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Cover Letter 4

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Madam,

According to you mail sent February 10 2009, I have the pleasure to submit the corrected version of the referred manuscript for publication in CONTRACEPTION.

Yours faithfully

Donat De Groote, PhD

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Detailed response to reviewers

Changes have been done according to the queries in the reviewed manuscript sent by Ms. Davenport on February 10 2009.

Donat De Groote, PhD

Effects of oral contraception with ethinylestradiol and drospirenone on oxidative stress in women 18-35 years old

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Short title: Oral contraception and oxidative stress

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Abstract

Background: Oral contraceptives (OCs) with estrogens and progestins may affect oxidative stress (OS) status.

Study Design: A group of 32 women using oral contraceptives (OCU) containing 0.03 mg ethinylestradiol and 3 mg drospirenone has been compared to a matched control group of 30 non-contraception users (NCU). Blood levels of antioxidants, trace elements and markers of lipid peroxidation were assessed by biochemical methods. A microarray analysis of whole blood mRNA levels of 200 genes involved in OS-dependant pathway was also performed.

Results: Levels of zinc, vitamin-E and antibodies to oxidized LDLs were not significantly different between the two groups. On the other hand, significant increases in the mean levels of lipid peroxides (+176%, $p<0.001$), oxidized LDLs (+145%, $p<0.002$), copper (+103%, $p<0.001$), Cu/Zn ratio (+100%, $p<0.001$) and a significant decrease in the mean level of β-carotene (-41%, $p<0.01$) were observed in the OCU compared to NCU. There was a highly significant positive correlation between the lipid peroxide levels and the copper to zinc ratio. From the 200 genes tested by microarray, one coding for HSP70 was significantly up-regulated ($\log^2$ fold change = + 0.45, $p<0.02$) and one coding for iNOS significantly down-regulated ($\log^2$ fold change = - 0.24, $p <0.05$) in the OCU compared to the NCU.

Conclusions: The recently-introduced combination of ethinyl oestradiol and drospirenone induced the heightening of lipid peroxidation correlated with high levels of copper, a situation that could be associated with increased cardiovascular risk.

Key words: Contraceptive pill; Estrogens; Progestins; Reactive Oxygen Species; Antioxidants.

1. Introduction

Reactive oxygen species (ROS) are metabolic products of the respiratory chain. It is estimated that approximately 1 to 3% of the inhaled oxygen produces ROS. Furthermore, the exposure to UV radiations, to air pollutants such as cigarette smoke or ozone, entails an increase of the production of ROS. ROS can have beneficial effects by participating, for example, in the mechanisms of immunological defenses against infections or detrimental effects linked to the oxidation of lipids, proteins and DNA. The body protects itself from noxious effects of ROS by a complex antioxidant system consisting of enzymatic antioxidants such as the catalase, the glutathione peroxidase (GPx) and superoxide dismutase (SOD) and non-enzymatic antioxidants such as, for example, vitamins A, C and E, glutathione (GSH) and uric acid. An imbalance between the production of ROS and the antioxidant defenses leads to a state of oxidative stress (OS) which is involved in the process of ageing and in various physiopathological processes such as cancer, diabetes and atherosclerosis [1-3]. The pro- or antioxidant effects of estrogens and progestins remain controversial. Various clinical studies evaluated the influence of estradiol therapy (ET) and of hormone replacement therapy (HRT) associating oral estrogens and progestins on the OS of postmenopausal women. Some of these studies showed protective effects on the OS [4-10] whereas others showed no significant effect on OS [11-15]. Surprisingly, only few studies have investigated the effects of oral contraceptives (OCs) on the OS. Some studies showed a significant increase of lipid peroxides in women using subdermal implants of levonogestrel or injectable depo-medroxy progesterone acetate (DPMA) [16-18]. An increase of blood lipid peroxides was observed in rats treated with estro-progestative OCs [19] and a decrease of the liposoluble antioxidants coenzyme Q10 and α-tocopherol was shown in women taking the OC compared to a control group not using OCs [20]. More recently, a study assessing the effect of OC on OS in a subgroup of women in the age ranged 40-48 years showed an important OC-related

heightening of lipid peroxides associated with a higher concentration of copper and altered liposoluble antioxidant defenses [21]. In the present study, we analyzed the effects on biochemical and molecular OS markers of the latest generation OCs in a representative group of women18-35 years old.

