ACTA ABP BIOCHIMICA POLONICA

THE 44th FEBS CONGRESS 6-11 JULY 2019 Krakow, Poland

Vol. 66, No 4/2019
567–576
https://doi.org/10.18388/abp.2019_2880

Regular paper

Effect of glycosaminoglycans accumulation on the non-oxidative sulfur metabolism in mouse model of Sanfilippo syndrome, type B*

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Lack of the N-alpha-acetylglucosaminidase gene is responsible for the occurrence of a rare disease – the Sanfilippo syndrome, type B. The result of this gene knock-out is accumulation of glycosaminoglycans (GAGs) – more specifically heparan sulfate – a sulfate rich macromolecule. The sulfur oxidative pathway is involved in the sulfate groups' turnover in the cells. In contrast, the non-oxidative sulfur pathway leads mostly to formation of sulfane sulfur-containing compounds. The aim of our research was to observe an interaction between MPS IIIB and non-oxidative sulfur metabolism. In this work, we examined selected tissues (livers, kidneys, hearts and spleens) of 3 month old mice with confirmed accumulation of GAGs. The activity and expression of three sulfurtransferases (components of non-oxidative sulfur metabolism): rhodanese, 3-mercaptopyruvate sulfurtransferase and cystathionine γ-lyase was determined, as well as the sulfane sulfur level and the level of other low molecular sulfur-containing compounds (reduced and oxidized glutathione, cysteine and cystine). In all tested tissues, the sulfane sulfur and/or sulfurtransferases' activities, as well as the cysteine content, underwent statistically significant changes. These correlations were also related to the sex of the tested animals. The obtained results indicated that accumulation of incompletely degraded GAGs in the tissues had affected the non-oxidative sulfur metabolism.

Key words: glycosaminoglycans; lysosomal storage disease; mucopolysaccharidosis type III; non-oxidative sulfur metabolism; sulfurtransferases

Received: 15 October, 2019; revised: 02 November, 2019; accepted: 06 November, 2019; available on-line: 05 December, 2019

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***Acknowledgements of Financial Support**:
The costs of the article published as a part of the 44th FEBS Congress Kraków 2019 – From molecules to living systems block are financed by the Ministry of Science and Higher Education of the Republic of Poland (Contract 805/P-DUN/2019).
This work was supported by the Polish National Science Centre [grant number: UMO-2017/01/X/NZ3/00346].

Abbreviations: BPDS, bathophenanthrolinedisulfonic acid disodium salt; CSH, cysteine; CSSC, cystine; CTH, cystathionine γ-lyase; GAG, glycosaminoglycan; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; KO, knock-out; MPS IIIB, Mucopolysaccharidosis type IIIB; MPST, 3-mercaptopyruvate sulfurtransferase; NAGLU, N-alpha-acetylglucosaminidase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PCA, perchloric acid; PCR, polymerase chain reaction; TST, rhodanese; WT, wild type

INTRODUCTION

Mucopolysaccharidosis type IIIB (MPS IIIB), also known as Sanfilippo syndrome B, is an autosomal recessive lysosomal storage disorder caused by a homozygous or compound heterozygous mutation in the gene encoding N-alpha-acetylglucosaminidase (*NAGLU*) (Andrade *et al.,* 2015; Zhao *et al.,* 1996). Sanfilippo syndrome B results from defective degradation and subsequent storage of heparan sulfate that is deposited in lysosomes (Kloska *et al.,* 2011; Andrade *et al.,* 2015). Heparan sulfate, a member of glycosaminoglycans (GAGs) family, is a linear polysaccharide found in all animal tissues (Dreyfuss *et al.,* 2009). This molecule is present on numerous cell surfaces as a proteoglycan or extracellular matrix component (Iozzo, 1998; Strott, 2002). GAGs, like most other biomolecules, are subject to turnover, being both synthesized and degraded. Proper catabolism of GAGs is dependent on a number of lysosomal enzymes, such as sulfotransferases and hydrolases. The same enzyme may be involved in degradation of more than one GAG, but the array of enzymes needed for the complete degradation of any one GAG is unique (Huxtable, 1986; Kloska *et al.,* 2011). Any deficiencies in the ability to synthesize and/or break down GAGs are associated with various metabolic diseases.

MPS IIIB is classified as a rare disease due to its a relatively low incidence. In scientific literature, there are numerous publications containing phenotypic descriptions and clinical features of this syndrome (Harris, 1961; van Schrojenstein-de Valk & van de Kamp, 1987; Yogalingam *et al.,* 2000; Andrade *et al.,* 2015). The molecular mechanism of this disease is known (Zhao *et al.,* 1996; Tessitore *et al.,* 2000; Tanaka *et al.,* 2002; Mangas *et al.,* 2008; Najmabadi *et al.,* 2011). However, there are many aspects where the biochemistry of MPS IIIB is still unclear or even unknown. Therefore, understanding the role of GAGs and their effect on various biochemical processes in normal and pathogenic states seems to be very important. Even more so, because no effective treatment for this disease is currently available (Gaffke *et al.,* 2018), despite several ongoing clinical trials with potential drugs (according to information on the EU Clinical Trials Register website).

Eight GAGs are known in the animal kingdom and most of them contain sulfate or sulfamate residues: chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparin, heparan sulfate, keratan sulfate (Huxtable, 1986). Moreover, all tissue-organized life forms contain sulfonated GAGs, but the abundancy, molecular

weight and type of GAGs are tissue-specific (Gomés & Dietrich, 1982; Prydz, 2015). Since glycosaminoglycans are sulfur compounds, they can be a source of sulfur compounds in an organism. Leaback (Leaback, 1970) estimated that sulfur or sulfate derived from turnover of GAGs in man might reach even 250 mg/day. Therefore, it can be assumed that there is a relationship between GAGs synthesis and/or storage and changes in the activity of some sulfuric enzymes. A number of sulfatases are involved in GAGs metabolism, e.g. induronate sulfate sulfatase and N-acetylglucosamine 6-sulfate sulfatase are involved in the catabolism of heparan sulfate, heparin or keratan sulfate; heparan sulfate N-sulfatase (a sulfuramidase) cleaves the N-sulfonyl group from heparan sulfate and heparin (Huxtable, 1986), but incorporation of the sulfate or sulfamate groups into GAGs is caused by post-polymerization modifications (N-sulfonation, 2-O-sulfonation of iduronate, 6-O-sulfonation of glucosamine) of unsulfonated GAGs (Rodén & Schwartz, 1975; Lindahl, 1976; Riesenfeld *et al.,* 1982). Interestingly, sulfate incorporation into GAGs and the ratio of N- to O-sulfonation are both age-related – they decrease when an organism gets older. A corresponding decrease in sulfotransferase activities is also observed (Rodén & Schwartz, 1975; Lindahl, 1976; Riesenfeld *et al.,* 1982). Sulfotransferases are part of the oxidative pathways of sulfur metabolism. They are responsible for the transfer of a sulfate moiety from the universal donor, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), to various acceptors containing a hydroxyl or an amine group. The changes in oxidative sulfur metabolism are accompanied by occurrence of a mutation in the *Naglu* gene that results in GAGs accumulation. However, the sulfur metabolism is more complex. Besides the oxidation pathway, there is also the non-oxidative pathway of sulfur and GAGs accumulation that could have secondary effects on the disturbances of this pathway. The study presented here aims at investigating whether GAGs accumulation accompanying the Sanfilippo syndrome may indirectly affect the non-oxidative sulfur metabolism and if so, in what way. To date, there has been no scientific research on this subject.

