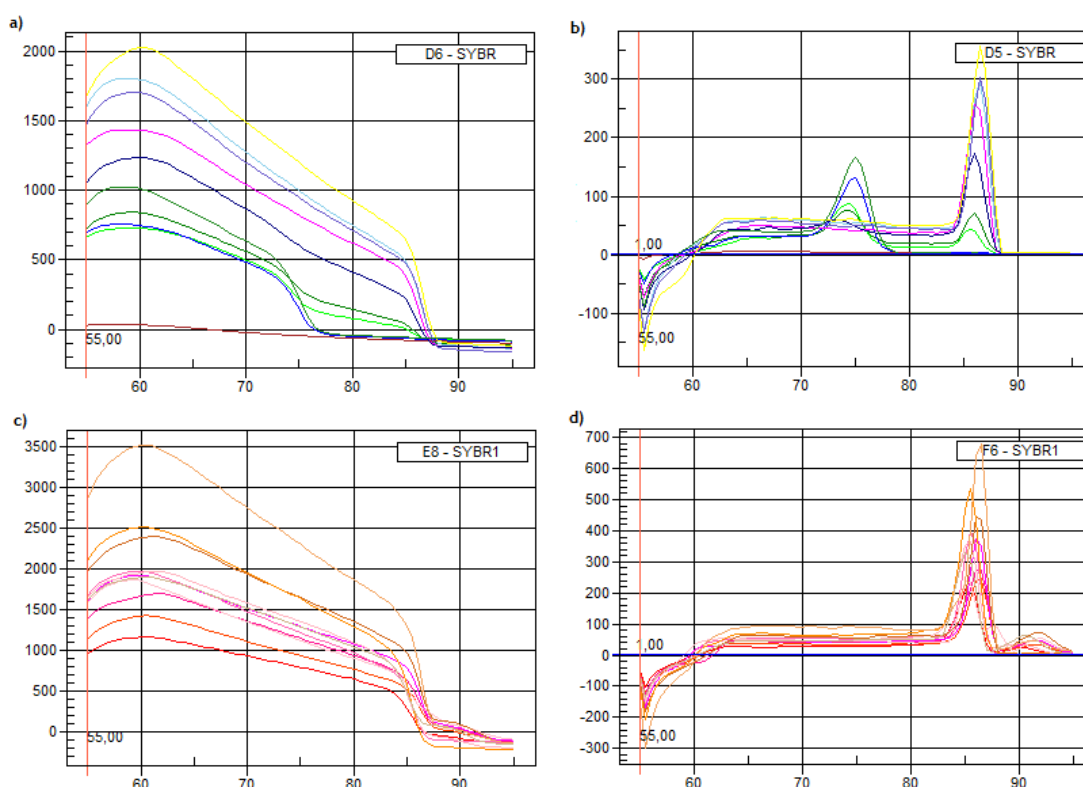


Figure 12: Melting curves for GSTT1 and  $\beta$ -Actin genes. Each line correspond to a sample well, and all graphics are expressed as fluorescence units by  $^{\circ}\text{C}$ . Correct amplification at a sample was considered when its melt peak was between the  $80^{\circ}\text{C}$  and  $90^{\circ}\text{C}$ . a) Dissociation lines for GSTT1 wells; b) Melting curve peaks for GSTT1; c) Dissociation lines for  $\beta$ -Actin wells; d) Melting curve peaks for  $\beta$ -Actin.

### 3.5.4. Relative expression calculus

The relative expression of target genes was performed recurring to the  $\Delta\Delta C_T$  Method, also known as the Double Approximation Method (69). This method does not account the amplification efficiency, considering it as a 100% efficient reaction. In spite of the existence of methods that are efficiency-sensitive, such as the Pfaffl Method (68, 70), the Double Approximation Method was chosen because it does not requires previous tests in dilution series, which would be forbidding due to the small amount of cDNA on the samples. The cycle threshold ( $C_T$ ) were evaluated by the plots traced by the software iQ5 v2.0 (Bio-Rad®, Munich, Germany) using a line in the beginning of the exponential phase of the reaction, linking all curves. This line is automatically traced by the software, but the user can move it to a more suitable position. The line in these tests was moved so both amplification curves (target gene and  $\beta$ -actin) were at exponential phase, because this line must define the  $C_T$  for all curves. After defining this line and the  $C_T$ 's, the equation was applied to get the values for the relative expression of target genes at a given moment. The first moment was defined as baseline, the moment before the application of any stimulus.  $C_T$ 's values were copied to an

Excel® 2013 sheet (Office®, Microsoft™, 2012), using the program for further calculations and data analysis. Since duplicates were made, it was used the mean of both  $C_T$ 's of a moment. When duplicates had too disparate  $C_T$ 's, if one of them were within the expected range, that  $C_T$  is used while the other is rejected.

The first step for this relative gene expression method is the normalization in function to the housekeeping gene ( $\Delta C_T$ ). Thus, the mean  $C_T$  for the target genes, at each moment, was subtracted by the mean  $C_T$  for the  $\beta$ -actin, at the corresponding moment (1):

$$\Delta C_T = C_{T(\text{target})} - C_{T(\text{Housekeeping})} \quad (1)$$

After that, the differences obtained at each moment were normalized relatively to the baseline ( $\Delta\Delta C_T$ ), which was defined, as previously mentioned, as the first moment. The reason for this choice was because these samples were collected on the first training week of the season, after holydays, a general preparation phase. That normalization is a second subtraction from the differences obtained by the difference obtained at the first moment (2):

$$\Delta\Delta C_T = C_{T(\text{stimulated})} - C_{T(\text{baseline})} \quad (2)$$

At last, all that is left is to apply the relative expression formula (3).

$$\text{Relative Expression} = 2^{(-\Delta\Delta C_T)} \quad (3)$$

## 3.6. Statistical analysis

### 3.6.1. Genotyping

For GSTM1 and GSTT1 null/present genotypes, swimmers were divided and compared to the results obtained for controls. Swimmers were further divided in sub-groups, according to their age, speciality and gender. Hardy-Weinberg Equilibrium (HWE) was tested by a Chi-square test to compare the observed genotype frequencies with the expected genotype frequencies in controls (71). To apply this ideal genetic model to a population, at least four assumptions have to be made: (I) mating is random; (II) allele frequencies are equal in both sexes; (III) migration is insignificant; (IV) there is not mutation nor selection.