2. Materials and methods

2.1. Subjects

Sixty-two healthy female subjects participated in the study. The study was approved by and performed under the guidelines of the Ethic Committee of the University Hospital of Liège, Belgium and informed consent was obtained from each of the subjects before blood sampling. The test group consisted in 32 women aged 23.0 ± 3.9 years (mean ± SD) who were regular OC users (OCU) and who were recruited during routine gynecology visits. The average duration of OC use was of 33 ± 26 months (mean ± SD), ranging from 3 months to 9 years. All the OCU were taking a contraceptive pill containing 0.03 mg ethinylestradiol and 3 mg drospirenone (DRSP). The control group consisted of 30 non-contraceptive users aged 29.4 ± 4.3 years (mean ± SD) (NCU) with no other hormonal treatments who were recruited during routine gynecology or medically assisted reproduction visits.

All the subjects were of normal body weight and were nonsmokers with no evidence of chronic disease. None of the subjects consumed >25 mL alcohol/d or were taking other medications, antioxidants, or vitamin supplements. The mean (± SD) body mass indexes (BMI) were, respectively, 23.7 ± 2.8 kg/m^2 for the NCU group and 21.3± 2.9 kg/m^2 for the OCU group.

Blood samples for the assessment of OS markers were taken between the third and fifth day of the menstrual cycle.

2.2. Samples processing

Blood samples were drawn on EDTA or Na-heparin as anticoagulant or clot activating gel according to the investigated parameter. Blood samples were centrifuged within 4 h after sampling and plasma or sera were immediately frozen and kept at -20°C until tested. For microarray analysis, whole blood samples were collected on PackGene™ tubes and immediately frozen and kept at -80°C until mRNA extraction.

2.3. Antioxidants and trace element determination

Plasma vitamin-E (α-tocopherol) (Vit-E) and β-carotene were determined simultaneously by HPLC procedure (Alliance Waters, USA) coupled with a diode array detector (PDA 2996, Waters, USA) according to the method of Zhao et al. [22]. EDTA plasma samples were immediately frozen at -20 °C until analyzed. The plasma concentrations of copper (Cu) and zinc (Zn) were determined by inductively coupled plasma-mass spectrometry [23].

2.4. Markers of lipid peroxidation

The analysis of lipid peroxides was performed with the OxyStat commercial kit (OxySat, Biomedica Gruppe, Austria). Briefly, the peroxide (-OOH) concentrations are determined by a spectrophotometric based method by reaction of the biological peroxides (LOOH) with peroxiydase and subsequent color reaction using 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. Oxidized low-density lipoproteins (ox-LDLs) in plasma samples were determined spectrophotometrically with a competitive enzyme-linked-immunoassay (ELISA) kit from (Immunodiagnostik, Germany). The titers in free antibodies (IgG) against oxidized LDLs were assessed with a commercial enzyme immunoassay (Biomedica Gruppe, Austria) using Cu^{2+} oxidized LDLs as antigen.

2.5. Microarray analysis

We designed and prepared a low-density (200 genes) oligonucleotide microarray for mRNA expression profiling in whole blood. Genes for this microarray were selected from oxidative

stress-sensitive pathways. Corresponding oligonucleotides were generated by ArrayDesigner 3 software (Premier Biosoft Intl, USA) using the Tm parameter = 75°C + 2°C. Probes were limited to a length of 60 nucleotides. All oligonucleotide sequences were designed using the thermodynamic model optimized for microarrays. To avoid cross-homology, the set of oligonuleotides was tested by performing a BLAST analysis against the NCBI "Human Ref Seq RNA" database. Oligonucleotide probes were deposited by a robot on chemically pre-treated glass slides. mRNA were purified from whole blood with the QIAamp RNA Blood Mini Kit from Qiagen (QIAGEN gmbH, Germany). A reverse transcription in the presence of probes containing the Genisphere 3DNA^{TM} capture sequence (Genisphere Inc, USA) was then realized. The resulting cDNAs were then hybridized on the microarray. The presence of the sample cDNAs were then detected by complementary 3DNATM Capture Reagents (Genisphere Inc, USA) that were Cy3-labeled. The acquisition and the analysis of the images were realized by means of a scanner GenePix and of the software GenePix Pro 5.0. (Axon Instruments, Molecular Devices, U.S.A). All values derived from the image analysis were background corrected and normalized by the variance stabilization method of Huber et al. [24], against a common reference slide. After normalization, outlier arrays were detected and removed when their Pearson correlation coefficient was in average lower than 70%. A pool of mRNA was used as standard control.