MATERIALS AND METHODS

Animals. The first mouse breeding pairs were donated by Antoni Matilla Dueñas from the Health Sciences Institute Germans Trias i Pujol (Barcelona, Spain). In the study presented here we used the C57BL/6- mice *wild type* strain (WT) and mice with mutation B6.129S6-Naglu^{tm1Efn}/J in the *Naglu* gene (*Naglu*$^{-/-}$) (Li *et al.,* 1999; jax.org). The animals were bred using heterozygotes which provided numerous litters. At the same time, the animals were not used for experiments, which guaranteed their constant accessibility. The animals were bred, kept and sacrificed in order to collect tissues in accordance with the Polish Ethical Law, and the consent of the relevant Ethics Committees (I Local Ethical Committee for Animal Experiments operating at the Jagiellonian University in Krakow, Poland). Selected tissues were collected from thirty one animals of both sexes being at a similar age, i.e. below 3 months. Four research groups were formed for this study: females (WT – 6 mice and *Naglu*$^{-/-}$ – 7 mice) and males (WT – 9 mice and *Naglu*$^{-/-}$ – 9 mice).

Mice genotyping. Genotyping procedure was carried out using the KAPA Mouse Genotyping Kit. In the first step, DNA for polymerase chain reaction (PCR) was isolated. For this purpose, 44 µl of water, 5 µl of 10× KAPA Express Extract Buffer and 1 µl of 1U/µl of KAPA Express Extract Enzyme were added to the 2 mm fragments of the mice tail. The samples were incubated for 10 minutes at 75°C, and then enzymes were inactivated for 5 minutes at 95°C. The samples were centrifuged and the supernatant containing DNA was ten-fold diluted. Subsequently, PCR reaction was carried out. The reaction mixture consisted of 7 µl of water, 10 µl of Fast Genotyping Buffer, 1.5 µl of DNA solution and 0.5 µl of each of the three primers: common forward primer – oIMR5056 (5'GTCGTCTCCTGGTTCTGGAC3'), wild type reverse – oIMR5057 (5'ACCACTTCATTCTGGCCAAT3') and mutant reverse – oIMR8162 (5'TGGATGTGGAATGTGTGCGAG3'). For genotyping, PCR cycling conditions were: 94°C (2 min) for the first cycle, 94°C (20 s), 65°C (15 s), and 68°C (10 s) for the next 10 cycles, and 94°C (15 s), 60°C (15 s), and 72°C (10 s) for further 28 cycles, with a final extension at 72°C (2 min). All of the PCR products were separated by gel electrophoresis on 2.0% agarose gel. The expected product size was as follows: mutant – 270 bp, heterozygote – 250 bp and 270 bp, wild type – 250 bp. Examples of the results are presented in Fig. 1.

Tissue collection. After the mice reached appropriate age, the animals were anesthetized using 2% isofuran, at a flow rate of 0.5 l/min. After successful anesthesia, the spinal cord was mechanically disrupted. The liver, kidney, heart and spleen were collected from each animal. The tissues were washed out in cold saline, weighed and immediately frozen in liquid nitrogen, and kept at –80°C for further use. Before the assays, the tissues were homogenized in four volumes of the appropriate solution (0.1 M phosphate buffer, pH 7.5; 1 mM BPDS/10% PCA or Trizol) and centrifuged. The supernatant was used for determinations of enzyme activities and sulfane sulfur levels, determinations of low molecular weight sulfur-containing compounds using RP-HPLC and studies of gene expression.

Glycosaminoglycans (GAGs) content determination in tissues. Frozen tissues (the liver and kidney) were homogenized in 5 ml of the solution consisting of 0.9% NaCl and 0.2% Triton X-100, and shaken overnight at 4°C, which allowed to completely lyse them. Subsequently, the samples were centrifuged (20 min, 3500 g, 4°C). To 50 µl of the kidney supernatant (20 µl for the liver), 50 µl of 8 M guanidinium chloride was added. The samples were incubated for 15 min at room temperature, and then 50 µl of a mixture containing 0.3% sulfuric acid and 0.75% Triton X-100 was added. The entire volume was shaken and incubated for the next 15 min. In the subsequent step, 750 µl of alcian blue solution (0.05% alcian blue, 1% sulfuric acid, 0.25% Triton X-100, 0.02M guanidine chloride) was added. To obtain a sediment, the samples were incubated for

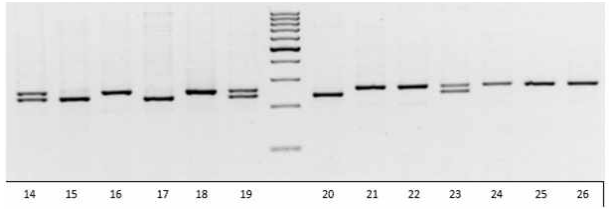

Figure 1. Exemplary gel used to determine the animal genotype.
For example, the animal with number 14 is a heterozygote, 15, WT; 16, *Naglu*$^{-/-}$ mutant, etc.

1 hour at 4°C. Subsequently, the samples were centrifuged (15 min, 12000×*g*, 4°C) and the supernatant solution was thoroughly removed. The pellet was resuspended in 500 µl of the solution consisting of 40% DMSO and 0.05 M magnesium chloride, and vigorously shaken to remove the dye that was not bound with GAGs in the pellet. The mixture was centrifuged (15 min, 12000×*g*, 4°C) to remove DMSO. Then, 500 µl of the solution containing 4 M guanidinium chloride and 33% propanol was added to the pellet to dissolve the precipitate; for this purpose, the mixture was shaken. The dye absorbance was measured at a wavelength equal to 600 nm. The standard curve was prepared in the same way using heparan sulfate instead of tissue homogenate at the concentration range of 2.5–400 µg/ml.

