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The Correlations of Levels of Main Thiol Substances of Serum of Adult Healthy and Ill Persons with the Levels of Typical Risk Factors of Cardiovascular Diseases (In Memory of Late Professor Edward Bald (1941-2019)

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Abstract

Introduction: The increase in the concentration of homocysteine in the blood plasma is usually associated with atherosclerosis and all kinds of cardiovascular [and other] diseases. We all know the classic risk factors for atherosclerosis and cardiovascular diseases in general: overweight (obesity), so high BMI, high total and „bad" cholesterol (LDL), and low concentration of „good" HDL cholesterol, triglycerides, glycemia, high systolic and diastolic blood pressure and homocysteine. However there are just a few studies that would explicite describe the possible correlations of the concentrations of homocysteine and other major plasma thiol compounds (cysteine, glutathione, cysteinylglycine) on the one hand, and „classic" risk factors on the other hand. However, recently we have had a real explosion of interest in these low molecular thiols, particularly glutathione, its level and metabolism [especially so called γ-glutamyl cycle]. Then the sexual dimorphism of these processes was discovered.

Objective of the Study: The main objective was to find out what correlations exist, if any, between the levels of above-mentioned thiol substances themselves but most of all the possible correlations of their levels and those of the above-mentioned „classic" risk factors for atherosclerosis and other cardiovascular diseases [and typical for so called metabolic syndrome.

Materials and Methods: 80 patients of the cardiology clinic of the Provincial Hospital John Paul II in Bełchatów [Poland]; including 53 women and 27 men; 45 of them were healthy, i.e. not yet diagnosed as ill, and 35 patients (diagnosed). Blood glucose, total cholesterol, LDL, HDL and triglycerides concentrations were determined using the analytical system Dimension R [Dade Behring].

The concentration of the thiols was determined after their separation by high performance liquid chromatography (HPLC) using ultraviolet detection after the suitable derivatization in the Department of Environmental Chemistry, University of Lodz.

Main Conclusions:

1. There exist but rather seldom significant differences between men and women and between healthy and sick people as far as the levels of both low molecular thiol substances and those of typical risk factors for cardiovascular diseases are compared.

2. However, there are quite a lot of significant correlations between: a. the serum levels of thiol substances themselves, b. thiol substances and typical risk factors, c. typical risk factors themselves.

These correlations, sometimes strong or quite strong, seem to be the matter of whole paper as far as the prognostic values are taken into account.

3. The observed correlations between thiol substances might be explained assuming so called sexual dimorphism of enzymes of both transulfuration and γ-glutamyl cycle ie an indirect activation of cystathionase [CTH] with estradiol and such an inhibition of gamma glutamyltranspeptidase with the male hormones.

4. Above mentioned effects and additionally prooxidative activity of cysteinylglycine explain the observed differences of correlations between thiolos and classical risk factors in men and women.

Keywords: *Low-Molecular Thiols; Transsulfuration-Gamma Glutamyl Cycle; Cardiovascular Diseases' Risk Factors; Antioxidants; Glutathione; Prooxidative Activity of Cysteinylglycine; Sexual Dimorphism*

Background

The increase in the concentration of homocysteine in the blood plasma is usually associated with atherosclerosis and all kinds of cardiovascular diseases in general [1], but also with diseases of the kidneys, nervous system and others. Homocysteine [Hcy] is sometimes called the cholesterol of the 21st century [2]. There are various theories explaining the biotoxicity of (higher concentrations) Hcy; most likely its torch homocysteine thiolactone [HcyTh] modifies various enzymatic and structural proteins [3,4]. High-energy homocysteine anhydride HcyTh is always formed, but especially in the case of hyperhomocysteinemia [3].

In patients with different macro- and microvascular complications, for instance in type 2 (but also 1) diabetes but not only then, an increase in the level of pathologically altered proteins is usually observed, resulting from: non-enzymatic glycation (the so-called Maillard reaction [5] and/or increased activity of free radicals (more precisely, reactive oxygen species [ROS]) [6] and/or homocysteinylation [7].

Protein homocysteinylation occurs both in cells and in extracellular fluids. This process leads to their deformation and inactivation and what is more, to the formation of autoantibodies [3]. Endothelial proteins may be subject to this process, which may be the first cause of atherosclerosis.

The factor that protects against the action of HcyTh is the enzyme homocysteine thiolactonase. Interestingly, this enzyme accompanies one of the proteins of the lipoprotein complex called „good cholesterol" or HDL [3]. Presumably, it is homocysteine thiolactonase that is responsible for the beneficial role of HDL. This enzyme [also known as paraoxonase] has other functions, including removing the lipid peroxides [8] (including those of LDL [9]) - formed under the influence of ROS.

We all know the classic risk factors for atherosclerosis and cardiovascular diseases in general: overweight (obesity), so high BMI, high total and „bad" cholesterol (LDL), and low concentration of „good" HDL cholesterol, triglycerides, glycemia, high systolic and diastolic blood pressure [10]. Homocysteine has been added to this group some time ago [3].

However there are just a few studies that would explicite describe the possible correlations of the concentrations of homocysteine and other major plasma thiol compounds (cysteine, glutathione, cysteinylglycine) on the one hand, and „classic" risk factors on the other hand [13]. However, in the last decade we have had a real explosion of interest in these low molecular weight thiol substances, particularly glutathione, its level and metabolism, and their relationship to various diseases and pathologies [10,11].

Special interest was focused on the level and type of γ-glutamyl transpeptidase [GGT] [12,13], being the enzyme decisive most about the glutathione degradation, i.e. its level. Azarova., *et al*. [14] found that *GGT* gene polymorphisms alone and in combination with those of glutathione synthetase are associated with type 2 diabetes risk regardless of sex, age, and body mass index, as well as correlated with plasma glutathione, hydrogen peroxide, and fasting blood glucose levels. Recently Mangoni., *et al*. [15] found that serum homocysteine, cysteine and glutathione levels are independently associated with cardiovascular risk scores at population level.

One has to remember that all mentioned small molecular weight thiol substances, even homocysteine, but first of all glutathione, are very potent antioxidants [16]. It is noteworthy that homocysteine is the main substrate [more important than cysteinyl glycine] in cysteine biosynthesis [17,18]. Glutathione and cysteinylglycine are the metabolites of so called γ -glutamyl cycle being the system claimed to be responsible, at least to a large extent, for the transport of aminoacids into the cells [19-21].