2.6. Statistical analysis

For the biochemical parameters, the differences between groups were analyzed by the non-parametric Mann-Whitney test. The differences between the median values of both groups were considered as significant at $p < 0.05$. The correlations between the various studied biochemical parameters were analyzed by using the Pearson coefficient of correlation. The correlations between the various parameters were considered as significant at $p < 0.05$. For the microarray, a gene by gene analysis of the normalized data was performed by the R package

limma[2] [25] on the two groups. This package makes use of an adapted version of the hierarchical model proposed by Lonnstedt and Speed [26]. The central idea is to fit a general linear model with arbitrary coefficients and contrasts of interest, to the expression data for each gene. The empirical Bayes approach shrinks the estimated sample variances towards a pooled estimate, resulting in a far more stable inference [27]. The differences in gene expression between the groups were considered as significant when Wilcoxon BH adjusted was $p < 0.05$.

3. RESULTS

3.1. Biochemical data

Levels of Zn (0.73 ± 0.15 versus 0.75 ± 0.10 mg/L) , Vit-E (11.26 ± 1.76 versus 11.46 ± 2.47 mg/L) and antibodies to oxidized LDL (Ab-ox-LDL) (555.0 ± 501.4 versus 530.5 ± 447.5 U/L) were not significantly different between the two groups. Significant increases in the mean levels of LOOH (478.2 ± 194.7 versus 1321.9 ± 356.9 μmol/L, $p < 0.001$), ox-LDL (503.9 ± 536.3 versus 1236.3 ± 1023.8 ng/mL, $p < 0.002$), Cu (0.86 ± 0.23 versus 1.75 ± 0.36 mg/L, $p < 0.001$), Cu/Zn ratio (1.18 ± 0.21 versus 2.37 ± 0.39, $p < 0.001$) and a significant decrease in the mean level of β-carotene (0.35 ± 0.22 versus 0.20 ± 0.07 mg/L, $p < 0.01$) were observed in the OCU compared to NCU (Table 1).

3.2. Microarray analysis

From the 200 genes tested by microarray analysis, only 2 were significantly differentially expressed in the OCU compared to NCU (Fig.1). The first was the gene coding for heat shock protein 60 kD (HSP60, + 0.45 $\log^2$ fold change between OCU and NCU, adjusted $p < 0.05$) and the second was the gene coding for the nitric oxide synthase 2A (NOS2A, - 0.24 $\log^2$ fold change between OCU and NCU, adjusted $p < 0.05$).

3.3. Correlations

Bivariate analysis of the different parameters showed positive significant correlations between the levels of lipid peroxides and the levels of ox-LDL ($r = 0.474$, $p < 0.001$), Cu ($r = 0.834$, $p < 0.001$) and Cu/Zn ratio ($r = 0.834$, $p < 0.001$); positive significant correlations between the levels of ox-LDL and the levels of Cu ($r = 0.423$, $p < 0.001$) and Cu/Zn ratio ($r = 0.408$, $p < 0.001$); positive correlations between the levels of Cu and the levels of Zn ($r = 0.352$, $p < 0.01$) and Cu/Zn ratio ($r = 0.905$, $p < 0.001$); and negative significant correlations between β-carotene and the levels of LOOH ($r = -0.304$, $p < 0.05$), Cu ($r = -0.261$, $p = 0.05$) and Cu/Zn ration ($r = -0.335$, $p < 0.05$) (Table 2).