Enzyme assay. The MPST activity was assayed according to the method of Valentine and Frankenfeld (Valentine & Frankenfeld, 1974) with some modifications described by Wróbel and others (Wróbel *et al.,* 2004). The enzyme units were defined as nmoles of pyruvate produced during 1 min incubation at 37°C per 1 mg of protein. The TST activity was assayed by the Sorbo's (Sorbo, 1955) method, following a procedure described by Wróbel and others (Wróbel *et al.,* 2004). The enzyme units were defined as nmoles of SCN^-, which formed during 1 min incubation at 20°C per 1 mg protein. The CTH activity was determined according to Matsuo and Greenberg (Matsuo & Greenberg, 1958) method that was modified by Czubak and others (Czubak *et al.,* 2002). The enzyme activity was expressed as nmoles of α-ketobutyrate produced during 1 min incubation at 37°C per 1 mg of protein.

Protein and sulfane sulfur level determination. Total protein content was determined by the method of Lowry and others (Lowry *et al.,* 1951). The crystalline bovine serum albumin was used as a standard. Sulfane sulfur level was determined by the method of Wood (Wood, 1987). This method is based on cyanolysis reaction and colorimetric detection of ferric thiocyanate complex ion. The level of sulfane sulfur was expressed as nmoles per 1 mg protein.

Low molecular sulfur-containing compounds determination using RP-HPLC. The RP-HPLC (reversed-phase high-performance liquid chromatography) method of Dominic and others (Dominic *et al.,* 2001) with the modifications described by Bronowicka-Adamska and others (Bronowicka-Adamska *et al.,* 2011) was used to determine the level of low molecular sulfur-containing compounds, such as reduced (GSH) and oxidized (GSSG) of glutathione, cysteine and cystine. Standard curves were generated in the supernatant obtained from tissue homogenates in the range from 13 to 75 nmol of each compound per ml.

Isolation of total RNA. Total RNA was extracted from tissues using the Trizol reagent, according to the protocol provided by the manufacturer. Extracted RNA was suspended in ribonuclease free-water and was quantified by measuring the absorbance at 260 nm. After the isolation procedure, purity of obtained RNA was checked. This parameter was determined as the ratio of the absorbance: A_{260} nm/A_{280} nm. The integrity of obtained RNA was also confirmed by separation of the 28S and 18S rRNA bands in agarose-gel electrophoresis. Until further studies, the RNA solutions were stored at –80°C.

Reverse transcription of RNA. Total RNA from a particular tissue was reverse-transcribed using the GoScript™ Reverse Transcriptase Kit according to the manufacturer's instruction. For reverse transcription (RT) reaction, 3 µg of total RNA was mixed with 1 µl Oligo d(T) primer (0.5 µg/µl) and water pretreated with diethylpyrocarbonate (DEPC-H_2O) and incubated for 5 min at 70°C. After preincubation, other components were added to this mixture: 4 µl GoScript™ 5x concentrated reaction buffer, 3 µl $MgCl_2$, 1 µl deoxyribonucleotide triphosphates (dNTPs, 10 mM) and 1 µl RNase inhibitor (20 U/µl) and 1 µl GoScript™ Reverse Transcriptase (160 U/µl) in a total volume of 20 µl. The mixture was first incubated for 5 min at 25°C, then for 60 min at 42°C and finally for 15 min at 70°C. If necessary, the solutions of complementary DNA (cDNA) were stored at –20°C.

Polymerase chain reaction. Expression of four genes (MPST, TST, CTH, GAPDH) was analyzed by PCR. Amplification of cDNA was run in a 25 µl reaction volume that contained the following: 2 µl of synthesized cDNA, 10 µM of each of gene-specific primer pair, 2 U/µl DNA polymerase in 10 mM buffer Tris-HCl, pH 8.8 (supplemented with 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 10 mM of each dNTPs and DEPC-H_2O. In each case, a similar reaction was also performed in the mixture without DNA (negative control) in order to confirm the specificity of the obtained reaction products. For the CTH and TST gene, PCR cycling conditions were 94°C (5 min) for the first cycle, 94°C (30 s), 60°C (30 s) and 72°C (2 min) for the next 28 cycles, with a final extension at 72°C (8 min). The primer sequences were as follows: CTH forward 5'CAGCAAGACCCGATGCAAAG3'; CTH reverse 5'CAAAGCAACACCTGCCACTC3' (304 bp), and TST forward 5'AACCTGGGCATAAGCAACGA3'; TST reverse 5'GGTCCACCTTCTTGTCCTGG3' (460 bp). For the MPST gene, after an initial 5 min denaturation at 94°C, amplification was performed under the following conditions: 94°C (30 s), 63°C (30 s), and 72°C (2 min) for 30 cycles, with a final incubation at 72°C for 10 min. The primer sequences were as follows: forward 5'AGCATTTATGAAGCCCGCCT3' and reverse 5'CCTGGTCACTGTCGTCGTAG3' (420 bp). As the reference gene (an internal standard), GAPDH was used. GAPDH is a gene encoding glyceraldehyde 3-phosphate dehydrogenase. PCR cycling conditions for the GAPDH gene were as follows: 94°C (5 min) – initial denaturation, and the amplification stages: 94°C (30 s), 59°C (30 s), and 72°C (2 min) for the next 28 cycles with a final extension at 72°C (8 min). The primer sequences were as follows: forward 5'GTCCCAGCTTAGGTTCATCAG3' and reverse 5'TTTGGCTCCACCCTTCAAGT3' (404 bp). All mRNA sequences of the tested genes were obtained from the National Center for Biotechnology Information (NCBI) and all of the primers were synthesized by the DNA Sequencing and Synthesis Service - IBB PAN in Warsaw, Poland. PCR conditions for these four genes were established and optimized specifically to meet the needs of the present study; they are published for the first time in this paper. All of the amplification reactions were performed at least 3 times to ensure the accuracy of results. All of the PCR products were analyzed by electrophoresis on a 2.0% agarose gel stained with ethidium bromide and directly visualized under UV light and photographed.

Statistical analysis. All of the presented experiments were repeated at least three times. The data were expressed as arithmetic means with standard deviations (S.D.). Statistical significance of the differences between the appropriate experimental groups and the controls were determined using the Mann-Whitney U-test or the Student's *t*-test. We assumed three levels of statistical

significance. The differences were regarded as statistically significant at the first level when $^{*}p<0.05$, at the second level when $^{**}p<0.01$ and at the third when $^{***}p<0.001$.

RESULTS

Selection of tissues and GAGs accumulation

Based on research previously conducted by Li and others (Li *et al.,* 1999), four tissue samples (the liver, kidney, heart and spleen) were selected for our study. The investigation was performed using the same mouse model of MPS IIIB as Li and others (Li *et al.,* 1999) had described. The accumulation of GAGs was confirmed in the liver and kidney. Data from measurements made with mice used in the experiment are presented in Table 1. The data demonstrated that in the liver and kidney, the accumulation of GAGs took place as it was assumed based on earlier publications (Li *et al.,* 1999). Comparing the values obtained for the *Naglu*$^{-/-}$ and WT mice, the GAGs content was larger by an order of magnitude in each case (Table 1).