There is a lot of papers dealing with the above mentioned risk factors of atherosclerosis [10,11,22]. However, the correlations between these factors are seldom presented [21]. Neither the correlations found between men and women nor between ill and healthy persons have been shown.

Recently there is quite a lot of papers on sexual dimorphisms in glutathione metabolism [the review in] and glutathione-dependent signaling, also for a number of human pathologies and diseases such as neurodegeneration, cardiovascular diseases and metabolic disorders By the way many biological processes and systems have shown sexual dimorphisms, including development and aging, mammalian phenotypic traits, social behavior, gut microbiome and the immune system [22,23].

Objectives of the Study

The objectives are following:

1. To determine the content of the main small molecular weight thiol substances (i.e. homocysteine, cysteine, glutathione and cysteinylglycine) in serum of healthy and sick adults (women and men).

2. To determine the content/level of typical risk factors of cardiovascular diseases [listed below] in serum of adults: total cholesterol, LDL ("bad") cholesterol, HDL ("good") cholesterol, triglyceridesas well as glucose [fasting], systolic and diastolic blood pressure and body mass index (BMI) in healthy and sick persons, men and women.

3. To find out what correlations exist, if any, between the levels of above-mentioned thiol substances themselves but most of all the possible correlations of their levels and those of the above-mentioned „classic" risk factors for atherosclerosis and other cardiovascular diseases [and typical for so called metabolic syndrome [10].

Materials and Methods

80 patients of the cardiology clinic of the Provincial Hospital John Paul II in Bełchatów [Poland]; including 53 women and 27 men; 45 of them were healthy, i.e. not yet diagnosed as ill, and 35 patients (diagnosed). All patients were weighed and measured and BMI was

calculated; systolic and diastolic blood pressure were measured twice. Blood samples were drawn in an empty stomach and serum was obtained immediately.

Blood glucose, total cholesterol, LDL, HDL and triglycerides concentrations were determined in the laboratory of the Diagnostics Laboratory of the Provincial Hospital of John Paul II in Bełchatów using the analytical system Dimension R [Dade Behring].

After 45 minutes (in dry ice), serum samples were transferred to -80°C in the laboratory of the Department of Environmental Chemistry, University of Lodz. The concentration of the thiols was determined [24] after their separation by high performance liquid chromatography (HPLC) using: 1) ultraviolet (UV) detection on a Hewlett-Packard instrument; 2) reduction (S-S to SH) with tri-n-butylphosphine; 3) deproteinization with perchloric acid ($HCLO_4$) and 4) derivatization with 2-chloro-1-methylpyridinium iodide.

Determination of thiols was performed in [unfortunately only] 68 patients-48 women and 20 men, including 38 healthy [essentially] and 30 ill persons [with a diagnosed condition].

After about half a year 62 new patients were examined in the general outpatient clinic in Rząśnia [Poland] exactly as above [unfortunately only classical risk factors not the thiols' levels]. So finally, 142 patients (43 men and 99 women; 94 of them healthy and 48 definitely ill) were examined but only on level of above mentioned classical risk factors, so the correlations between them.

The results were analyzed statistically. So, the t Student test was performed after analysis of homogeneity of variances with test F [35]. Statistical significance was for $p < 0,05$; but in case $0,05 < p < 0,1$ it was considered as "border of statistic significance [B]. We checked if there exist linear Pearson correlations; so we calculated values of correlation coefficients $r_{X/Y}$ and checked if they are significant [25,26].

Results

The results are shown on four table 1-4. Only one figure (Figure 1) just describes thiol metabolism (γ-glutamyl cycle).

No	Level of	Unit	Mean value X (standard deviation s)		X_M/X_W x 100% [ev. stat. signif.]	Mean value x (standard deviation s)		$X_{I/}X_H$ x 100% [ev. stat. si-gnif.]
			Women (W) [n = 48]	Men (M) [n = 20]		Healthy (H) [n = 38]	Ill (I) [n = 30]	
1	Homocysteine (Hcy)		9,49 (1,90)	11,0 (4,26)	115,9	9,24 (2,24)	10,52 (5,12)	119,6
2	Glutathione (G)		6,90 (2,45)	6,49 (1,65)	94,1	6,62 (1,23)	7,05 (2,87)	106,5
3	Cysteine (C)		194,4 (35,3)	191,2 (40,3)	98,4	191,8 (35,1)	193 (38,7)	100,6
4	Cysteinyl-glycine (CG)		28,5 (5,93)	29,9 (6,25)	104,9	30,5 (6,16)	27,4 (10,24)	89,8 [B]
5	Hcy/G	------	1,57 (0,51)	1,90 (0,69)	121,0	1,52 (0,42)	1,81 (0,98)	119,1 [B]
6	Hcy/C	------	0,050 (0,013)	0,059 (0,024)	118,0 [B]	0,050 (0,015)	0,054 (0,029)	108,0
7	Hcy/CG	------	0,344 (0,088)	0,377 (0,143)	109,6	0,342 (0,111)	0,368 (0,204)	107,6
8	G/C	-------	0,034 (0,011)	0,033 (0,011)	97,1	0,032 (0,011)	0,036 (0,023)	112,5
9	G/CG	-------	0,234 (0,072)	0,204 (0,051)	87,2	0,216 (0,066)	0,237 (0,139)	109,7
10	CG/C	-------	0,149 (0,033)	0,163 (0,046)	109,4 [B]	0,154 (0,041)	0,146 (0,083)	94,8

Table 1: *Levels of main thiol substances in serum for A. women and men and B. healthy and ill people.*
[B] means that 0,05 < p < 0,1 ["border of statistical significance"]
[*] " " " " " " 0,01 < p < 0,05 [statistical significance].

Sick people (Table 1) have [on the "borderline of significance"] in serum 10,2% lower concentration of cysteinylglycine [CG] [and higher by 19,1% ratio of concentration homocysteine [Hcy] to glutathione [G].

Men (Table 1) have higher [on the "borderline of significance"] serum ratio of Hcy to cysteine [C] by 18% and CG by 9,4%.