4. DISCUSSION

The effects of estrogens and progestin on oxidative stress are controversial. Estrogens display an antioxidant activity by inhibiting the expression and function of the NADP+/NADPH oxidase [28, 29], by increasing the expression and level of activation of the endothelial isoform of the nitric oxide synthase (eNOS) [30] and by stimulating the expression and activity of the manganese SOD (MnSOD) and of the extracellular SOD (ecSOD) [31]. These antioxidant activities of estrogens are counteracted by progestins via the activation of the NADPH oxidase and the inhibition of the expression and activity of the MnSOD and of the ecSOD [32, 33]. A direct pro-oxidant effect of estrogens was shown in experimental models in the rat [34] and the Syrian hamster [35]. These pro-oxidant effects of estrogens can partially be explained by their metabolism. Indeed, estrogens can be metabolically activated into catechol estrogens by the enzymes of cytochrome P450. These last ones are easily auto-oxidized to ortho-quinone by-products which are powerful oxido-reducing agents capable of generating ROS [36].

The increase of serum Cu related to OC use is known and has been attributed to the induction by estrogens of the hepatic synthesis of the acute phase protein ceruloplasmin, the main Cu carrier protein [37-39]. Cu and Zn are trace elements which play a vital role as catalytic co-

factors for a variety of enzymes including CuZn-SOD, an enzyme which participates actively in the elimination of ROS [40]. An imbalance between these two trace elements could result in a dysfunction of CuZn-SOD and, consequently, a decrease of the protection of lipids against oxidation by ROS. There is numerous evidence which suggests that Cu plays a direct role in lipid peroxidation [41, 42]. Cu is a pro-oxidant redox-active transition metal while Zn, which is redox-inactive, is capable of counteracting the pro-oxidant activity of Cu [43]. An association between high serum Cu/Zn ratios and aging on one hand and aging-related chronic degenerative diseases on the other hand has been shown [44]. In the same study, the Cu levels were positively correlated (and Zn negatively correlated) to the lipid peroxides levels. The different OS-sensitive pathways that are influenced by estrogens and/or progestins are shown in Fig.2.

Surprisingly, only two genes were differentially expressed in leucocytes of OCU compared to NCU, the heat shock protein 60kD (HSP60) and the inducible nitric oxide synthase (iNOS). The increased expression of HSP60 in OCU can be explained by a direct effect of estrogens. Indeed, it has been shown that 17β-estradiol (E2) and two metabolites (16α-hydroxysterone and 2-methoxyestradiol) were able to increase the levels of HSP60 transcripts in MCF-7 breast cancer cells [45]. E2 and the E2 receptor-β agonist diarylpropiolnitrile (DNP) have been shown to restore the levels of HSP60 in the heart of Sprague-Dawley rats undergoing trauma-hemorrhage, a condition where the heat shock protein was impaired [46]. Cu could also be involved in the over-expression of HSP60. Indeed, HSP60 mRNA expression was specifically reduced in the atria of Cu-deficient rats [47] and the induction of HSP60 in response to Cu in a fish hepatoma cell line (PLHC-1) has been shown [48]. Our results showed for the first time an in vivo elevation of HSP60 in OCU. The respective role of each hormone on the expression of HSP60 remains to be elucidated.

Different observations suggest that estrogens and progestins regulate the expression of iNOS in different ways, but their conclusions are controversial [49, 50, 51, 52].

Most of the genes involved in the OS-sensitive pathway that are influenced by estrogens and/or progestins (Fig. 2) were detected on the microarray but were not significantly changed in OCU compared to NCU. This suggests that these pathways are not the main sources of OS in OCU. On the contrary, the good correlation of the Cu/Zn ratio with the levels of lipid peroxides and with oxidized LDLs suggest that the estrogen-mediated increase of Cu in the liver could be a key factor of OS production in OCU. One possible mechanism could be that the combination of a mild activation of some OS-sensitive pathways with a important Cu release initiates a chain reaction involving the production of superoxide ($^{\cdot}O_2^-$), Cu reduction, formation of hydroxyl radicals and lipid peroxidation [53, 54].

The significant decrease in β-carotene levels in the OCU compared to NCU is in agreement with the study of Palan et al. [20] and Pincemail et al. [21] and can be attributed to the estrogen induction and activation of the retinol binding protein leading to increased conversion of β-carotene into retinol [55].

Several experimental models and numerous clinical observations suggest a major role of OS in the process of atherogenesis and cardiovascular diseases [56-59].