Calculations were done using the computer software IBM® SPSS® Statistics version 20.0, for Windows®-based 64-bit Operating Systems. It was used the Chi-square Test with Pearson coefficient (p-values) calculation to correlate the GSTM1 and GSTM1 null/present genotypes in our test sample, in comparison to our controls. In the cases where a class was

expected to count less than 5 cases and the conditions permitted to, it was applied the Fisher's Exact Test to calculate the p-values. Results were considered statistically significant when  $p \leq 0.05$ .

### 3.6.2. Relative Expression

Results from relative expression analysis were treated individually for each swimmer, since results for gene expression are very variable from one individual to another. The comparison of the results was done accounting to the tendency of each swimmer's gene expression after a stimuli.

To do so, the results were grouped in a line graph to compare the evolution of gene expression along our data collection points. Two graphs were done, both for GSTT1 gene expression. In the first, three expression profiles, from three swimmers, were analysed for the totality of our samples (5 moments). The second graph joined the expression profiles for the 8 athletes who participated in all our sample collection moments, but only evaluates the post-competition recovery sample moments (at 48h and 72h after competition), comparing to the baseline (first data collection moment).

All the calculations for relative expression analysis (mean  $C_T$ 's, normalizations and formula application), and graphic representation as well, were done in an Excel® 2013 sheet (Office®, Microsoft™, 2012), as mentioned in section 3.5.4.

## 4. Results

### 4.1. Genotyping

Genotype determination of all individuals in the sample test group and in the control group was done to verify the frequency of the GSTM1 and GSTT1 null/present genotype. The results were discriminated by agarose gel electrophoresis after PCR amplification, and detected by UV exposure. The presence of GSTM1 was identified when a band was present near the 300bp ladder, as the amplified fragment for this gene was 215bp long. The presence of GSTT1 gene was considered when a band near the 400bp ladder, since its fragment was 473bp long. When a band was absent, it was considered the null genotype, for that gene, for that individual. This method does not distinguish individuals into homozygote or heterozygote, because it is only needed an allele to express these genes.

An example of the resulting bands for the GSTM1 and GSTT1 null/present genotypes is depicted on figure 12.

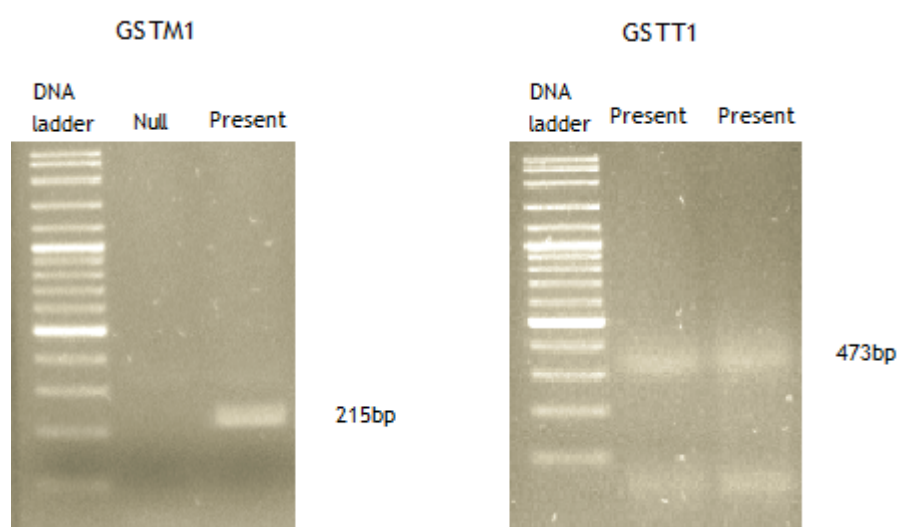


Figure 13: Gel images captured with UV light, for genotype analysis. On left side, an example of a null and a present genotype for GSTM1. On the right side, two examples for present GSTT1 genotype. In this case, bands migrated further than expect, so their length doesn't match with the DNA ladder. That situation is likely due to partial DNA degradation by haemoglobin contamination.

The results were treated according to the possible group divisions. Controls were under the HWE. The results for the GSTM1 null/present genotypes, comparing our test sample with the control group, are depicted on table 5.

Table 5: GSTM1 null/present genotype distribution comparison between swimmers and controls.

GSTM1	Swimmers	Controls	P-value
Null	7 (35.0%)	22 (42.3%)	0.321
Present	13 (65.0%)	30 (57.7%)	

The results for the GSTT1 null/present genotypes, comparing our test sample with the control group, are depicted on table 6.

Table 6: GSTT1 null/present genotype distribution comparison between swimmers and controls. † P-value calculated by Fisher's Exact Test, since one class count less than 5 cases.

GSTT1	Swimmers	Controls	P-value
Null	0 (0.0%)	13 (25.0%)	†0.098
Present	20 (100.0%)	39 (75.0%)	

The results for the combined GSTM1 GSTT1 null/present genotypes, comparing our test sample with the control group, are depicted on table 7.

Table 7: Combination of GSTM1 and GSTT1 null/present genotypes distribution between swimmers and controls.

GSTM1/GSTT1	Swimmers	Controls	P-value
Present/Present	13 (65.0%)	27 (51.9%)	0.285
Null/Present	7 (35.0%)	12 (23.1%)	
Present/Null	0 (0.0%)	3 (5.8%)	
Null/Null	0 (0.0%)	10 (19.2%)	

In the distribution according to the speciality, the results for GSTM1 and GSTT1 null/present genotypes were the following, depicted on table 8.