No	Level of:	Unit	Mean value X (standard deviation s)		X_M/X_W x 100% [ev. stat. . signif.]	Mean value X (standard deviation s)		X_I/X_H x 100% [ev. stat. si-gnif.]
			Women (W) [n = 53]	Men (M) [n = 27]		Healthy (H) [n = 45]	Ill (I) [n = 35]	
1.	BMI		26,6 (6,19)	25,8 (4,32)	97,0	24,6 (3,62)	27,6 (5,67)	112,2 [*]
2.	Systolic pressure (Sypr)		113,8 (12,9)	121,9 (14,3)	107,1 [B]	114,2 (11,0)	120,7 (16,3)	105,7 [B]
3.	Diastolic pressure (Diapr)		69,4 (10,3)	77,1 (8,30)	111,1 [*]	71,7 (8,3)	74,3 (9,9)	103,6
4.	Total cholesterol (TCh)		188,6 (28,5)	195,5 (36,3)	103,7	198,8 (36,3)	187,6 (31,1)	94,4
5.	HDL cholesterol (Ch- HDL)		53,2 (13,1)	60,5 (9,29)	113,7 [*]	52,4 (11,6)	50,7 (13,1)	96,8
6.	LDL cholesterol (Ch- LDL)		113,0 (30,2)	123,0 (32,9)	108,8 [B]	125,2 (35,0)	114,1 (28,1)	91,1
7.	Triglyce-rides (TG)		107,7 (64,6)	101,3 (51,6)	94,1	88,4 (32,7)	119,9 (25,7)	135,6 [***]
8.	Glucose (Gl)		88,0 (12,9)	87,5 (11,0)	99,4	85,1 (8,2)	90,5 (13,9)	106,3 [B]

Table 2: *Levels of typical risk factors of cardiovascular diseases in serum for A. women and men and B. healthy and ill people.*
[B] means that 0,05 < p < 0,1 ["border of statistical significance"].
[*] " " " " " " 0,01 < p < 0,05 [statistical significance].
[***] " " " " " " p < 0,001 [very high statistical significance].

Sick persons (Table 2) have significantly higher contents of triglycerides [TG] then healthy ones by 35,6% [p < 0,001] and BMI by 12,2% [0,01 < p < 0,05]. Sick people (Table 2) have [on the "borderline of significance"] in serum higher contents of fasting glucose and systolic pressure.

Men (Table 2) have significantly higher contents of HDL cholesterol [Ch-HDL] [by 13,7%; 0, 1 < p < 0,5] and systolic pressure [by 11%; 0,01 < p < 0,05]. Men (Table 2) have higher [on the "borderline of significance"] systolic pressure and contents of LDL cholesterol.

A. Correlations between serum thiols [X/Y] themselves					
No	X versus Y	Correlation coefficient $r_{X/Y}$			Interpretation
		All group (n = 68)	Women only (n = 48)	Men only (n = 20)	
1.	Homocysteine/glutathione [Hcy/G]	0,30***	0,32***	---------	Only for women
2.	Homocysteine/cysteine [Hcy/C]	0,18	- 0,04	0,56!	Only for men-[quite strong corr.]
3.	Homocysteine/cysteinyl-glycine Hcy/ CG	0,26*	0,11	0,30**	Only for men
4.	Glutathione/cysteinę [G/C]	0,23*	0,22*	----------	Only for women
5.	Glutathione/cysteinylglycine [G/CG]	0,43***	0,47***	----------	Only for women-[quite strong corr.]
6.	Cysteine/cysteinylglycine [C/CG]	0,18	0,18	----------	Only for women If any?
B. Correlations between serum thiols [X] and typical risk factors [Z] of cardiovascular diseases					
1.	Total cholesterol/Hcy	0,14	0,13	----------	Only for women If any?
2.	Total cholesterol/CG	-0,08	-0,15	----------	Only for women If any?
3.	LDL cholesterol/Hcy	0,16	0,17	----------	Only for women
4.	LDL cholesterol/CG	-0,04	-0,13	----------	Only for women If any?
5.	HDL cholesterol/G	0,00	0,28*	- 0,15	Contradiction of correlations for wo-men [positive] and men [negative]

6.	HDL cholesterol/C	-0,17	0,09	-0,36**	Only for men [negative]
7.	HDL cholesterol/CG	0,03	0,21*	- 0,19	Contradiction of correlations for women [positive] and men [negative]
8.	Triglycerides/G	0,20*	0,19	0,01	Only for women
9.	Glucose/G	-0,29*	-0,30**	0,02	Only for women [negative]
10.	Systolic pressure/G	-0,21*	-0. 19	----------	Only for women [negative]
11.	Systolic pressure/CG	----------	- 0,18	----------	Only for women [negative]
12.	Diastolic pressure/CG	0,11	-0,12	0,50!	Only for men [quite strong corr.] or –less probably- contradiction of correlations for women [positive] and men [negative]
13.	BMI/G	-0,21*	-0,20*	---------	
14.	BMI/C	0,13	0,18	----------	

Table 3: *Linear Pearson correlations between: A. serum thiols themselves and B. serum thiols and typical risk factors of cardiovascular diseases.*

------ Means that $/r_{X/Y}/ < 0,08$ *[if such values were for all people tested, for men and women separately either,such a story was not shown in the table].*

In some cases values of $/r_{X/Y}/ < 0,08$ *were shown for comparison.*

Values of r > 0,22 mean that for certain such a correlation is statistically significant [0,01 < p< 0,05]; they are marked as bold. Values of 0,017 < /r/ < 0,2 are marked as yellow.

$/r_{X/Y}/ > 0,50$ *are marked with !.*

$0,4 < /r_{X/Y}/ < 0,50$ *are marked with***.*

$0,3 < /r_{X/Y}/ < 0,40$ *are marked with**.*

$0,2 < /r_{X/Y}/ < 0,3$ *are marked with*.*

Significant correlations (Table 3-part A] -only positive] were found:

1. Only for women G/CG [Strong- see figure 1], G/C, Hcy/G [quite strong] and C/CG.
2. Only for men between Hcy and C [strong] and Hcy/CG.

Only for women exist positive correlations (Table 3-part B] between: LDL cholesterol/Hcy, total cholesterol/Hcy, Ch-HDL/G and Ch-HDL/CG, TG/G and BMI/C. Negative correlations exist between both total and LDL cholesterol and CG, both fasting glucose [quite strong]] and systolic pressure [Sypr] with G, diastolic pressure [Diapr] with CG and BMI with G.

Correlations existing only for men are more seldom than for women [6 versus 13 on 32 all possible correlations; 4 are significant for men whereas 6 for women]. They are mostly weak except two cases 1] between Diapr and CG [positive, strong] and 2] Ch- HDL with C [negative, quite strong].

In two cases there is the contradiction as the correlation for women is negative whereas for men is positive. Such a situation takes place between Ch-HDL and G and Ch-HDL/CG.