It is well known that the cardio vascular side effects of OCs occur mainly in women who present other risk factors such as dyslipidemia, smoking, above 35 years of age, excess body weight and high blood pressure [60]. The mechanisms by which the physiological and environmental factors as well as the lifestyle and food habits act on the state of OS are complex and influenced by genetic factors such as the polymorphism of genes coding for proteins having pro-oxidant activities (myeloperoxidase-MPO-, the receptor of the type 1

angiotensin) or antioxidant activities (Cu/ZnSOD, MnSOD, ecSOD, PON-1, GPX, etc.) [61-64].

In the present study, we provide evidence for a relationship between OC use and the state of OS. The disturbance of the OS markers was documented by a very significant increase of lipid peroxides and ox-LDLs, probably in conjunction with an increase in circulating Cu induced by estrogens. The OCU group was about 5 years younger in the mean than the NCU group. Nothing is known about an age-related effect on the studied parameters in this range of age. Increased plasma levels of lipid peroxides and Cu have manly been shown in elderly [65-67]. If there is a positive correlation between age and the variables measured, the effect we have seen might be underestimated. Nevertheless, due to the extent of the differences we observed, that should have no impact on the conclusions. These observations are based on a cross-sectional comparison. They will need to be confirmed in longitudinal studies in which women act as their own control or better still, with randomization to non-steroidal contraception or active treatment.

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Fig. 1.

Volcano graph of gene expression in OCU. Log^2 fold changes of OCU versus NCU (x axis) are plotted against 1/p (y axis) where p is the Wilcoxon BH adjusted p value between OCU and NCU. Differences are considered statistically significant for $p < 0.05$ ($1/p > 20$).

Fig. 2.

Schematic representation of estrogens and progestins metabolic pathways involved in oxidative stress and lipid peroxidation.

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Table 1
Biochemical values in non-contraceptive users (NCU) and oral contraceptive users (OCU)

	NCU (n = 30)	OCU (n = 32)	p value between groups
Lipid peroxides (μmol/L)	478.2 (194.7)	1321.9 (356.9)	< 0.001
Oxidized LDL (ng/mL)	503.9 (536.3)	1236.3 (1023.8)	0.0015
Ab-ox-LDL (U/mL)	555.0 (501.4)	530.5(447.5)	ns
α-tocopherol (mg/L)	11.26 (1.76)	11.46 (2.47)	ns
β-carotene (mg/L)	0.35 (0.22)	0.20 (0.07)	0.0086
Copper (mg/L)	0.86 (0.23)	1.75 (0.36)	< 0.001
Zinc (mg/L)	0.73 (0.15)	0.75 (0.10)	ns
Cu/Zn ratio	1.18 (0.21)	2.37 (0.39)	< 0.001

Values are means (S.D.). Differences between groups are statistically significant for $p < 0.05$.

Table2_2

Table 2
Bivariate analyses of oxidative stress parameters

		LOOH	Ox-LDL	Anti-ox-LDL	Vit-E	β-car	Copper	Zinc	Cu / Zn
LOOH	Pearson Correlation	1							
	Sig.(2-tailed)								
	N								
Ox-LDL	Pearson Correlation	**.474****	1						
	Sig.(2-tailed)	**.000**							
	N	**54**							
Anti-ox-LDL	Pearson Correlation	-.136	.062	1					
	Sig.(2-tailed)	.308	.645						
	N	58	58						
Vit-E	Pearson Correlation	.057	-.048	.000	1				
	Sig.(2-tailed)	.681	.728	.997					
	N	55	55	59					
β-carotene	Pearson Correlation	**-.304***	-.228	.010	.157	1			
	Sig.(2-tailed)	**.024**	.098	.941	.253				
	N	**55**	54	58	55				
Copper	Pearson Correlation	**.834****	**.423****	-.102	.226	**-.261 ***	1		
	Sig.(2-tailed)	**.000**	**.000**	.435	.087	**.050**			
	N	**57**	**57**	61	58	**57**			
Zinc	Pearson Correlation	.155	.171	-.003	.108	.128	**.352****	1	
	Sig.(2-tailed)	.250	.203	.982	.421	.344	**.006**		
	N	57	57	61	58	57	**60**		
Cu/Zn	Pearson Correlation	**.834****	**.408****	-.068	.203	**-.335***	**.905****	-.018	1
	Sig.(2-tailed)	**.000**	**.000**	.602	.124	**.010**	**.000**	.892	
	N	**58**	**58**	62	59	**58**	**61**	61	