The weight of the animals ranged from 20–25 g (without statistical differences between groups), however, an interesting effect concerning the mass of particular tissues was observed. The liver and spleen of the female and male *Naglu*$^{-/-}$ mice had greater mass in comparison with the same tissues obtained from the group of WT animals (Table 2). These differences were statistically significant. Additionally, the appearance of the spleens collected from the *Naglu*$^{-/-}$ mice group was different in comparison with the spleens collected from the WT mice (Fig. S1 in the supplement at https://ojs.ptbioch.edu.pl/index.php/abp/). The *Naglu*$^{-/-}$ spleens were larger and alteration in the red color tone of the tissue was visible – the *Naglu*$^{-/-}$ spleens were darker as compared to the WT spleens. In macroscopic assessment of the state of the organs, such manifestations may be a sign of pathology (Bhaumik *et al.,* 1999), therefore, the spleens were also included in the study. Selection of the heart was associated with the low GAGs accumulation in this tissue as compared to the previously mentioned tissues (only 2–3 fold) according to the relevant literature (Li *et al.,* 1999). Nevertheless, that tissue is also interesting from another point of view, namely the heart is strongly dependent on oxidative metabolism.

Table 1. GAGs accumulation in the selected tissues of both, the WT and *Naglu*$^{-/-}$ animals.
Values represent an arithmetic mean ±S.D. of 8–10 animals.

Experimental group		GAG [mg/g of wet tissue]
Liver		
Female	WT	0.049±0.003
	Naglu$^{-/-}$	0.498±0.096
Male	WT	0.057±0.005
	Naglu$^{-/-}$	0.615±0.251
Kidney		
Female	WT	0.082±0.017
	Naglu$^{-/-}$	1.159±0.252
Male	WT	0.116±0.016
	Naglu$^{-/-}$	1.017±0.044

Table 2. Average tissue weight collected from individual research groups.
Values represent an arithmetic mean ±S.D. of 6–9 animals. *$p<0.05$; (Mann-Whitney U-test)

Experimental group		Average tissue weight [g]
Liver		
Female	WT	1.053±0.112
	Naglu$^{-/-}$	1.384±0.140*
Male	WT	1.246±0.130
	Naglu$^{-/-}$	1.703±0.173*
Kidney		
Female	WT	0.275±0.035
	Naglu$^{-/-}$	0.264±0.038
Male	WT	0.329±0.032
	Naglu$^{-/-}$	0.359±0.067
Heart		
Female	WT	0.132±0.018
	Naglu$^{-/-}$	0.121±0.012
Male	WT	0.146±0.021
	Naglu$^{-/-}$	0.156±0.026
Spleen		
Female	WT	0.103±0.017
	Naglu$^{-/-}$	0.123±0.019*
Male	WT	0.082±0.014
	Naglu$^{-/-}$	0.105±0.019*

The low molecular weight sulfur-containing compounds' level

The results obtained from the RP-HLPC measurements (Table 3) showed that in the *Naglu*$^{-/-}$ mice of both sexes, the level of cysteine had increased almost in all cases: in the liver – by 2 folds (female) and 1.8 fold (male), in the heart – by 1.2 fold (female) and 1.1 fold (male) and in the spleen – by 1.6 fold (male) in comparison with the WT individuals. Only in the female *Naglu*$^{-/-}$ kidneys and spleens did the cysteine level remain unchanged. A contrary result was obtained in the *Naglu*$^{-/-}$ male kidneys, where the cysteine level was decreased (1.2 fold) (Table 3). Additionally, the total cysteine level in the tissue was also decreased in the same way (Table 3). Another type of change was observed in the female *Naglu*$^{-/-}$ mice kidneys, where cysteine and cystine levels, as well as the total cysteine level, were unchanged, except that the cysteine/cystine ratio (CSH/CSSC) was by 1.4 fold lower in comparison with the female WT kidneys (Table 3). In the female *Naglu*$^{-/-}$ kidneys, along with a decrease in the CSH/CSSC ratio, the depletion (1.3 fold) of the reduced to oxidized glutathione GSH/GSSG ratio (Table 3) followed. The GSH/GSSG ratio depletion (1.1 fold) was also observed in the female *Naglu*$^{-/-}$ spleens, but in this case it was caused by a reduction in the level of reduced and oxidized glutathione, as well as the total glutathione level by about 1.3, 1.2 and 1.3 fold, respectively. In the female *Naglu*$^{-/-}$ kidneys, the depletion of the GSH/GSSG ratio was not associated with fluctuations in these parameters (Table 3). Our results (Table 3) indicated that in two cases a GSH/

Table 3. Changes in the level of oxidized and reduced forms of glutathione, cysteine and cystine, total glutathione and total cysteine and the ratio between the reduced and oxidized form of glutathione/cystine in selected tissues of both WT and *Naglu*$^{-/-}$ mice.
Values represent an arithmetic mean ±S.D. of 3–4 animals, with each determination consisting of 1–2 assays. *p<0.05; (Mann-Whitney U-test)

Experimental group		GSH	GSSG	Total glutathione (2GSSG+GSH)	GSH/GSSG	CSH	CSSC	Total cysteine (2CSSC+CSH)	CSH/CSSC
		nmole/mg protein				nmole/mg protein			
					Liver				
Female	WT	56.52±5.43	2.27±0.17	61.06±5.41	24.99±3.15	0.60±0.09	< LOQ	NA	NA
	Naglu$^{-/-}$	62.57±2.09	2.89±0.15*	68.35±1.79*	21.72±1.81	1.25±0.18*	ND	NA	NA
Male	WT	46.25±2.71	2.78±0.35	51.20±2.77	20.71±4.13	0.81±0.05	<LOQ	NA	NA
	Naglu$^{-/-}$	49.89±4.00	2.09±0.17*	54.07±4.35	23.85±0.16	1.42±0.10*	<LOQ	NA	NA
					Kideny				
Female	WT	9.47±0.79	1.06±0.19	11.59±1.03	9.13±1.41	10.09±3.20	1.53±0.29	13.16±3.81	6.30±1.24
	Naglu$^{-/-}$	8.69±3.32	1.24±0.44	11.16±4.20	6.97±0.28*	7.36±0.75	1.68±0,09	10.72±0.87	4.38±0.36*
Male	WT	9.03±2.71	1.07±0.23	11.18±3.17	8.31±0.77	24.50±1.29	2.02±1.04	28.55±1.91	15.09±7.81
	Naglu$^{-/-}$	7.63±0.11	0.97±0.17	9.56±0.24	8.07±1.36*	20.75±0.34*	1.50±0.37	23.57±1.29*	16.18±3.70
					Heart				
Female	WT	6.72±0.43	1.55±0.08	9.83±0.27	4.34±0.50	1.93±0.01	<LOQ	NA	NA
	Naglu$^{-/-}$	9.95±0.32*	1.55±0.12	13.04±0.56*	6.45±0.30*	2.38±0.01*	<LOQ	NA	NA
Male	WT	6.56±1.04	2.44±0.32	11.44±0.40	2.73±0.78	0.87±0.01	<LOQ	NA	NA
	Naglu$^{-/-}$	8.46±0.99	1.86±0.42	12.17±1.29	4.73±1.37	0.95 ±0.02*	0.46±0.12	1.59±0.51	2.16±0.56
					Spleen				
Female	WT	27.52±1.86	3.60±0.03	34.72±1.81	7.65±0.57	1.18±0.13	< LOQ	NA	NA
	Naglu$^{-/-}$	20.11[a]	2.95[a]	26.00[a]	6.82[a]	1.17[a]	< LOQ	NA	NA
Male	WT	15.49±1.15	3.01±0.10	21.50±1.36	5.15±0.21	0.86±0.07	<LOQ	NA	NA
	Naglu$^{-/-}$	17.86±0.63*	3.07±0.04	23.69±0.55*	5.73±0.27*	1.37±0.16*	<LOQ	NA	NA