Table 8: Combination of GSTM1 and GSTT1 null/present genotypes distribution between within swimmers, according to their distance speciality. <sup>(1)</sup>SDS vs MDS; <sup>(2)</sup>SDS vs Controls; <sup>(3)</sup>MDS vs Controls

GSTM1/GSTT1	SDS	MDS	Controls	P-value
Present/Present	11 (73.3%)	2 (40.0%)	27 (51.9%)	<sup>(1)</sup> 0.226
Null/Present	4 (26.7%)	3 (60.0%)	12 (23.1%)	<sup>(2)</sup> 0.396

Present/Null	0 (0.0%)	0 (0.0%)	3 (5.8%)	<sup>(3)</sup> 0.291
Null/Null	0 (0.0%)	0 (0.0%)	10 (19.2%)	

Distribution by gender for our Swimmers, when compared to Controls, was also done to verify if there was a preferential genotype drift linked to physical practice in a gender. Results are shown on table 9.

Table 9: Combination of GSTM1 and GSTT1 null/present genotypes distribution between swimmers and controls according to their gender. <sup>(1)</sup>Male vs Male; <sup>(2)</sup>Female vs Female; <sup>(3)</sup>Male vs Controls; <sup>(4)</sup>Female vs Controls.

GSTM1/GSTT1	Swimmers		Controls		P-value
	Male	Female	Male	Female	
Present/Present	8 (61.5%)	5 (71.4%)	20 (52.6%)	7 (50.0%)	<sup>(1)</sup> 0.631
Null/Present	5 (38.5%)	2 (28.6%)	9 (23.7%)	3 (21.4%)	<sup>(2)</sup> 0.480
Present/Null	0 (0.0%)	0 (0.0%)	2 (5.3%)	1 (7.1%)	<sup>(3)</sup> 0.573
Null/Null	0 (0.0%)	0 (0.0%)	7 (18.4%)	3 (21.4%)	<sup>(4)</sup> 0.520

## 4.2. Relative expression

The results for relative expression were grouped in the form of chart to facilitate the analysis. Only 8 swimmers in total were access in this analysis. Their characteristics can be consulted at table 10.

Table 10: Individual characteristics for the 8 swimmers eligible for GSTT1 relative expression analysis. All of them compared on the 5 sample collect moments. The nr of races and best position are related to the winter short course National championships. \*Number of events (number of eliminatories); #Classifications achieved in all events, from the best to the worst.

Swimmer	Gender	*Nr of races	#Classification	Genotype (GSTM1/GSTT1)
F1	Male	4 (3)	1 <sup>st</sup> , 2 <sup>nd</sup> , 2 <sup>nd</sup> , 3 <sup>rd</sup>	Null/Present
F5	Female	3 (1)	16 <sup>th</sup> , 26 <sup>th</sup> , 27 <sup>th</sup>	Present/Present
F6	Male	0	-	Present/Present
F11	Female	0	-	Present/Present
F12	Female	0	-	Present/Present
F14	Male	3 (1)	11 <sup>th</sup> , 18 <sup>th</sup> , 19 <sup>th</sup>	Present/Present
F15	Male	0	-	Null/Present
F22	Female	2	17 <sup>th</sup> , 26 <sup>th</sup>	Present/Present

Three swimmers (F1, F4 and F5) were evaluated for their expression at the total number of moments, so, for their expression throughout the complete Winter season. The results are pointed on figure 14.

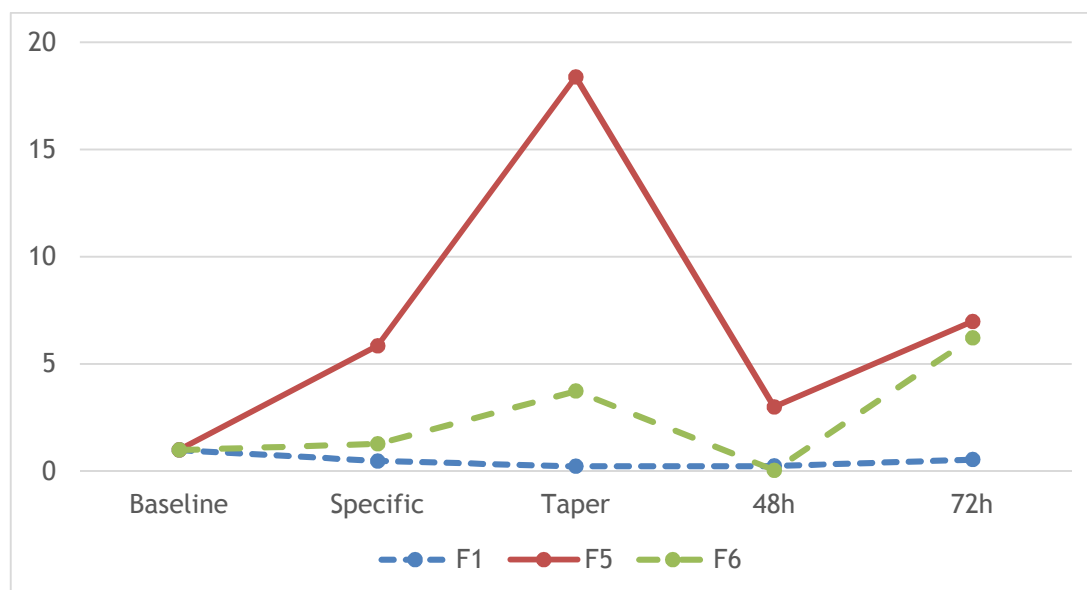


Figure 14: GSTT1 expression throughout the Winter season, for three different swimmers. Units are expressed in expression fold, comparing to the housekeeping-gene,  $\beta$ -actin. Baseline is the moment 1, Specific is the moment 2, Taper is the moment 3, and 48h and 72h are the fourth and fifth moments, relative to the post-competition recovery.

From the above graph we can see that, apart from the swimmers F1, the expression of this antioxidant gene increases as the season goes by. The amount of available GSTT1 drops dramatically after a competition, but it gradually recuperates to prepare the organism for a new oxidative assault.

The previously three swimmers (F1, F5 and F6) and five more who had also participated in all sample collection (F11, F12, F14, F15 and F22), were investigated for their GSTT1 expression during the recovery after a competition. The baseline was defined as the first sample collected. The results are shown in figure 15.