There were either practically no correlation or at least very weak ones between thiol substances themselves and between thiols and typical risk factors when correlations were evaluated separately for group of a. healthy and b. ill persons.

Correlations exclusively between different typical risk factors of cardiovascular diseases [without homocysteine] were evaluated on quite big group of people, both sexes and both healthy and ill. They are presented in table 4.

No	Z versus W	Correlation coefficient $r_{z/w}$		Interpretation	Correlation coefficient $r_{z/w}$		Interpretation
		Healthy people (n = 94)	Ill People (n = 48)		Women (n = 99)	Men (n = 43)	
1.	TG/Gl	0, 339**	0, 238*	Ilness weakens correlation	0, 414***	0, 181	Corr. for women stronger
2.	Ch-LDL/Gl	0, 204*	0, 158		----------	----------	
3.	Ch-LDL/TG	- 0, 189	0, 175	Contradic-tion of corr. for healthy [negative] and Ill people [positive]	0, 021	0, 334**	Only for men
4.	Ch-HDL/Gl	- 0, 162	- 0, 209*		----------	----------	Lack of correlation
5.	Ch-HDL/TG	- 0, 243*	-0, 382**	Ilness strengthens correlation	- 0, 280*	0, 057	Only for women
6.	TCh/Gl	0, 333**	0, 130	Ilness weakens correlation	0, 237*	0, 370**	Corr. for men stronger
7.	TCh/TG	0, 315**	0, 200*	Ilness weakens correlation	0, 257*	0, 365**	Corr. for men stronger
8.	TCh/Ch-LDL&	0, 813!!	0, 940!!	Super strong correlation	0, 907!!	0, 679!!	Super strong correlation
9.	TCh/Ch-HDL	0, 152	0, 120		0, 131	0, 108	
10.	DiaPr/Gl	0, 165	0, 169		0, 197	0, 308**	Corr. for men stronger
11.	DiaPr/TG	0, 182	0, 227*		0, 295*	0, 020	Only for women
12.	DiaPr/Ch-LDL	0, 198*	0, 314**	Ilness strengthens correlation	0, 226*	0, 483***	Corr. for men stronger
13.	DiaPr/Ch-HDL	-0, 302**	-0, 085	Ilness weakens correlation	- 0, 213*	- 0, 012	Only for women
14.	DiaPr/TCh	0, 183	0, 349**	Ilness strengthens correlation	0, 245*	0, 452***	Corr. for men stronger
15.	SyPr/Gl	0, 219*	0, 278*		---------	----------	Lack of correlation
16.	SyPr/TG	0, 132	0, 334**	Ilness strengthens correlation	0, 294*	0, 030	Only for women
17.	SyPr/Ch-LDL	0, 231*	0, 508!	Ilness strengthens correlation	0, 143	0, 502!	Corr. for men stronger
18.	SyPr/Ch-HDL	- 0, 245*	- 0, 044	Ilness weakens correlation	- 0, 154	0, 099	? Lack of correlation
19.	SyPr/TCh	0, 218*	0, 534!	Ilness strengthens correlation	0, 168	0, 447***	Corr. for men stronger
20.	BMI/Gl	0, 232*	0, 294*		0, 274*	0, 280*	
21.	BMI/TG	0, 218*	0, 451***	Ilness strengthens correlation	----------	----------	Lack of correlation
22.	BMI/ Ch-LDL	0, 053	0, 228*	Ilness strengthens correlation	----------	----------	Lack of correlation
23.	BMI/ Ch-HDL	- 0, 261*	- 0, 267*		----------	----------	Lack of correlation

Table 4: *Linear Pearson correlations between typical risk factors [Z/W] of cardiovascular diseases-themselves [additional studies].*

------ Means that / $r_{X/Y}$ / < 0, 06 [if such values were for all people tested, for men and women separately either, such a story was not shown in the table].

In some cases values of / $r_{X/Y}$ /< 0, 06 were shown for comparison.

Values of r > 0, 14 mean that for certain such a correlation is statistically significant [0, 01 < p< 0, 05]; they are marked as bold. This limit value is lower than that in table 3 [seeexplanations under table 3]ie 0, 22 because N=n_1+ n_2Is much bigger [142, not 60]. Negative correlations marked yellow.

/ $r_{X/Y}$ / > 0, 50 are marked with!.

*0, 4 </ $r_{X/Y}$ /< 0, 50 are marked with***.*

*0, 3 </ $r_{X/Y}$ / < 0, 40 are marked with**.*

0, 2 </ $r_{X/Y}$ /< 0, 3 are marked with.*

& there is obvious almost full very high correlation; only one similar is for diastolic versus systolic pressure.

Such correlations exist both in group of healthy and ill persons. There is 23 of them on 28 all possible correlations. Quite often an illness seems to strengthen the correlation [rise of value of $r_{X/Y}$] as the following [only positive]: Diapr/Ch- LDL, Diapr/TCh, Sypr/TG, Sypr/Ch-LDL, Sypr/TCh, BMI/TG, BMI/Ch-LDL and only one negative: Ch-HDL/TG.

More seldom are the cases when it seems that an illness weakens the correlation [decrease of $r_{X/Y}$]: positive for TG/Gl, TCh/TG, TCh/Gl and negative for Diapr/Ch-HDL and Sypr. Ch-HDL.

Correlations only for women (Table 4-right side] might be observed for Diapr/TG and Sypr/TG-both positive and Ch-HDL/TG, Diapr/Ch-HDL-both negative. Only in case TG/Gl correlation is more explicit for women than in men group.

Correlations for men are usually stronger than for women (Table 4-right side] for TCh/Gl, Tch/TG, Diapr/Gl, Diapr/Ch-LDL, Diapr/TCh, Sypr/Ch-LDL and Sypr/TCh. But only in case Ch-LDL/TG correlation it looks as it exists only for men. It is noteworthy that all these correlations for men are positive and are strong or at least quite strong. Just take in mind that according the formula [26] shown below:

$$t = r\sqrt{\frac{n-2}{1-r^2}}$$

Even for such a low value of r [$r_{X/Y}$] as 0,14 values of t [Student's test coefficient] are so high, that such a correlation is statistically significant [all because relatively big values of n = 140 [not 66 as for correlations shown in table 3]. So, for r > 0,2 let alone r > 0,3 such correlations are strong.

However generally there is less sex dependent correlations than those depending on state of health.