[a]standard deviation was not calculated because of a low number of results; <LOQ, lower than the limit of quantification of the method; NA, not applied; ND, not detected; The data presented are arithmetic means of 2–8 determinations. The limit of detection for glutathione (GSH) in the RP-HPLC method is equal to 0.01 [nM·ml^{-1}] and for oxidized form of glutathione (GSSG) – 0.1 [nM·ml^{-1}]. The limit of quantification for GSH is 0.1 [nM·ml^{-1}] and GSSG: 1 [nM·ml^{-1}] (Dominic *et al.,* 2001). The limit of detection for cysteine (CSH) was defined by the RP-HPLC method and is equal to 0.01 [nM·ml^{-1}] and for cystine (CSSC) – 0.1 [nM·ml^{-1}]. The limit of quantification for CSH: 0.1 [nM·ml^{-1}] and the CSSC – 1 [nM·ml^{-1}] (Dominic *et al.,* 2001).

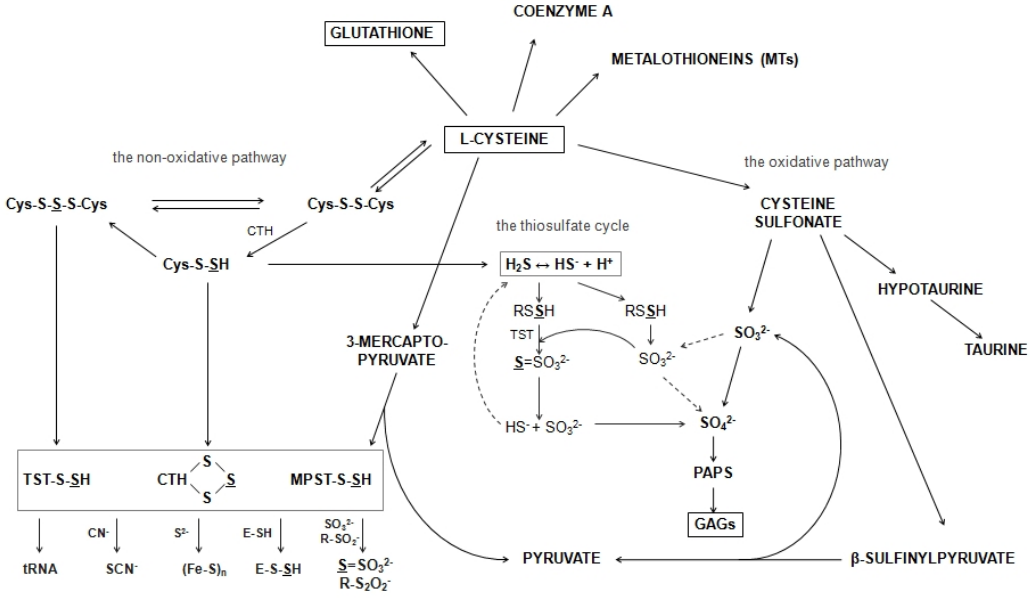

Figure 2. L-cysteine metabolism in mammalian cells (Cys-S-S-S-Cys, thiocystine; Cys-S-S-Cys, cystine; Cys-S-SH, thiocysteine; sulfane sulfur in the picture is underlined and bold).

Table 4. Level of sulfane sulfur and sulfurtransferases activity in selected tissues of both the WT and *Naglu*$^{-/-}$ animals.
Values represent the arithmetic mean ±S.D. of 3–4 animals, which each determination consisting of 4–20 assays. *p<0.05; **p<0.01; ***p<0.001 (Student's t-test). ND – not detected

Experimental group		Sulfane sulfur [nmol/mg protein]	TST [nmol/mg protein·min]	MPST [nmol/mg protein·min]	CTH [nmol/mg protein·min]
			Liver		
Female	WT	120.99±15.98	520.16±47.72	345.37±80.70	3.268±0.524
	Naglu$^{-/-}$	115.91±8.22	548.80±43.20	283.05±73.24	2.026±0.051***
Male	WT	109.79±18.00	433.33±28.91	285.28±112.64	2.945±0.303
	Naglu$^{-/-}$	96.56±7.26*	459.64±58.44	262.14±68.19	1.961±0.098***
			Kideny		
Female	WT	142.15±17.27	182.95±5.60	339.83±24.55	0.919±0.182
	Naglu$^{-/-}$	135.45±12.59	191.03±25.40	258.82±49.46**	1.080±0.143*
Male	WT	145.20±21.24	224.75±30.00	323.56±88.76	0.718±0.114
	Naglu$^{-/-}$	127.07±13.21*	214.65±32.54	244.34±60.73*	0.779±0.124
			Heart		
Female	WT	193.19±30.68	37.61±7.55	78.75±18.73	0.099±0.073
	Naglu$^{-/-}$	199.26±29.93	41.18±3.51	89.93±12.72	ND
Male	WT	204.32±21.57	32.90±4.24	67.56±15.49	0.156±0.032
	Naglu$^{-/-}$	214.23±16.98	38.87±2.33***	69.57±19.45	0.206±0.040
			Spleen		
Female	WT	77.16±5.87	31.65±1.12	52.10±9.93	0.134±0.036
	Naglu$^{-/-}$	67.03±13.50	24.11±2.36***	34.99±13.49	0.237±0.048**
Male	WT	60.39±1.24	13.09±1.27	59.56±6.41	0.041±0.000
	Naglu$^{-/-}$	70.94±10.13*	16.35±0.00**	41.28±7.91**	ND

GSSG ratio increase was observed: in the female *Naglu*$^{-/-}$ hearts (1.5 fold) and male *Naglu*$^{-/-}$ spleens (1.1 fold). This may be a secondary effect of an increase, in both cases, of the reduced form of glutathione and total glutathione level (female *Naglu*$^{-/-}$ hearts: GSH 1.5 fold, total glutathione level – 1.3 fold; male *Naglu*$^{-/-}$ spleens: GSH 1.2 fold, total glutathione level – 1.1 fold) (Table 3). In the female *Naglu*$^{-/-}$ livers, an increased level of the oxidized (1.3 fold) and total glutathione (1.1 fold) could be also observed, but in this case, the changes did not cause any alterations in the GSH/GSSG ratio (Table 3).