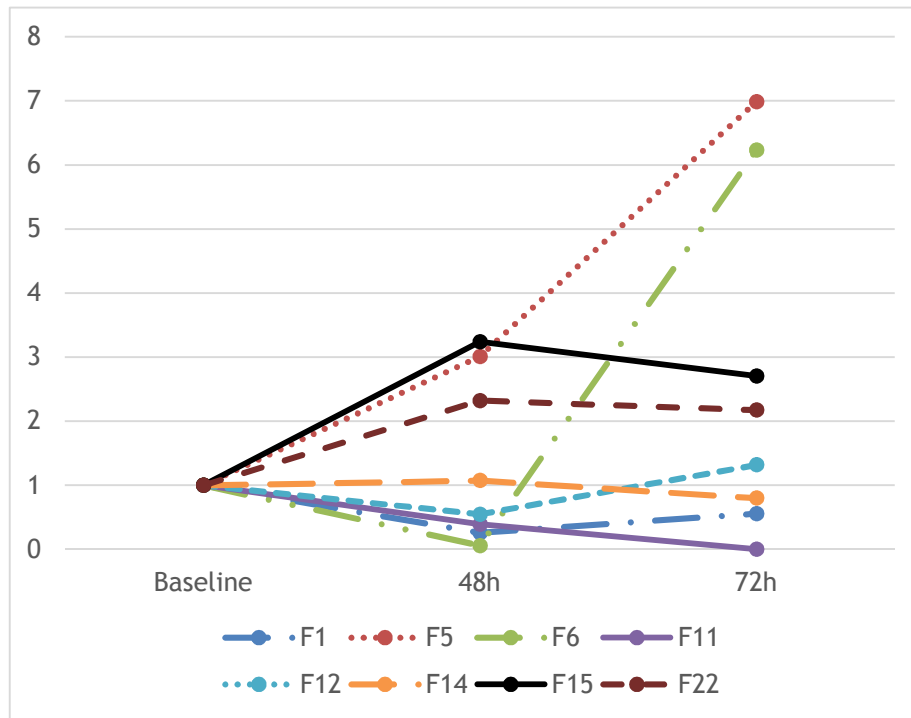


Figure 15: GSTT1 expression tendency after a competition, for eight different swimmers. Units are expressed in expression fold, comparing to the housekeeping-gene,  $\beta$ -actin. Our baseline is the first collected sample, then 48h and 72h are the samples collected at that time after the competition.

From this graph we can noticed that swimmers had very different responses, yet it is possible to figure out a pattern on GSTT1 relative expression. A filtering from the above information had to be done. First, swimmers were divided into 2 groups according to their tendency in GSTT1 relative expression at the 0h to 48h period:

- 1) Increased expression (F5, F14, F15 and F22);
- 2) Decreased expression (F1, F6, F11 and F12).

The results of this first division can be seen at the figure 16.

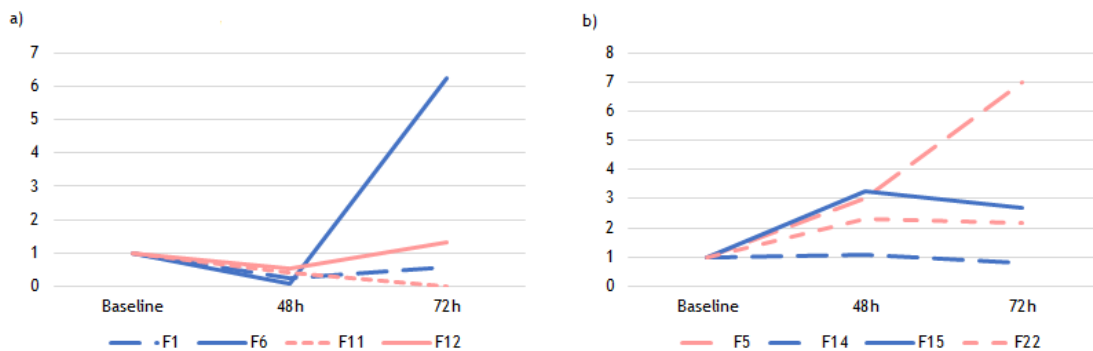


Figure 16: GSTT1 expression lines after the division by 2 groups, according to their evolution at the first 48h. Lines in blue correspond to male swimmers, and those in rose to female swimmers. a) swimmers who dropped their GSTT1 expression after the first episode; b) swimmers who had increased expression after the same episode.

Besides, we can divide swimmers into 3 groups, according to their expression behaviour at the second time interval (48-72h):

- 1) Very intense expression increase (F5 and F6);
- 2) Lesser increase in expression (F1 and F12);
- 3) Expression decrease or maintenance (F11, F14, F15 and F22).

The results for the division into 3 groups for the recovery at the 48h to 72h interval can be accessed on figure 17.