Discussion

There are-in our group of people- very few differences between the serum parameters tested, and if they are, they are relatively low. This applies to the differences between women and men as well as those between sick and healthy people. However, there are much more correlations between the parameters studied, and a number of them are quite strong.

This applies to linear Pearson correlations between 1. the levels of low molecular weight thiol substances themselves [6 possibilities, because 4 thiols]; 2. between the levels of these thiols and those of typical risk factors for cardiovascular diseases - there are 32 possible correlations [because there are 4 thiols and 8 analyzed risk factors]; 3. between various common risk factors themselves [28 possible correlations].

Of course, the size of the surveyed group is small, this is especially true of the number of surveyed men [this is due to the fact that evidently women seem to "care more about their health" and go to the doctor more often and therefore they dominate among clinic patients/clients]. Moreover [as concerned tests are relatively expensive and require special methods and devices], the content of thiol substances was determined just in a small number of people [only 68, including only 20 men].

We have to note that among people qualified as healthy there may be some people who are already sick - simply so called healthy people were people who have not been diagnosed [yet?] with any disease. On the other hand, people - classified as sick, i.e. with diagnosed disease - were not in the advanced stage of the disease.

Therefore, it is not surprising that among ill persons there were not many clear changes in parameters evaluated. However, when the total number of examined patients about doubled, there were much more significant differences between the group of healthy and ill people [not shown, however, this applies to the correlations shown in table 4]. And thus significant differences "appeared" for diastolic

blood pressure, total and LDL cholesterol [while the significance of the difference in triglyceride levels decreased slightly]. As our many years of experience show, usually extremely seldom the correlations found with a relatively small number of respondents "disappear" when tested on a larger group.

Therefore, we consider the correlations, and not just the mean values, to be the most important results of our research. We believe that this gives a greater prognostic value for many of the parameters studied, especially at their rather low and high levels.

A slightly similar role as correlations is played by the [mean] values of the ratios of certain parameters' levels. And here it is necessary to recall [as also related to our present research] the recent work of Feng., *et al* [13]. They suggest that ratio of γ -glutamyl transferase [GGT] activity to the content of HDL cholesterol [GGT/Ch-HDL] can be used as a predictive factor for prevalence of nonalcoholic fatty liver after adjustment for confounding variables, but its increase is observed in a number of other medical conditions. Note that increased GGT activity can lead to either a marked decrease in the level of G or an increase in the level of CG [or both]. This can be seen from the analysis of the reaction pattern of the so-called gamma γ-glutamyl cycle-Meister cycle, about which see the next text.

We should always remember that for any two correlated variables, A and B, the following relationships are possible: 1) A causes B or vice versa (direct causation); 2) A causes C which causes B or the other way round (indirect causation); 3) A and B are consequences of a common cause but do not cause each other and finally 4) the correlation is just coincidence [27].

Before we proceed to the interpretation of the obtained correlations, mainly those between the levels of various thiols in the serum, let us emphasize the role of glutathione and its formation and degradation [to the extent that allows us to interpret the correlations found by us].

Glutathione (GSH) [note that in our text it was marked simply as G] is a tripeptide, γ-L-glutamyl-L-cysteinyl-glycine [28], and the most abundant soluble thiol antioxidant and low molecular weight peptide in cells [28]. G is critical for the maintenance of redox homeostasis of cells and tissues and is intimately involved in the regulation of redox signaling pathways and detoxification reactions [20,28].

As an antioxidant, glutathione reduces/eliminates ROS in enzymatic and non-enzymatic reactions; regenerates other oxidized, low molecular weight antioxidants, such as like vitamin C and vitamin E; is involved in the repair of protein, nucleic acid and lipid molecules damaged in the peroxidation process and maintains protein thiol groups in a reduced state.

In addition, glutathione is involved in the folding and ubiquitinylation of proteins, detoxification of xenobiotics, the synthesis of cysteinyl leukotrienes and the formation of deoxyribonucleotides from ribonucleotides. Glutathione also plays an important role in regulating the activity of metabolic pathways and the processes of cell growth and differentiation [29,30].

Besides glutathione is storing cysteine - due to the high demand in cysteine in the body, GSH acts as a continuous source and transporter of cysteine throughout the body [20,29].

Glutathione is the most important cell thiol buffer. The ratio R (of the concentrations of the reduced form to the oxidized form of glutathione [GSH]/[GSSG]) under physiological conditions is 300 - 400, but during strong oxidative stress it may drop even to 2 [31].

G concentrations range from 0. 1 mM to 10 mM in the cytosol of cells, with around 1 - 2 mM in most cell types [20]. In hepatocytes, G concentrations can reach 10 mM as the liver is the major producer and exporter of GSH [21].

A typical eukaryotic cell is dominated by the reduced form of G (GSH), with the oxidized form (GSSG) accounting for less than 1% of the total pool. Extracellular glutathione concentrations, with the exception of bile, which contains glutathione even at a concentration of 10

mM, are much lower (e.g. in blood plasma the concentration of glutathione is less than 20 μM) and the oxidized form is the dominant [21].

Cellular G concentrations are controlled exclusively by GSH synthesis as cells cannot import G [32]. G synthesis requires glutamate, cysteine and glycine and occurs via two steps: 1] the formation of the dipeptide γ-glutamyl-cysteine from glutamate and cysteine via glutamate-cysteine ligase (GCL, the ATP-requiring and rate-limiting step of G synthesis), and 2] the subsequent addition of glycine via GSH synthetase (GS) [29,32].

The γ-glutamyl cycle (Figure 1) comprises the enzymatic reactions involved in the extracellular glutathione degradation and the intracellular GSH synthesis: The enzymes γ-glutamyl transpeptidase and dipeptidase, localized in plasma membrane of cells [in spite dipeptidase might be inextracellular fluids as well], cleave the extracellular GSH to their constituent amino acids. Within the cells the GSH is synthesized de novo by two reactions, that consume ATP, that are catalyzed by GCL and glutathione synthetase, sequentially. In the maintenance of the cellular redox state participate also the enzymes glutathione peroxidase and glutathione reductase [20].