The sulfane sulfur level

In the liver, kidney and heart of the WT mice (both female and male), the content of sulfane sulfur was at a similar level, in the range of 110–205 nmol/mg protein (Table 4), and only in the WT spleens was the determined sulfane sulfur level different, amounting to one-half of the above values: 77 nmol/mg protein for the female and 60 nmol/mg protein for the male mice (Table 4). Taking into account sex of the animals, the WT spleens and *Naglu*$^{-/-}$ livers differed significantly in the level of sulfane sulfur – in the males, the level was lower than in the females (Table 4). To make data more clearly arranged, Table 4 has been rearranged to show differences concerning sex in a better way and added in supplemental information as Table S1 (at https://ojs.ptbioch.edu.pl/index.php/abp/). However, a comparison of the WT and *Naglu*$^{-/-}$ groups of mice indicated that only in spleens of the male *Naglu*$^{-/-}$ mice was there a statistically significant (p<0.05) increase in the sulfane sulfur level observed (Table 4). On the other hand, a statistically significant (p<0.05) decrease in the sulfane sulfur level was noted only for the male *Naglu*$^{-/-}$ mice in the liver and kidney (Table 4). The knockout of the *Naglu* gene in the female individuals did not affect the sulfane sulfur level changes. In all of the examined female *Naglu*$^{-/-}$ tissues, the sulfane sulfur content was at the level comparable to the WT group (Table 4). The changes in the level of sulfane sulfur in particular tissues affect the fluctuation of sulfurtransferases' activities, as well as cysteine availability to glutathione synthesis (Fig. 2) – the most important antioxidant produced by the body.

Changes in activity and expression of three enzymes participate in non-oxidative sulfur metabolism in the selected mice tissues

Rhodanese (TST, EC: 2.8.1.1)

Based on the results presented in Table 4, it could be stated that the highest activity of TST was noted in the liver, lower - in the kidney and heart, and the lowest activity was observed in the spleen. Moreover, there were significant differences between the sexes in the values of TST activity in all tested tissues (Table S1 in the supplement at https://ojs.ptbioch.edu.pl/index.php/abp/). In the liver, heart and spleen of the female mice, both WT and Naglu$^{-/-}$, the TST activity was higher in comparison to the activity of the enzyme determined in the male mice (Table S1 in the supplement at https://ojs.ptbioch.edu.pl/index.php/abp/). Only in WT and *Naglu*$^{-/-}$ kidneys were these results divergent – the male mice were characterized by a higher activity of TST than the female (Table S1 in the supplementat at https://ojs.ptbioch.edu.pl/index.php/abp/). Statistically significant differences in the TST activity between the WT and *Naglu*$^{-/-}$ mice were

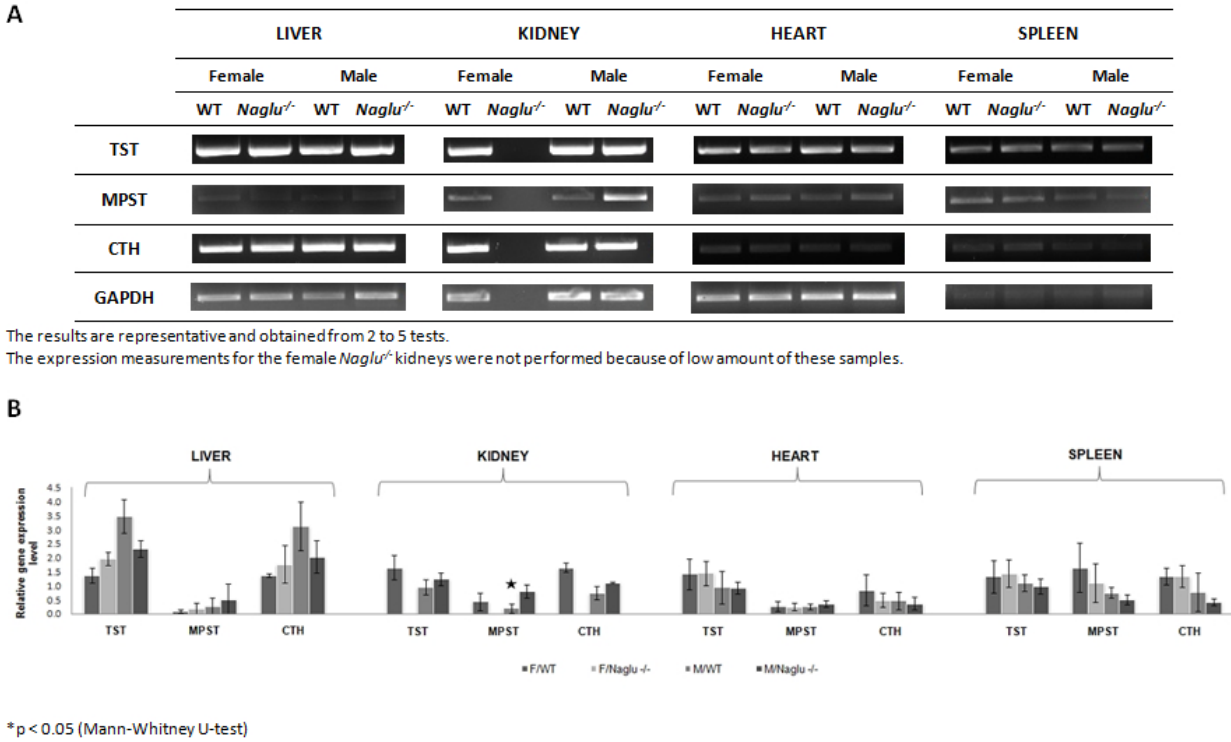

Figure 3. RT-PCR analysis.
(**A**) Genes expression in various tissues of mice. (**B**) The relative expression level of TST, MPST and CTH in selected tissues of mice. Densities of bands were normalized using signal for GAPDH gene.

observed only in the heart and spleen. The TST activity in the male *Naglu*$^{-/-}$ hearts (p<0.001), as well as in the male *Naglu*$^{-/-}$ spleens (p<0.01), were higher in comparison to appropriate WT control group (Table 4). Interestingly, it was observed that in the female *Naglu*$^{-/-}$ spleens the TST activity was decreased and that result was statistically significant (p<0.001). However, the expression measurements (Fig. 3) demonstrated no differences in the TST expression in the tested tissues.