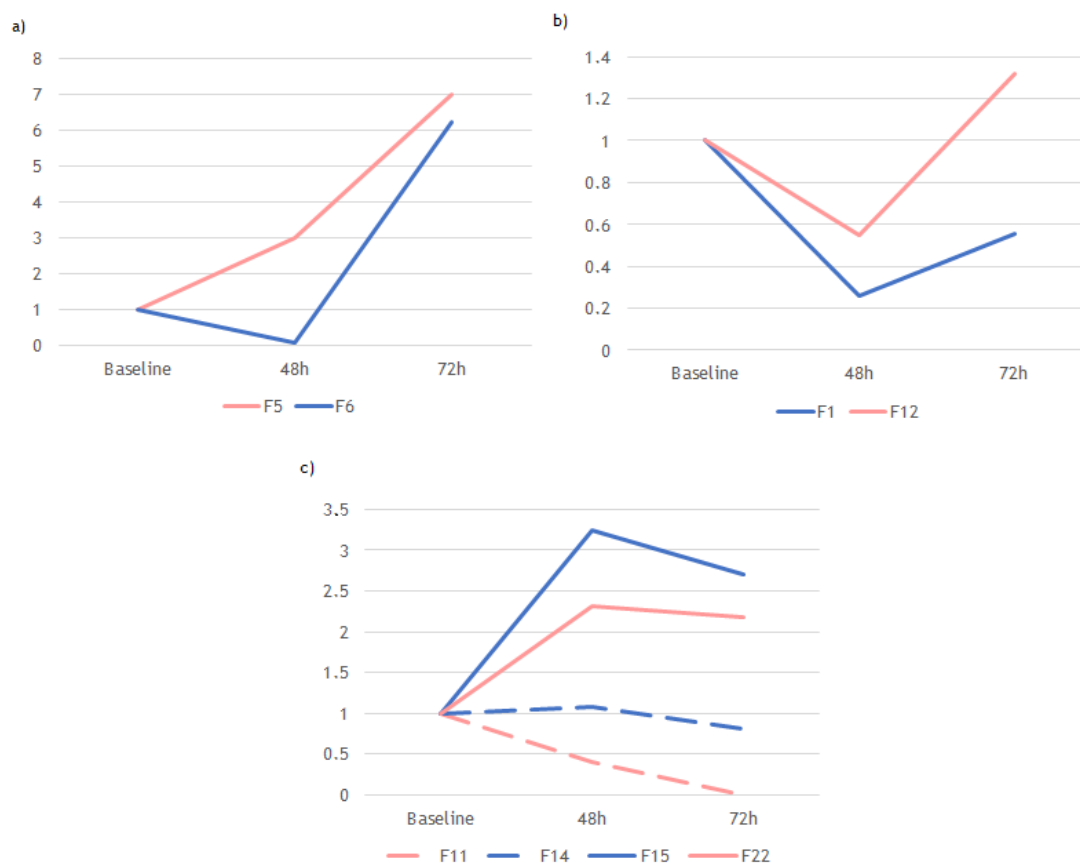


Figure 17: GSTT1 expression lines after the division by 3 groups, according to their expression evolution at the 48h-72h period. Lines in blue correspond to male swimmers, and those in rose to female swimmers. a) Swimmers who greatly increased GSTT1 expression after the second episode; b) Swimmers with moderate expression increase; c) Swimmers who dropped or maintained their GSTT1 relative expression at this interval.

To better understand the behaviour of our swimmers' GSTT1 relative expression lines, they were divided in terms of gender, genotype and if they participated at the winter short course national championship. This data was compared to the expression tendency at the first 48h, at the 48h to 72h interval and from the baseline to the expression at 72h. The first step was evidence whose subjects stood for two exercise episodes (competition and training) within this 72h time course, and those who only stood for the second episode (training).

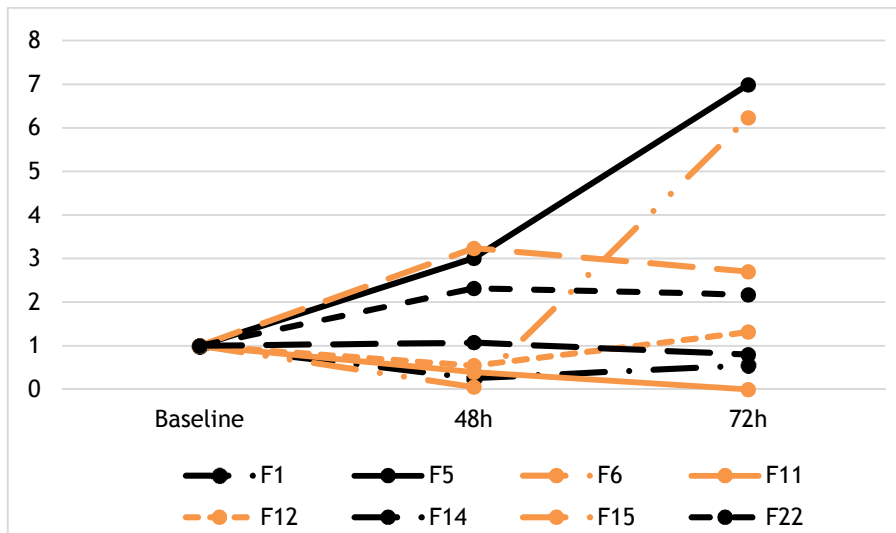


Figure 18: Expression tendency lines for our sample's swimmers, evidencing the groups divided by number of physical episodes. The orange lines are for swimmers with only one episode (the training), while black lines stand for swimmers who had two episodes (the competition 48h before the training).

Since only 4 out of our 8 swimmers with GSTT1 expression data participated in two episodes, they were divided in order to the amount of stimulus during the 72h, to check if it had an impact on expression. The charts for the influence of gender in GSTT1 expression are depicted on figure 19 a) for the two episode group, and on figure 19 b) for the single episode group.

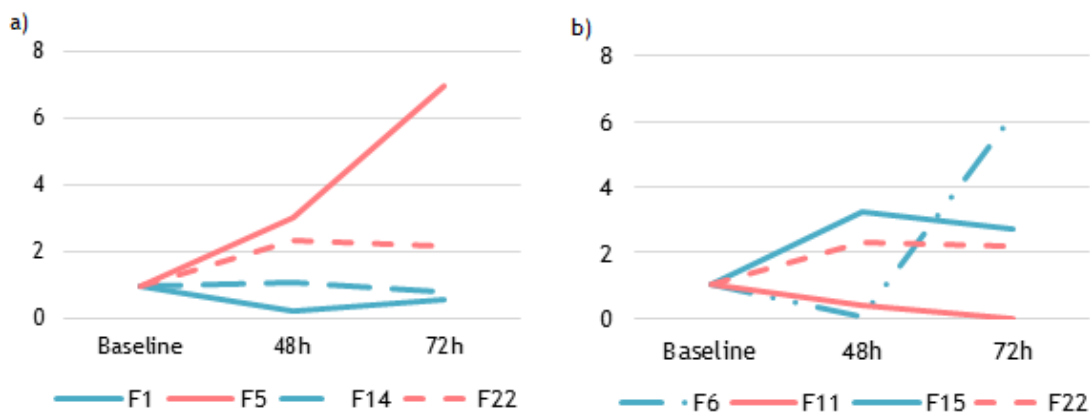


Figure 19: Gene expression comparison between the one episode group and the two episodes group, in matter of gender influence. The rose lines mark female swimmers, while the blue lines correspond to male swimmers. a) Swimmers submitted to 2 episodes (competition + training); b) Swimmers submitted to only one episode (training).

The expression of GSTT1 increases more in females than in males, in the 48h after an exercise episode. For those who hadn't such episode, there isn't a tendency in favour of a gender, in the same time interval. In the following interval, after the second exercise episode, we can notice that woman, in those who were submitted to the first episode, are

consistently expressing more GSTT1 relatively to  $\beta$ -Actin than males. However, in the single episode group, the opposite occurs.