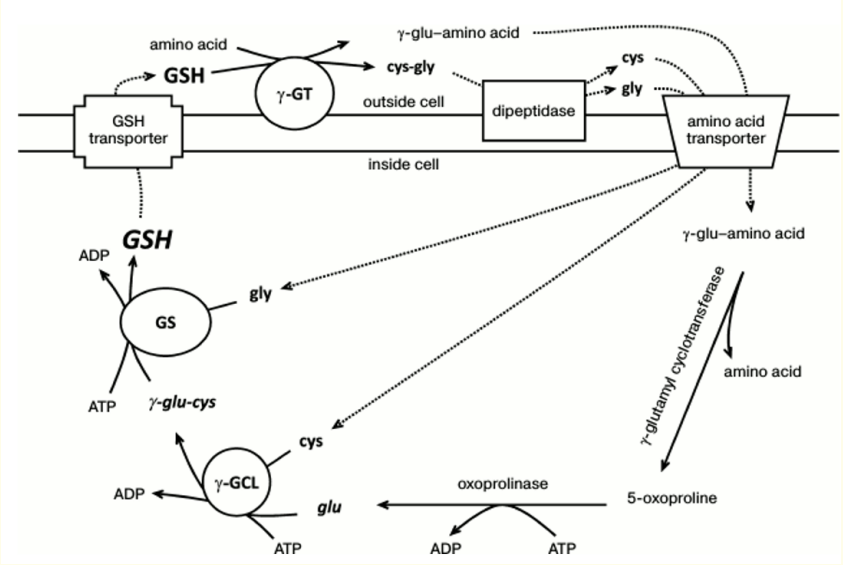

Figure 1: *γ-Glutamyl cycle of glutathione synthesis and degradation and its localization. γ-GCL, γ-glutamyl cysteine ligase; GS, glutathione synthetase; γ-GT, γ-glutamyl transferase.*

Kannan., *et al*. [33] already in 1996 discovered a hitherto unreported Na (+) -dependent, GSH transporter [inhibited with bromosulphophtaleine [BSP] in the lens epithelium. Transport of glutathione from the cells outside might, take place with oxidized form of glutathione ie GSSG.

We want to emphasize that the thiol substances determined by us in the serum do not come from the outflow from damaged cells. Either as glutathione are transported to the extracellular space by special transporters, or they are formed in this space like cysteinylglycine or they come mainly from this space like cysteine.

But what about cysteine origin? It comes mostly from cystine of blood plasma [CySSCys] coming first of all from liver where it is formed from homocysteine in reaction of so called transsulphuration. It does not exclude formation quite a big part of cysteine from glutathione.

Cystathionine-β-synthase [CBS] catalyzes the first step of the transsulfuration pathway [17], from homocysteine to cystathionine: L-serine + L-homocysteine →{\displaystyle \rightleftharpoons } L-cystathionine + H_2O.

CBS uses the cofactor pyridoxal-phosphate (PLP) and can be allosterically regulated by effectors such as the ubiquitous cofactor S-adenosyl-L-methionine (AdoMet) [24].

In the second step of transsulfuration cystathione gamma lyase [CTH] converts cystathionine to cysteine [18]: Cystathionine + H_2O → NH_4^++ α-ketobutyrate + cysteine.

One of the alternate reactions involving CBS is the condensation of cysteine with homocysteine to form cystathionine and hydrogen sulfide (H_2S) [24]. H_2S in the brain is produced from L-cysteine by CBS. This alternative metabolic pathway is also dependent on AdoMet [18]. However, H_2S is formed first of all not with CBS but with CTH and what is more both from cysteine and homocysteine [18].

CBS is regulated at the transcriptional level by transciption factors NF-Y, SP-1, and SP-3 and downregulated by insulin. Methionine upregulates CBS at the post-transcriptional level [24].

γ-Cystathionase (CTH, EC: 4.4.1.1), also withPLP, an enzyme widely distributed in the eukaryotic organisms plays a pivotal role in the L-cysteine desulfuration pathway. A decrease of the expression of CTH entails a drop in the level of C and G and hydrogen sulfide (H_2S) in the cells [18] H_2S, endogenously formed by CTH, affects the vasodilation and regulation of blood pressure. H_2S plays a role in protection of neurons against oxidative stress, and stimulates γ-glutamylcysteine synthetase [GCL] and thereby an increase in the level of G [i.e. GSH].

So finally when we have taken into consideration both transsulfuration and γ–glutamyl cycle as one entity we can see that the key stages deciding on redox metabolism [and not only that] consist in activity of three enzymes. They are following: 1) CTH [forming C and H2S as well] -from the transsulfuration route 2) GCL [the key step in formation of glutathione-! not glutathione synthetase itself] and 3) γ-glutamyl transferase (γ-glutamyl transpeptidase-GGT) [deciding on glutathione degradation]; both 2) and 3) enzymes are parts of γ-glutamyl cycle.

Women have lower blood and plasma levels of Hcy because they have higher betaine levels in tissues (because estrogen upregulates their biosynthesis) and betaine activates CBS [24,26].

In uterine artery endothelial cells, CBS mRNA and protein levels increase upon treatment with estriadiol-17β [17]. Recently Lechuga., *et al*. [34] found that $E_2β$ stimulates H_2S production by upregulating CBS and CTH mRNA and protein expressions through specific estrogene receptors *CBS* and *CTH* transcription in ovine uterine artery endothelial cell *in vitro* [compare also [35]].

In the liver of male C57BL/6 mice, GCL protein levels are downregulated with age, and these changes correlated with the decline in GSH content of the spleen, lymphocytes and the brainstem [36]. Interestingly, these decreases in GSH content were more dramatic in male mice than female mice [37]. Female mice show significantly higher hepatic GCL activity than males [37].

The expression of GGT is upregulated in response to oxidative stress [38] GGT is a well-established predictive biomarker for liver dysfunction and biliary tract diseases. A more recent Korean study reported that GGT activity is associated with an increased level of arterial stiffness in both genders [39]. Ha and colleagues showed that serum GGT levels correlate with blood pressure, but the correlation was only significant in men, not in women [40].

But it is also noteworthy that: a. gluthathione reductase activity is higher for women than for men in endothelial cells and erythrocytes [41], b. glutathione peroxidase of serum is higher for female mice than male ones [42], c. there was found the associations of some polimorphisms of GGT, but that of glutathione synthetase [GS] as well, with the increase of glutathione level, only in females [14].

Now we are ready to interprete as simply as possible observed correlations.

Initially, we thought that the existence of a correlations between the thiol X and Y, especially these particularly clear and significant, is simply a reflection of the „resulting" or formation of Y from X. And yet Hcy → C → G → CG [we omit intermediate metabolites here]. It may or may not be so. Firstly, we sometimes have negative or positive feedback loops, and secondly, metabolites may be involved in other processes, thirdly: the level of metabolites in each multistage process, and particular cycle, may be approximately constant ["steady state conditions"], that is, undergo relatively insignificant changes. So there is not necessarily a correlation between metabolites' concentrations.