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Figure 1

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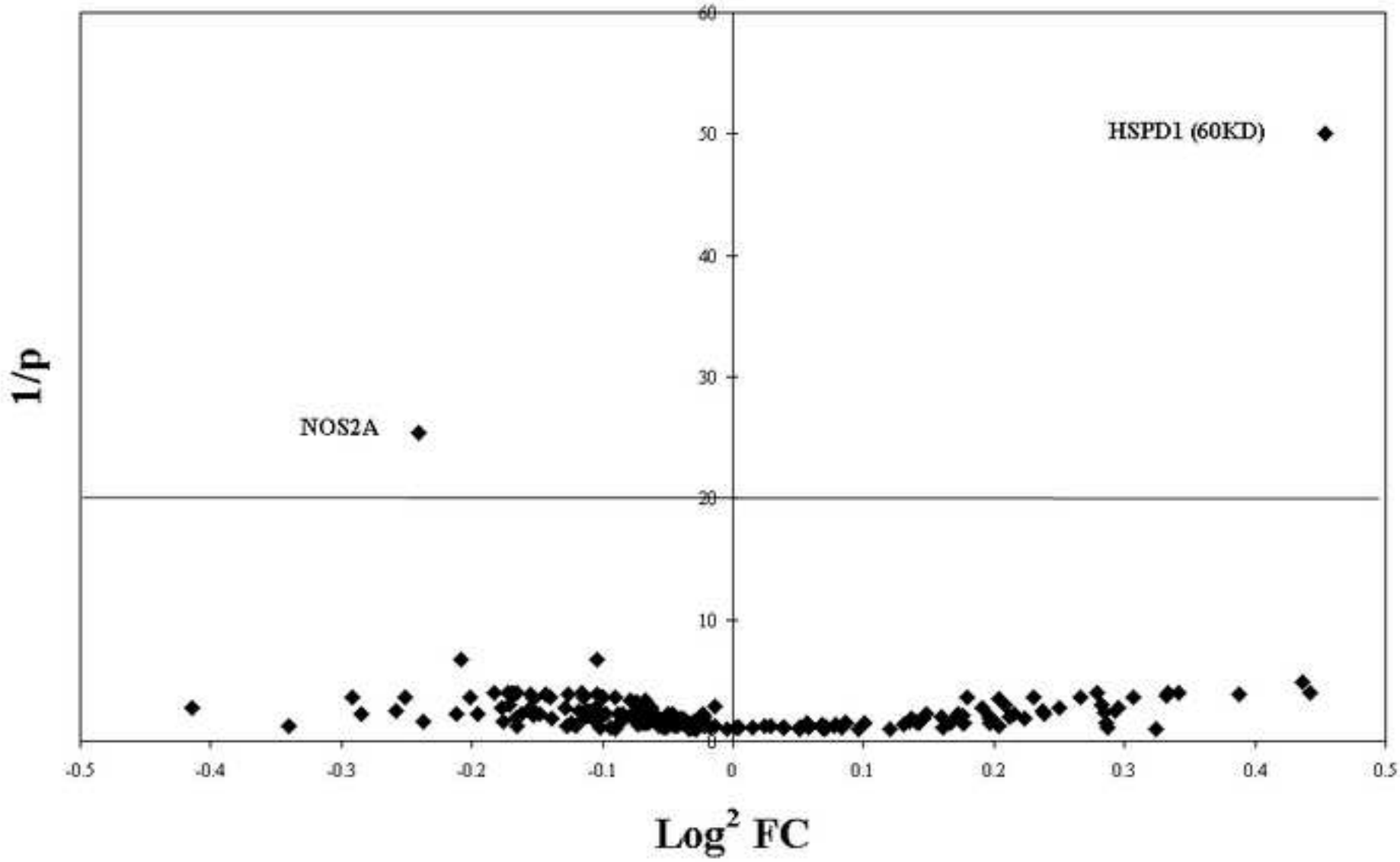

Figure2

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