3-mercaptopyruvate sulfurtransferase (MPST, EC: 2.8.1.2)

According to the data collected in Table 4, the highest activity of MPST was shown in the WT kidney and liver, the lowest – in the spleen. There were no major differences associated with the MPST activity in the selected tissues between the female and male mice, except one case – the *Naglu*$^{-/-}$ kidneys – where the TST activity in female mice was higher in comparison to the activity of the enzyme determined in the male individuals (Table S1 in the supplement at https://ojs.ptbioch.edu.pl/index.php/abp/). A statistically significant decrease in the MPST activity was observed in the *Naglu*$^{-/-}$ kidneys, both female (p<0.01) and male (p<0.05), as well as in the male *Naglu*$^{-/-}$ spleens (p<0.01) (Table 4). In other tissues, the MPST activity remained unchanged (Table 4). Taking into consideration the expression measurements illustrated in Fig. 3, it could be noted that there were no significant differences in the expression of the MPST gene in the liver, heart and spleen, but the results obtained for the male kidney indicated that the MPST expression in the *Naglu*$^{-/-}$ mice was higher in comparison to the level of the expression determined in the WT male kidneys (Fig. 3).

Cystathionine γ-lyase (CTH, EC: 4.4.1.1)

Based on the data shown in Table 4, it can be said that the highest CTH activity was detected in the mice liver. In general, the CTH activity in the selected tissues was very low in comparison to the TST or MPST activity (Table 4), therefore, it is difficult to postulate that there was any difference between the females and males, although the results demonstrated a slight decrease in the CTH activity in the male WT and *Naglu*$^{-/-}$ livers and kidneys, as well as the WT spleens (Table S1 in the supplement at https://ojs.ptbioch.edu.pl/index.php/abp/). However, in both the female and male *Naglu*$^{-/-}$ livers, the decrease in the CTH activity was demonstrated (p<0.001). In contrast, in the female *Naglu*$^{-/-}$ kidneys and spleens, the activity of CTH had significantly increased (p<0.05 and p<0.01, respectively) (Table 4). In other tissues, the CTH activity remained unchanged (Table 4). Furthermore, the expression studies (Fig. 3) did not demonstrate any differences in the level of the CTH gene expression in the tested tissues.

DISCUSSION

In our experiments, we used mice that did not exceed 3 months of life. They were healthy and have not shown any pathological and behavioral changes to date, although such changes may become more visible with time. The *Naglu*$^{-/-}$ mice, both female and male, revealed the accumulation of GAGs, more specifically heparan sulfate (Table 1), that are characteristic for some lysosomal storage diseases. These results are in accordance with the results previously obtained by Li and others (Li *et al.,* 1999). The highest accumulation of GAGs occurred in the liver and kidney. Li and others (Li *et al.,*

1999) also observed that the spleen and heart were able to accumulate GAGs, although the GAGs level in these tissues was only a small percentage of the level measured in the liver and kidney. Interestingly, we noted that the liver and spleen collected from the *Naglu*$^{-/-}$ mice had a statistically significant larger mass in comparison to the appropriate WT tissues (Table 2). In macroscopic assessment of the state of the organs, we also observed that the appearance of the spleen (Fig. S1 in the supplement at https://ojs.ptbioch.edu.pl/index.php/abp/) was different, depending on the experimental group the mouse belonged to (*Naglu*$^{-/-}$ or WT group). A similar observation was made by Bhaumik and others (Bhaumik *et al.,* 1999), but they used older mice (aged 9 months). However, Bhaumik and others (Bhaumik *et al.,* 1999) showed that an adult mouse affected by MPS III suffered of liver enlargement and hepatosplenomegaly. The age of the mice that were used by Bhaumik and others (Bhaumik *et al.,* 1999) could reflected the final MPS III stages, in contrast to our animals. From the point of view of the knowledge on MPS, the most important disease stages are the earliest ones (this is correlated with the average life-span of a MPS sufferer) and thus they were the ones we examined.

The deficiency of one enzyme involved in degradation of GAGs is often accompanied by an increase in other enzymes in other enzymatic pathways (Li *et al.,* 1999), but we are not able to state how many other biochemical processes may be disturbed by only one change that causes a pathogenic state. Therefore, in this work, we tried to establish the effect of accumulation of incompletely degraded sulfur-containing compounds (GAGs) on some parameters involved in non-oxidative metabolism of sulfur. According to the results presented in Table 3, it could be said that GAGs accumulation in particular tissues caused an increase in the cysteine level in the majority of cases. Cysteine is a component and/or substrate for a variety of anabolic and catabolic enzymes (Stipanuk, 2004), as well as biologically important factors, such as glutathione, coenzyme A or metallothioneins (Ruttkay-Nedecky *et al.,* 2013). Apart from this observation, in the male *Naglu*$^{-/-}$ liver and heart, the remaining low molecular weight sulfur-containing compounds were unchanged (Table 3). Nevertheless, the increase in cysteine level contributes to an increase in the content of cell reductants, such as glutathione or thioredoxin, that can effectively counteract a locally occurring oxidative stress (Nagahara *et al.,* 2007). Jacquez and others (Jacquez *et al.,* 2016) observed that the disturbance of redox balance could be GAGs- and pro-inflammatory cytokine-related. We documented the redox imbalance in two cases. In the female *Naglu*$^{-/-}$ hearts and male *Naglu*$^{-/-}$ spleens (Table 3), the GSH/GSSG ratio was increased. The GSH/GSSG ratio is the major redox couple that determines the antioxidative capacity of the cells (Wu *et al.,* 2004). The higher the GSH/GSSG ratio value, the more stressors appear in the cell environment. However, based on the results (Table 3), it could be said that GAGs accumulation in the heart (*Naglu*$^{-/-}$, female) and spleen (*Naglu*$^{-/-}$, male) boosts the cysteine production (elevated level) and this cysteine is used to produce or restore the reduced form of glutathione (elevated level). This process seems to be efficient, because the total glutathione level is also elevated in both tissues and despite the unfavorable conditions in the cells (the increased GSH/GSSG ratio), the number of cellular reductants (GSH, CSH) increases. In the female *Naglu*$^{-/-}$ livers, the total glutathione level was also increased (Table 3), but in this particular case it was caused by the increased level of oxidized glutathione. The cysteine residue of GSH can be readily oxidized nonenzymatically to disulfide (GSSG) by e.g. free radicals and reactive oxygen/nitrogen species (Wu *et al.,* 2004), but under our experimental conditions the oxidation of reduced glutathione did not adversely affect the GSH/GSSG ratio (Table 3). Slightly different results – in comparison with the male *Naglu*$^{-/-}$ spleens – were obtained in the female *Naglu*$^{-/-}$ spleens (Table 3), where all of the gutathione-related parameters (GSH, GSSG, total glutathione and GSH/GSSG ratio) were decreased. Despite the cysteine level that remained unchanged, in the female *Naglu*$^{-/-}$ spleens, GAGs accumulation causes some unknown processes that lead to consumption of glutathione resources and a decreased GSH/GSSG ratio (Table 3). Similarly, in the female *Naglu*$^{-/-}$ kidneys, the GSH/GSSG as well as CSH/CSSC ratio had decreased, but these changes were not associated with any disturbances in the reduced and oxidized glutathione content, as well as the level of both cysteine and cystine (Table 3). Only in one case was a decrease in the cysteine level noted (Table 3). That result was obtained in the male *Naglu*$^{-/-}$ kidney. As a consequence, the total cysteine level was also decreased (Table 3). The results are closely related to the results obtained from measurements of sulfane sulfur level and activities of enzymes that participate in formation (CTH mostly) and/or transport of sulfane sulfur atom-containing compounds (TST, MPST) (Table 4) in the non-oxidative sulfur pathway.