After analyzing this matter, we came to the conclusion that the existence of a correlation must result primarily from significant changes in the activity of enzymes, especially the key ones, of the process consisting of transsulfuration and the γ - glutamyl cycle, i.e. their activation and inhibition.

We especially mean the [inhibitory or stimulating] effect exerted by male sex hormones [testosterone], and especially by female sex hormones [estrogens], because in our research presented here we found that very often the correlation is stronger in one sex [even only in one sex it takes place]. We remind you that only women have positive correlations: I] Hcy/G [$r_{X/Y}$ = 0. 32]; II] G/C [$r_{X/Y}$ = 0. 22]; III] G/CG [$r_{X/Y}$ = 0. 22] i IV] C/CG [$r_{X/Y}$ = 0. 18]. Only in men there are correlations: V] Hcy/C [$r_{X/Y}$ = 0. 56] and VI] Hcy/CG [$r_{X/Y}$ = 0. 30].

Estradiol activates subsequent transsulfuration enzymes, both CBS and CTH. This increases the level of cysteine. Although this mainly concerns the liver [and these processes do not occur at all in a number of cells/organs], it indirectly increases the availability of cysteine to the γ-glutamyl cycle of many cells throughout the body. Moreover, both of these enzymes, especially CTH, produce H_2S, which activates GCL, a key enzyme of the γ-glutamyl cycle. The concentration of γ-glutamylcysteine [GC], so next G and then [owing to GGT enzyme] CG dipeptide increases. This explains the existence of an I-III correlations.

Correlation IV seems to be the result of the overlap of the correlations I-III, because when: Hcy ↑ → C ↑ (and H2S ↑) → G ↑ → CG ↑, then C ↑ → CG ↑.

However, in order for this sequence, and especially the III and IV correlations, to be fully plausible, it would be necessary to either assume that another glutathione synthesis enzyme, e.g. glutathione synthetase [GS], is also activated with estradiol [or with some Z generated in the process activated by estradiol] or that the enzyme responsible for the degradation of CG, i.e. dipeptidase, is inhibited. Then the level of CG would not decrease, and could even increase slightly, so III and IV correlations would be maintained.

Perhaps, however, this dipeptidase is inhibited [so its level decreases] not by estradiol, but by homocysteine thiolactone causing N-homocysteilation of lysine residues in many proteins. This phenomenon is actually the main reason for the harmfulness of even not so much elevated homocysteine concentration to the body.

And what about the correlations V and VI, quite clear in men - the only ones that occur in men? And why do I-IV correlations not occur in men? Neither CBS nor CTH nor GCL are activated with male hormones. However, as in women, transsulfuration, and thus the formation of cysteine from Hcy [note the Hcy level in men is slightly higher than in women], and the formation of H_2S, and thus activation of GCL, takes place.

So we already know why there correlation V takes place [but neither II: G/C, so C/G nor I nor III and thus IV]. But again: to make the V correlation [and the lack of correlations II and III] more probable only in men it would be necessary to find the reason for the accumulation –in men- of C [so that Hcy ↑ → C ↑ (and H_2S↑) but not ↑ G. It would be the inhibition of the GCL enzyme by testosterone. However this has not been proved [so far]. But there are reports of a decrease in G levels with age in male mice [men?], suggested by the authors to cause a decrease in GCL activity.

But why is there no correlation III: G/CG? Because the G level drops, as it was found that male hormones activate GGT, the activity of which in the serum of men increases [and is higher than in women]. And GGT degrades glutathione to CG. We suggest that additionally homocysteine thiolactone inhibits dipeptidase [as in women according to our suggestion-compare above text] and the CG level goes up. So summarizing in men: Hcy ↑ → C ↑ (and H2S ↑), but still not ↑ G, because: 1. ↓ GCL [?] and 2. GGT ↑ → G ↓, and CG ↑, especially since DP ↓.

For comparison in women analogical sum of relations might be as follows:

Hcy ↑ → C ↑ (and H2S ↑)/ because CBS↑ and CTH ↑/ → GCL ↑ → G ↑ → CG ↑, especially since DP ↓.

(The efects of stimulation due to estradiol are marked as green, that of inhibition/enhancement due to testosterone as red, and inhibition with homocysteine thiolactone as yellow).

Now about "bad face" of cysteinylglycine [besides the fact that its formation lowers glutathione content]: Oxidative stress accompanies extracellular glutathione biodegradation to cysteinylglycine [CG] catalyzed by GGT [43]. This thiol dipeptide [CG] is characterized by a relatively low pK_a value (8,3), so it dissociates easily to thiolate ion ($-S^-$), which is able to reduce metal ions ie. $Fe^{3+}$to Fe^{2+} thereby leading to generation of superoxide anionoradicals ● O- then H_2O_2. Resulting CG radicals RS ● might bind forming SS ie. oxidized form of CG.

What is worse [in spite it was not told explicite by authors]: this might cause formation of hydroxyl radicals ● OH, the most dangerous from ROS ever [due so called Fenton reaction] [44].

This means that extracellular glutathione degradation might be one of most serious reasons of oxidative stress. This process is an example of how reductive properties of thiols can change into prooxidant. For this reason, cells characterized by high GGT activity [and so bigger CG content] are more vulnerable to oxidative dangers.

Above mentioned phenomena might explain the existence of some correlations between thiol substances and the level of typical risk factors of cardiovascular diseases (Table 3 part B], being visible only in men. One can name them as the prooxidative correlations. Three of them concern the level of "good cholesterol" ie Ch-HDL and all of them are negative. They are as follows: 1) Ch-HDL/C [$r_{X/Y}$ =- 0,36] and two weaker/even questionable: 2) Ch-HDL/G [$r_{X/Y}$ =- 0,15] and 3) Ch-HDL/CG [$r_{X/Y}$ =- 0,19]. The same prooxidative character concerns the last correlation, this time positive [only in men]: 4) Diapr/CG [$r_{X/Y}$ = 0,50]. Let's begin with the last strong correlation i.e. 4): increased ROS formation [even ● OH, compare earlier text] initiated with CG might cause some damage in blood vessels' walls and so an increase of diastolic pressure. But because CG comes from G and this from C, both CG directly and G and C rather indirectly result in prooxidative action, so among others lowering of activities of paraoxonase [against harmful effects of superoxides] and homocysteine thiolactone hydrolase [against those of Hcy thiolactone] contained in HDL complexes.