The last comparison done for GSTT1 relative expression included the genotype factor. Since all our swimmers who underwent relative expression evaluation were positive for GSTT1, this genotype was not included because it is not a differentiation factor. Therefore, the swimmers were evidenced in terms of their GSTM1 Null/Present genotype. Furthermore, data continued to be divided into two exercise episodes swimmers and single episode swimmers, to facilitate the comprehension. The results are draw on figure 20.

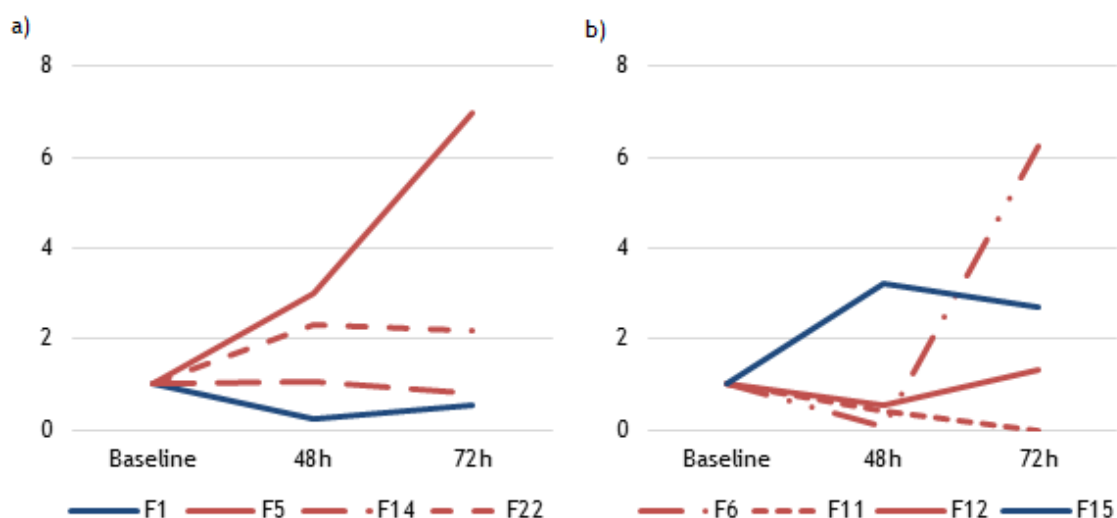


Figure 20: Comparison of the GSTT1 expression in the two episode group versus the single episode group, for the influence of the GSTM1 genotype. GSTT1 genotype has not included because it not a differentiation factor, as all our swimmers were GSTT1 Present. Blue lines represent GSTM1 Null individuals and magenta lines are GSTM1 Present. a) Swimmers submitted to 2 episodes (competition + training); b) Swimmers submitted to only one episode (training).

In the group who had two physical episodes in 72h (figure 20a), swimmers who were GSTM1 Present had consistently higher levels of GSTT1 relative expression than the swimmer without GSTM1. The opposite occurred in the single moment group (figure 20b), where the GSTM1 Null swimmer had the highest expression levels at the moment of his exercise episode (48h). Nevertheless, 24h after that (at 72h mark), his expression levels decreased slightly, while the GSTM1 Present swimmers suffered more drastic changes in their expression levels.

## 5. Discussion

### 5.1. Genotype determination

The results for the GSTM1 and GSTT1 Present/Null genotypes lack in power due to the small sample size and the absence of a selective force acting on our test sample. The amount of swimmers often led to classes with less than 5 cases when we intended to study subgroups in our sample.

The selection of the sample may also be criticised because the requisites for being a national level swimmer are not so difficult to fulfil that only individuals with genetic advantage are prepared to complete them. Due to this, our selection may include more swimmers who overcome their genotype limitations by hard and dedicated training, which do not favours a genetic study.

The most significant result we had for genotype distributions was the higher prevalence of the GSTT1 genotype in the test sample ( $p=0.098$ ), supporting a hypothesis that this gene may confer an advantage at the level of physical performance. We also noticed a higher incidence of the GSTM1/GSTT1 Present/Present and GSTM1/GSTT1 Null/Present genotypes in swimmers than in the control population, but there was no statistical significance of these results, as we expected small number of cases.

The brightest side of our results is that they prove it is possible to collect blood samples in filter paper in a non-invasive manner, store it easily and safely, extract DNA from those blood samples with enough integrity to permit amplification by PCR and see well-defined, strong stained DNA bands after an agarose gel electrophoresis. The possibility of sample contamination, either by the paper or by the storage conditions or even by the low amount of DNA, did not seemed to affect the quality of the results. The low amount of DNA was reflected in dimed or blurred bands, punctually, but never affected the verdict of a swimmer's genotype.

### 5.2. Gene Expression

The relative gene expression for GSTT1 revealed that this variable is individualized. However, there is a tendency to gene expression, along the recovery time, the values obtained seems to approximate the baseline expression. We drew two types of expression tendency lines: one for the expression throughout the season, for 3 swimmers who participated on all our data collection moments; and another for the gene expression after a competition to access recovery capacity (one physical exercise episode), for the whole 8

swimmers who completed all our data collection moments. Four of these swimmers performed two physical exercise episodes at different days. The first is the winter short course national championship, and the second is the training 48h after the competition day. This is a crucial premise in the analysis of expression results, because every swimmer is an individual case, under different conditions.

The information about the performance at the winter short course National Championships was also taken into account. It was very interesting to notice in the expression tendency lines that the swimmer who had the most stable GSTT1 relative expression throughout the season (F1) was also the swimmer with the better performance at the Championship, grabbing the title of National Champion. This may indicate that stable levels of GSTT1 expression along the season, without significant variance, are linked to a better sport performance. In fact, if the cells are adapted to a constant oxidative assault, their antioxidant defences are build up consistently to prevent damage. Thus, it is expected that a well-trained athlete don't have their antioxidant factor's expression greatly interfered by an episode of strong physical activity.