Stipanuk (Stipanuk, 2004), Stipanuk and Ueki (Stipanuk & Ueki, 2011) and Wróbel and others (Wróbel *et al.,* 2000) described in detail oxidative and/or non-oxidative metabolic pathways of sulfur, but to better understand the processes described in the paper presented here, Fig. 2 can be also useful. Based on the results presented in Table 4, it could be said that in the male *Naglu*$^{-/-}$ spleens, an increased cysteine level was accompanied by an increased level of sulfane sulfur-containing compounds. It means that cysteine – apart from glutathione synthesis (Table 3) – is also redirected to a non-oxidative pathway, in which sulfane sulfur-containing compounds (Table 4) and hydrogen sulfide can be formed. In the male *Naglu*$^{-/-}$ spleens, the TST activity is increased (Table 4), and TST is responsible for transferring sulfur atoms from various donors (sulfane sulfur-containing compounds) to various acceptors (Nagasawa *et al.,* 2007), while the MPST activity is decreased (only in this case a difference in gene expression was observed – the level of the MPST gene expression increased in relation to the level marked in the control group; Fig. 3). MPST catalyzes transfer of the sulfane sulfur atom from 3-mercaptopyruvate to various acceptors, producing sulfane sulfur-containing compounds (e.g. thiosulfate) and also releases it as hydrogen sulfide (Nandi *et al.,* 2000; Williams *et al.,* 2003; Nagasawa *et al.,* 2007). Possibly, under experimental conditions used here, the reaction catalyzed by MPST had a slower course (occurred at a lower frequency) or GAGs accumulation in the cells contributed to producing other, not yet fully defined chemical compounds and/or substances that would be able to inhibit the enzyme activity by covalent bonds with the redox-active cysteine residue (-SH) located in the active site of the enzyme or by oxidation of its -SH residues to inactive -SOH groups. Changes in the sulfane sulfur level (decrease in that level) were also observed in the male *Naglu*$^{-/-}$ livers and kidneys (Table 4). Additionally, in the kidney, a decrease in the MPST activity was observed (Table 4), while in the liver, the MPST activity remained at the same level as determined in the male WT liver (Table 4). On the other hand, in the liver, a dimin-

ished CTH activity was observed, while in the kidney, it remained unchanged (Table 4). CTH is involved in sulfane sulfur generation in the cells (Toohey, 1989). Decreasing the activity of this enzyme, when it is associated with decreasing the sulfane sulfur level, may be the result of a decrease in the available pool of sulfane sulfur-containing compounds that are substrates for reactions catalyzed by CTH. A drop in the CTH activity was also observed in the female *Naglu*$^{-/-}$ livers, but the reduction was not related to changes in the sulfane sulfur levels (Table 4). On the other hand, it is directly or indirectly related to GAGs accumulation – in the cells, undigested GAGs might contribute to formation of some substances that subsequently might be related to a decrease in the CTH activity (inhibition of the enzyme), however, further studies are necessary. Contrariwise, an increase in the CTH activity was noted in two cases: the female *Naglu*$^{-/-}$ kidneys and spleens (Table 4). Despite the fact that sulfane sulfur remained unchanged in these tissues, the differences in the CTH activity provided us with the information that a turnover of sulfane sulfur-containing compounds occurred in the cells (Table 4). The same information was obtained from the male *Naglu*$^{-/-}$ hearts, where only the TST activity was elevated, but this enzyme participates in turnover of sulfane sulfur-containing compounds (Table 4). It means that cysteine was used in the non-oxidative sulfur pathway, although reductions in the glutathione level and GSH/GSSG were observed (Table 3). With the boosting activity of CTH, a decrease in the MPST activity (female *Naglu*$^{-/-}$ kidneys) and a decrease in the TST activity (female *Naglu*$^{-/-}$ spleens) were observed (Table 4). Decrease in the above activities may be caused by the same factors that have been presented above in the description of the possible MPST inhibition that took place in the male *Naglu*$^{-/-}$ spleens. In the case of the studied enzymes, we did not observe any changes in the levels of their expression (Fig. 3).

Furthermore, the literature provides information addressing the level of a particular sulfurtransferase (TST, MPST, CTH) activity levels in selected tissues, and based on the above data, the sulfurtransferases activities are the highest in the liver and kidney (Aminlari *et al.,* 2002; Nagasawa *et al.,* 2007) – our results (Table 4) are in accordance with these findings.

CONCLUSIONS

Accumulation of incompletely degraded GAGs in particular tissues contributes to increasing cysteine levels in the majority of cases: the *Naglu*$^{-/-}$ livers (both female and male), *Naglu*$^{-/-}$ hearts (both female and male) and *Naglu*$^{-/-}$ male spleens; this may result in changes in non-oxidative sulfur metabolism.

Excess of GAGs in the cells can lead to inhibition of PAPS formation from cysteine. An elevated level of cysteine in the cells can in turn accelerate its non-oxidative metabolism.

In all tissues tested, the sulfane sulfur and/or sulfurtransferases activities (components of non-oxidative sulfur metabolism) underwent statistically significant changes, which can also suggest that GAGs accumulation affects sulfane sulfur-containing compounds level and the level and/or activity of enzymes participating in their turnover.

Changes observed in non-oxidative metabolism of sulfur are related to the sex of the tested animals.

The obtained results confirm the assumed hypothesis and demonstrate that GAGs accumulation affects the non-oxidative sulfur metabolism.

Acknowledgements

The authors would like to thank Antoni Matilla Dueñas from the Health Sciences Institute Germans Trias i Pujol (Barcelona, Spain) for providing the first mouse breeding pairs.

Conflict of interests

The authors declare no conflict of interest.

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