But so we substantiate the negative correlation between CG [and indirectly other thiols mentioned] and HDL activity but not Ch-HDL content. But in fact, there are no papers ever explaining why, in general, with an increase in Ch-LDL, there is often a decrease in Ch-HDL and why it is associated [in a cause-and-effect manner] with an increase of ROS level. It is the case in spite there is a flood of papers on the leading role of HDL in myocardial infarction and generally cardiovascular diseases [45], deep relationships between oxidative stress and metabolic syndrome [10] taking in mind even the participation of microRNA in evidently epigenetic influence on HDL relations with ROS.

Kronenberg [2018] [46] strongly emphasizes that an HDL particle is highly complex and carries more than 80 proteins and several hundred lipid species. Simply, measuring cholesterol of HDL might not reflect the variety of biologic effects of heterogeneous HDL particles; sometimes [as after oxidation] HDL might not be "good".

In spite of these doubts, we can assume that the changes in the average HDL cholesterol level are parallel to the changes in their functionality.

Let's come back to the analysis of an origin of our correlations, now in group of women. There are many more of them than in men, but they are generally weak [0,18 < $r_{X/Y}$ < 0,30], maybe even a few of them are not actually present [$r_{X/Y}$ < 0,18]. Intriguingly, unlike men, the correlations found in women are never „pro-oxidative" [like those found in men]. They are consistent with the images of the metabolic syndrome described in the literature and the dependences existing at that time and their relationship with the intensity of the harmful effects of ROS; even if we can't explain them precisely at the molecular level.

So there is often overweight [and even obesity], hyperglycemia, increased levels of triglycerides, total cholesterol, LDL cholesterol, and thus blood pressure [systolic and diastolic]. At the same time, we have an increase in the concentration of ROS [and even the so-called oxidative stress], often a decrease in the concentration of antioxidants, such as glutathione and other thiols [apart from homocysteine, we wrote about earlier]. As a rule, then is lower level of „good cholesterol" HDL.

Therefore, it is not surprising that we found [in women] positive correlations: Ch-HDL/G [$r_{X/Y}$ = 0,28] and Ch-HDL/CG [$r_{X/Y}$ = 0,21], as well as negative correlations of fasting glucose and glutathione [G/Gl], [$r_{X/Y}$ = - 0,30], BMI/G [$r_{X/Y}$ = - 0,20], systolic blood pressure with glutathione [$r_{X/Y}$ = - 0,19] and with CG [$r_{X/Y}$ = - 0,18] as well as TCh/CG [$r_{X/Y}$ = - 0,15] and Ch-LDL/CG [$r_{X/Y}$ = - 0,13].

One can add the positive correlations TCh/Hcy [$r_{X/Y}$ = 0,13] and Ch-LDL /Hcy [$r_{X/Y}$ = 0,17], as usually enhanced levels of homocysteine [being independent risk factor] accompany increased levels of cholesterol. Once again neither we nor anybody are able to explain this on the molecular level.

There are only two positive correlations which we are not able to explain [BMI/C; $r_{X/Y}$ = 0,18 and TG/G; $r_{X/Y}$ = 0,19] unless they will be considered as "prooxidative [like those in men; compare earlier].

But why in group of women generally "prooxidative" correlations do not take place ? We don't know. But it looks like there somehow prooxidative action of CG [compare earlier] are attenuated or simply CG formation with GGT enzyme is not clearly increased [as is in case of men].

A lot of correlations exist between typical risk factors of cardiovascular diseases themselves. They were showed with quite a big group of people despite this group is very small compared to that in a number of papers]. But they were not our main concern. Rather, they were established for comparative purposes, including to show that in general the correlations found for smaller groups of subjects can be demonstrated [„do not disappear"] also for many more numerous groups. They generally confirm what we do know on the metabolic syndrome even at its beginnings. Let us confine ourselves here only to the strongest correlations [$r_{X/Y}$ ≥ 0,40]: TG/Gl, Diapr/TCh, Sypr/TCh, Sypr/Ch-LDL, BMI/TG. Negative are [usually weaker] correlations only with participation of HDL:with glucose, triglycerides, diastolic and systolic pressure and BMI.

So: T-Ch↑, Ch-LDL↑, Ch-HDL↓, TG↑ →Gl↑ and Diapr↑ and Sypr↑ and BMI↑ [with also some feedbacks existent-not shown].

Puzzling is the correlation Ch-LDL/TG negative in healthy and positive in ill persons' group-and practictically only for men.

Evidently the disease, even very undeveloped, strengthens some correlations but weakens other ones. Also some correlations are only in women/men or at least are more pronounced with one sex. We will not even try to interpret these puzzling, and potentially important, dependencies now. It requires further in-depth research on numerous groups of healthy and sick people of both sexes.

It seems that the sexual dimorphism in terms of common risk factors for cardiovascular diseases and the metabolic syndrome exist. We believe that it largely depends on the oxidation-antioxidant balance, including thiol substances, especially glutathione and the dimorphism that occurs there [about which we wrote earlier].

Main Conclusions

1. There exist but rather seldom significant differences between men and women and between healthy and sick people as far as the levels of both low molecular thiol substances and those of typical risk factors for cardiovascular diseases are compared.

2. However, there are quite a lot of significant correlations between: a. the serum levels of thiol substances themselves, b. thiol substances and typical risk factors, c. typical risk factors themselves.

These correlations, sometimes strong or quite strong, seem to be the matter of whole paper as far as the prognostic values are taken into account.

3. An in-depth analysis of our data along in combination with the data from the literature allowed us to suggest what might be the origins of observed correlations between the levels of thiol substances in serum and why some correlations take place only in women and other only in men. a. Thus in women estradiol activates cystathionine synthase [CBS] and first of all cystathionase [CTH] and those produce H_2S which in its turn activates cysteinyl- glycine ligase [CGL]. b. In men there is no activation of mentioned above enzymes of the transsulfuration route and first of all male hormones activate γ-glutamyltranspeptidase.

4. a. In women almost all correlations between thiols and the classical risk factors have very antioxidative characters showing the thiols, especially glutathione, are potent antioxidants [and antioxidative defence is deeply interconnected with the weakening of widely known metabolic syndrome]. b. In men almost every correlation found has a pro-oxidative nature; it might be explained first of all with the formation of H_2O_2 and hydroxyl radicals due to cysteinylglycine, which level is elevated as γ-glutamyltranspeptidase is activated by male hormones.

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