The other two swimmers with this complete analysis (F5 and F6) support this hypothesis. Their GSTT1 relative expression profile was very similar, consistently increasing the expression to a peak at the end of the taper phase, right before the date of the most important winter competition. The swimmer F5 participates in those championships but her best classification was a 16<sup>th</sup> place. F6 doesn't even participates in this event. Their levels drop drastically at 48h after the date of the competition, and after that day's training, at the 72h sample collection, GSTT1 relative expression greatly increases once more, revealing a severe oxidative stress condition, probably due to the training the previous day. In their cases, the cells weren't properly adapted to face the assault caused by one or two episodes of physical activity, ROS and other free radicals caused serious cell damage, and GSTT1 expression was activated almost as an emergency mechanism to mitigate the ongoing aggression.

In the post-competition charts, for the analysis methodology we decided to divide the swimmers by increased or decreased GSTT1 relative expression on the first 48h after the exercise episode. Doing this separation, it wasn't found any significant difference from males to females, at this period of time.

Secondly we analyse the expression profile in the 48h to 72h interval, after the second episode. Here, differences were greater, and 3 different groups had to be made. A group whose GSTT1 gene expression greatly increased, other group with a moderate expression increase, and a group whose expression decreased or maintained after the second stimuli. However, no tendency was found within the groups towards gender.

Afterwards results were analysed by condition and individually. Two groups were performed; between swimmers submitted to two exercise episodes (competition and training) within the 72h, and the swimmers who had done only one episode (training). The influenced of gender in expression lines was verified by comparative analysis between those groups. The

results obtained for the group of swimmers who had only an exercise episode at our 48h mark (figure 18b), shows once more that no relation can be pointed out between gender and expression tendency, at the first 48h. In the 48h to 72h interval, after their exercise episode, female athletes seem to stabilize their expression better, yet the difference it's not significant. The group who was submitted to two exercise episodes (figure 18a) revealed an overall and significant tendency of female swimmers to have greater values of GSTT1 relative expression than males. Besides, males present much more stable GSTT1 expression. This difference is greater in the first 48h, where both females had a great increase in expression, while males almost have no difference. At the 48h to 72h interval, the female swimmer F22 stabilizes her expression too, but F5 swimmer continues to increase her GSTT1 expression.

This difference amongst gender should indicate that males at included in this study are better prepared to compete, since their antioxidant defences readily dampen the oxidative effects of the competition, preventing most cellular damage and not triggering the overexpression of antioxidant genes, such as GSTT1. Females, on the other hand, react later to oxidative stress, and thus their GSTT1 expression increased later. It is possible that males also get a peak expression, but that should be before the 48h, when we collected the sample, and due to it we are unable to notice it. The swimmer F5 increases even more her expression probably due to lack of physical preparation, as her expression profile throughout the season (figure 13) is very irregular, it seems that she is continuously under uncontrolled oxidative stress and the training is not providing adaptation to protect her from such aggressions.

The last comparison included the influence of GSTM1 genotype in the behaviour of GSTT1 relative expression curves. There were just a swimmer (F1) at the two episodes group and another (F15) at the single episode group who had the GSTM1 Null genotype. A tendency was found for both swimmers: in the double episode group, the swimmer without GSTM1 had the lowest GSTT1 expression levels; in the single episode group, the GSTM1 Null swimmer had the highest. However, in both swimmers, their GSTT1 expression has little fluctuations comparing to GSTM1 Present cohorts, especially in the 48h to 72h interval. This tendency seems to evidence that GSTM1 Null genotype promotes a more constant GSTT1.

Looking at the swimmers' performance at the competition, we found that the GSTM1 Null or Present genotype does not affect directly the physical performance of individuals. For instance, the swimmer F1, GSTM1 null, had a great performance at the winter short course National championships, but the other GSTM1 null swimmer, F15, did not participated at that competition. The second best performing swimmer in our sample, the swimmer F14, is GSTM1 Present, and his GSTT1 expression profile was constant, similarly to the profile for GSTM1 Null swimmers. This seems to indicate that training is the main responsible for physical performance, and that this kind of expression analysis can indicate if an athlete is in shape, well prepared and trained, or not. Nevertheless, if GSTT1 relative expression is more stable in GSTM1 null individuals, this can also indicate that those individuals can adapt faster and more efficiently to training physical demands, which would help them achieve better performances.

## 6. Conclusion

This work traces a pilot-model for oxidative post-competition recovery.

Innovation brought by this work, in our point of view, is quite valuable. We evidence a new method to collect and analyse samples for genetic studies. We even evidence that epigenetic studies are possible to carry out from those almost non-invasive sample collection techniques. This is a real breakthrough because it greatly widens the availability of individuals for studies which need blood samples. A whole new protocol had to be implemented to treat those minimal samples and get trustworthy results, and we were able to fulfil this task, evidencing the quality and the meaning of the data collected.

The relations found in the expression of endogenous antioxidant defences, represented by the GSTT1, throughout the season and after a bout of intense exercise, are not statistically significant but gives us a clue about the individualization of recovery from swimmer to swimmer. We verified that high fluctuations of GSTT1 relative expression are not desirable, being associated to lower performance levels. The opposite, on the other hand, seems to be extremely beneficial, meaning that the athlete is well-prepared to face the exigency of an intense exercise, is able to quickly recover of it, and is able to accomplish great performances.

Further investigation on this subject is needed, though, as it can open the gates to new training principles. The size of our sample may not be wide enough for reliable conclusions about the influence of GSTM1 and GSTT1 Null/Present genotypes and expression over the physical performance, but we have found the tools to explore it to a new level. Our next step is to include the Nrf2 expression study.

We are aware of the limitations of the actual study. The power of the test sample is limited due to its small dimension. Besides, the requirements for study eligibility may also be too broad, since Portugal's swimming national level is not so difficult to obtain without the proper genetic background.

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## 8. Annexes

### 8.1. Authorization Chart

Where follows a copy of the free and elucidated consent term given to our athletes. Our sample collection served for other works in progress, and it is in Portuguese because it is the mother language of our sample swimmers.

**ESTUDO:**  
“Alterações fisiológicas e psicológicas decorrentes da competição”

#### **TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Nome:

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#### **1. ESCLARECIMENTO DAS AVALIAÇÕES:**

Está a participar numa pesquisa com o objectivo de estudar as alterações fisiológicas e psicológicas decorrentes do treino e competição. O estudo integra ainda outro objectivo suplementar que pretende analisar a relação entre o perfil dos estados de humor do atleta com a frequência de alguns polimorfismos de genes candidatos e sua expressão. Para o efeito, será submetido a um conjunto de testes, pouco invasivos, em alguns momentos de avaliação (ver quadro resumo na página seguinte).

Nos momentos que compõem o estudo, será necessário para a correcta avaliação hormonal efectuar testes mais do que uma vez por dia, com vista a registar flutuações decorrentes quer do ritmo biológico diário, quer do efeito da carga de competição. Em qualquer momento poderá desligar-se da presente pesquisa, sem qualquer constrangimento. Para que possa decidir sobre a sua participação, descrevemos seguidamente o teste que será conduzido:

#### **Avaliação hormonal:**

A avaliação hormonal será realizada através de uma amostra de saliva, directamente para um tubo *ependorf*. A recolha propriamente dita será realizada pelo participante em 3 períodos do dia (manhã, imediatamente ao levantar e antes do pequeno almoço - 7h30/8h00; final da manhã, antes do almoço (entre 11h00 e as 12h00); tarde, antes do jantar – 17h00 a 19h00) e em todos momentos que compõem o estudo. Cada amostra de saliva será conservada em frigorífico a uma temperatura de 4°C, aguardando os procedimentos seguintes.

#### **Avaliação psicológica:**

A avaliação Psicológica centra-se no estudo das relações humor-desempenho (comparação entre os estados de humor dos atletas antes e depois da competição). Será aplicado o questionário de avaliação do Perfil dos Estados de Humor (POMS), adaptado por Viana, Almeida e Santos (2001) e o Questionário de Stress e Recuperação para Atletas (RESTQ-Sport), validado para a língua portuguesa por Costa e tal. (2005). Ambos os questionários são rápidos de preencher.

#### **Avaliação genética:**

A avaliação genética será realizada através de uma amostra de sangue, retirada através de picada indolor no dedo da mão. Para este procedimento não será necessário realizar qualquer inserção de agulha no

antebraço, uma vez que apenas será recolhida uma quantidade pequena de sangue (mancha). A recolha propriamente dita será realizada através impressão em papel de filtro. Este papel será conservado em frigorífico a uma temperatura de 4°C, aguardando o procedimento de isolamento do ADN genómico. Todos os equipamentos serão esterilizados e/ou descartáveis e o procedimento efectuado por um técnico devidamente treinado.

**Avaliação metabólica:**

A avaliação metabólica será realizada através de uma amostra de sangue retirada através de picada indolor no dedo da mão. A quantificação da creatina quinase (CK) será realizada pelo aparelho *Reflotron*, com recorre às comuns tiras de quantificação de parâmetros bioquímicos. Todos os equipamentos serão esterilizados e/ou descartáveis e o procedimento efectuado por um técnico devidamente treinado.

**Avaliação da performance:**

Esta avaliação corresponde ao registo do melhor tempo de cada nadador para o evento competitivo de especialidade, sendo calculado depois a variação percentual desse registo ao longo do período de treino em estudo. Será usado, sempre que possível, o registo do melhor em competição.

**2. RISCOS E DESCONFORTOS POSSÍVEIS**

Não são esperados riscos nem desconfortos durante a realização das avaliações indicadas.

**3. BENEFÍCIOS ESPERADOS**

Caso o pretendam, todos os participantes neste projecto serão informados sobre os resultados recolhidos em todos os domínios de avaliação que participaram.

**4. RESPONSABILIDADE DO PESQUISADOR E DA INSTITUIÇÃO**

O investigador responsável suspenderá a pesquisa imediatamente ao perceber algum risco ou dano à saúde do participante, mesmo riscos não previstos neste termo de consentimento.

**5. RESPONSABILIDADE DOS PARTICIPANTES**

Os participantes terão de comparecer no local da avaliação, no dias e horário marcados. Qualquer impossibilidade ou desconforto que venha a perceber, deverá ser comunicado ao pesquisador responsável.

**6. RESULTADOS OBJECTIVOS:**

As informações obtidas nesta pesquisa, por meio dos resultados de todas as avaliações, poderão ser utilizadas como dados de pesquisa científica, podendo ser inclusive publicados e divulgados, sendo sempre resguardada a identidade dos indivíduos participantes.

**LIBERDADE DE CONSENTIMENTO**

A sua permissão para participar nesta pesquisa é voluntária. Poderá negá-la ou desistir em qualquer momento, se assim desejar.

Declaro ter lido este termo de consentimento e compreendido os procedimentos nele descritos. Informo também que todas as minhas dúvidas foram respondidas de forma clara e de fácil compreensão. Estou de acordo em participar na presente pesquisa.

\_\_\_\_\_, \_\_\_\_/\_\_\_\_/\_\_\_\_

Assinatura do participante ou encarregado de educação no caso de ser menor

\_\_\_\_\_

**ANEXO A**  
**QUESTIONÁRIO GERAL**

Nome:

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Data de Nascimento:

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Qual é a sua técnica e distância (s) de especialidade?

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Em que país e cidade nasceu?

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Em país os seus pais nasceram?

---

Em que país os seus avós nasceram?

---

Tem o hábito de fumar? Se sim, há quanto tempo?

---

Qual a cor da sua pele (branca, negra, amarela, vermelha)?

---

Sofre de alguma doença (hipertensão, diabetes, etc.)? Se sim, qual?

---

Está actualmente a tomar algum medicamento? Se sim, qual?

---

Consome algum tipo de suplemento ergogénico? Se sim, qual?

---

Serão excluídos de ambas as amostras os indivíduos: medicados com substâncias de impacto conhecido no músculo esquelético, como os corticoides; com algum tipo de restrição para prática física; em condições médicas crónicas, como a diabetes; que consumem mais do que duas bebidas alcoólicas por dia; que usam suplementos ergogénicos indicados para a hipertrofia muscular, como os suplementos proteicos, a creatina e os precursores androgénicos; em restrição calórica e/ou dieta especial.

Referência: