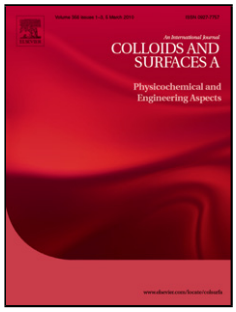

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PII: S0927-7757(13)00401-9
DOI: http://dx.doi.org/doi:10.1016/j.colsurfa.2013.05.022
Reference: COLSUA 18420

To appear in: *Colloids and Surfaces A: Physicochem. Eng. Aspects*

Received date: 28-11-2012
Revised date: 29-4-2013
Accepted date: 5-5-2013

Please cite this article as: G. Siegel, E. Ermilov, A.R. Pries, K. Winkler, A. Schmidt, L. Ringstad, M. Malmsten, B. Lindman, The Significance of Lipid Peroxidation in Cardiovascular Disease, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* (2013), http://dx.doi.org/10.1016/j.colsurfa.2013.05.022

The Significance of Lipid Peroxidation in Cardiovascular Disease

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Abstract

Background: The metabolic syndrome describes a cluster of cardiovascular risk factors that frequently appear together. Its diagnosis is generally based on several well-recognized indicators in clinical practice, such as abdominal obesity, elevated triglycerides, reduced high-density lipoprotein, raised blood pressure, and elevated fasting plasma glucose. Today, decisive importance must be attached to the metabolic syndrome since it leads to increased morbidity and mortality, and thus to a decreased life expectancy, and to higher direct and indirect healthcare costs. This is also due to the fact that its symptomatology irradiates on many organs of the body, which may thereby be damaged. *Methods:* In the present clinical trial on 11 metabolic syndrome patients treated with *Ginkgo biloba* (EGb 761, 2 × 120 mg/d) for two months, ellipsometry, fluorescence microscopy, photometric methods, ELISAs and EIAs were applied for biosensor profiling of metabolic syndrome risk, status and treatment outcome. *Results:* A spectrum of more than 20 arteriosclerotic, cytokinic, inflammatory, lipidic, and oxidative stress biomarkers served for a detailed diagnosis and therapy monitoring. After medication, the ratio oxLDL/LDL was reduced by 21.0%, 8-*iso*-$PGF_{2\alpha}$ 39.8%, MPO 29.6%, IL-6 12.9%, hs-CRP 39.3%, Lp(a) 26.3%, MMP-9 32.9%, insulin 9.4%, HOMA-IR 14.0%, ALP 14.8%, CREA 11.3%, URAC 10.6%, *in vitro* modelled nanoplaque formation 14.3% and size 23.4%, whereas SOD was augmented by 17.7%, GPx 11.6%, cAMP 43.5%, and cGMP 32.9%. Special focus was concentrated on the significance of lipid peroxidation for cardio-cerebro-vascular diseases. Through multiple correlations between the biomarkers and clinical parameters, their significance for and involvement in several clinical pictures could be elucidated. *Conclusion:* The present clinical observational study was helpful in unravelling this network of biomarker interactions and demonstrated its usefulness for theranostics. For personalized medicine, the selection of the biomarkers is of decisive importance. On the background of a growing obesity among children and adolescents with an increase in prevalence of the metabolic syndrome, diagnosing this syndrome in young subjects may be helpful in identifying a population of risk for increased subclinical arteriosclerosis.

Key words: Clinical trial; ellipsometry; Ginkgo biloba; metabolic syndrome; biomarkers; personalized medicine

Abbreviations

ALP	alkaline phosphatase
BP_{dias}	diastolic blood pressure
BP_{sys}	systolic blood pressure
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
CREA	creatinine
CS	chondroitin sulfate
GPx	glutathione peroxidase
HOMA-IR	homeostasis model assessment of insulin resistance
hs-CRP	high-sensitivity C-reactive protein
HS	heparan sulfate
HS-PG	heparan sulfate proteoglycan
IDL	intermediate-density lipoprotein
IL-6	interleukin-6
8-*iso*-$PGF_{2\alpha}$	8-*iso*-prostaglandin $F_{2\alpha}$
LDL	low-density lipoprotein
Lp(a)	lipoprotein(a)
MMP-9	matrix metalloproteinase-9
MPO	myeloperoxidase
oxLDL	oxidized low-density lipoprotein
ROS	reactive oxygen species
SOD	superoxide dismutase
URAC	uric acid
VLDL	very low-density lipoprotein

1 Introduction

The metabolic syndrome with its multifacet symptoms is an excellent example for targeting polyorganic impairment such as cardiovascular (cardiac infarction), cerebral (stroke, Alzheimer's), pancreatic (diabetes), hepatic (cholestasis), and gastrointestinal (irritable bowel syndrome) diseases. In the present clinical trial, photometric methods, ELISAs, EIAs and ellipsometry were applied. A wide spectrum of more than twenty oxidative stress, cytokinic, inflammatory, lipidic and arteriosclerotic biomarkers, all interconnected with each other, and navigated by reactive oxygen species (ROS) served for a detailed diagnosis and point-of-care therapy monitoring [1]. Special focus is concentrated on the significance of lipid peroxidation for cardiovascular diseases. As one example, correlations between oxLDL/LDL ratio as well as *in vitro* modelled nanoplaque formation on the one side and various biomarkers on the other are presented.

At an HS-PG-coated silica surface representing a receptor site for specific lipoprotein binding through basic amino acid-rich residues within their apolipoproteins, the binding process was studied by ellipsometric techniques [2]. LDL proved to be deposited strongly at the proteoglycan-coated surface, particularly in the presence of Ca^{2+}, apparently through complex formation 'lipoprotein receptor (HS-PG)–LDL–calcium'. This ternary complex build-up may be interpreted as arteriosclerotic nanoplaque formation on the molecular level before any cellular reactivity, possibly responsible for the arteriosclerotic primary lesion. HDL privileged by a high binding affinity compared with LDL and therefore strongly bound to HS-PG, protected against LDL deposition and completely suppressed calcification of the proteoglycan-lipoprotein complex. In addition, HDL was able to decelerate the ternary aggregational complex deposition. Therefore, HDL attached to its proteoglycan receptor sites is thought to raise a multidomain barrier, selection and control motif for transmembrane and paracellular lipoprotein uptake into the arterial wall.

The processes described take place under *in vitro* and *in vivo* conditions. *In vivo*, the polyanionic and hydrophilic glycosaminoglycan chains dominate the physical properties of the proteoglycans such as endothelial and vascular smooth muscle cell membrane syndecan (HS/CS-PG) and vascular matrix perlecan (HS-PG). Both these macromolecules belong to a class of transmembrane and interfacial matrix polyelectrolytes that control and regulate the transendothelial and paracellular trafficking of blood lipids at the strategic boundary layers of blood–cell (glycocalyx), cell–cell (paracellular adhesive ground and cement substance) and cell–matrix (basal lamina and subbase matrices) barriers [3].

From a series of previous preclinical and clinical studies it appeared, that many biomarkers, e.g., hs-CRP, cytokines, and liver values are up- or down-regulated in the subthreshold range, but so much the more have an important early diagnostic, preventive and prognostic significance [4-9]. The picture is the more complicated in that some inflammatory biomarkers, e.g., MPO and Lp(a) behave inversely with regards to cytokine balance. Nevertheless, it is a general rule for the metabolic syndrome that oxidative stress with its lipid peroxidation dominates decisively nanoplaque formation and size as well as all biomarkers. Our clinical trials with statins [3,4], ginkgo [6,10], ω3-fatty acids [11] and blackcurrant extract [12] unravelled this network of biomarker interactions and demonstrated its usefulness for theranostics.

Moreover, these clinical studies allowed us to characterize the clinical picture of the metabolic syndrome in its facets as well as to represent and develop its clinical sequelae till the cardio-cerebro-vascular events on a time axis (cf. Fig. 1). The importance of oxidative stress (ROS) for this faulty development is clearly recognizable. Nanoplaque formation, precursor of a fully-blown arteriosclerotic plaque, is a biomarker which cannot be measured directly in the patient. Therefore, we tried to prove nanoplaque build-up ellipsometrically, first of all on living human endothelial cells (primary culture) after incubation with oxLDL, and we were successful (cf. Fig. 2). These endothelial cells express the syndecan superfamily as natural lipoprotein receptor. As a next step, we coated the silica surfaces for ellipsometry with the isolated lipoprotein receptor (HS-PG), and could measure atherogenic nanoplaque formation with the lipoprotein fraction VLDL/IDL/LDL derived from the blood of patients

[13]. Finally, the determination of additional biomarkers from the blood of the patients as well as the calculation of their interrelations made a further step to personalized medicine feasible.

2 Material and methods

2.1 Subjects and study design

A preventional, randomized, 3-month study comprising a 1-month dietary run-in phase followed by a study treatment period of 2 months was conducted in the Phase I-II study clinic of the UMHAPT "Zaritza Johanna" University Hospital, Sofia, Bulgaria. The project has been reviewed and approved by the local Ethics Committee and the Bulgarian Drug Agency. Eleven patients (2 male, 9 female) with metabolic syndrome aged 26-48 years were recruited, provided that they fell within the additional inclusion criteria smoking (all 11 patients were smokers) and blood lipoprotein(a) [Lp(a)] concentration >30 mg/dL (9 patients). The inclusion criterion smoking was enclosed to clearly demonstrate the antioxidative effect of ginkgo, and Lp(a) > 30 mg/dL was enclosed to confirm the Lp(a) lowering by 23.4% from a preceding ginkgo study in aortocoronary bypass patients (8 patients) [10]. After the first taking of a 45 mL blood sample, the standard therapy of the patients was 2 × 120 mg/d *Ginkgo biloba* special extract EGb 761 (Rökan novo®: Spitzner Arzneimittel, Ettlingen; Tebonin®: Schwabe Pharmaceuticals, Karlsruhe, Germany) over 2 months. No statins, no calcium antagonists and no nitrate compounds were given. No adverse events occurred and all the patients felt well during and after ginkgo intake [6]. After 2-month medication, the second 45 mL blood sample was taken. 1 mL of blood was required for the determinations in duplicate of all biomarkers together, the remaining 44 mL for the preparation of the lipid fraction needed in the ellipsometry measurements.

2.2 Preparations and solutions

All experiments were carried out in a blood substitute solution. The normal blood substitute solution consisted of a Krebs solution simulating the extracellular ionic microenvironment of the proteoglycans and lipoproteins: Na^+ 151.16, K^+ 4.69, Ca^{2+} 2.52, Mg^{2+} 1.1, Cl^- 145.4, HCO_3^- 16.31 and $H_2PO_4^-$ 1.38 mmol/L (25°C, pH 7.3). The Krebs solutions were gassed by 93% N_2/7% CO_2 to prevent VLDL/IDL/LDL oxidation [2]. The HS-PG preparation had a molecular weight of about 175 kDa as assessed by chromatography on Sephacryl S 400

columns calibrated with reference proteoglycans. Analytical data revealed an HS proportion of 82 g/100 g dry weight corresponding to four HS chains (~4 × 36 kDa) with an average sulfate content of 0.5 sulfate groups/disaccharide unit covalently linked to the protein core. The protein content (~35 kDa) was 18 g/100 g dry weight. Lipoproteins were isolated by sequential preparative ultracentrifugation essentially as described previously [4].

2.3 Surface preparation

Hydrophilic and negatively charged silica surfaces were prepared from polished silicon slides by thermal oxidation followed by annealing and cooling in argon, which resulted in an oxide layer thickness of about 30 nm. The slides were then cleaned in a mixture of 25% NH_4OH, 30% H_2O_2, and H_2O (1:1:5, by volume) at 80°C for 5 min, followed by cleaning in a mixture of 32% HCl, 30% H_2O_2, and H_2O (1:1:5, by volume) at 80°C for 5 min. Methylated silica surfaces were prepared from the silica surfaces by rinsing with, in order, water, ethanol, and trichloroethylene (pro Analysi, Merck), and a subsequent treatment with a 0.1 wt% solution of $Cl_2(CH_3)2Si$ (Merck) in trichloroethylene for 90 min. The surfaces thus prepared had a surface potential of -40 mV and a contact angle of 90° [14]. Surface force measurements served for characterization of the HS-PG coated model surfaces [15].

2.4 Fluorescence microscopy

Vascular endothelial cells of the human umbilical vein were grown on methylated silica surfaces to form a confluent monocellular layer. Human umbilical cords from three normal term deliveries were obtained within the first minutes of birth. Cord segments were cut at 30 cm length, clipped at both ends, and placed into a sterile PBS–Dulbecco solution (Seromed L1825, Biochrom, Germany) containing penicillin/streptomycin (Seromed A2212) and glucose (Gibco, Life Technologies, Germany). Umbilical vein endothelial cells were treated for 5 min with collagenase (0.05 v/v%, Seromed C2-28, temperature 37°C), rinsed with and washed out into endothelial cell growth medium (PromoCell, Germany), centrifuged for 10 min at 900 rpm, and finally disseminated and cultured on methylated silica surfaces placed in plastic petri dishes (3001, Falcon, Germany). The dishes were incubated at 37°C under 5% CO_2. The cells were fed twice a week with a complete change of fresh culture medium. The cell-coated surfaces were taken from the culture medium and placed in an ellipsometer cuvette filled with normal blood substitute solution (Krebs solution) of the following

composition: Na^+ 151.16, K^+ 4.69, Ca^{2+} 2.52, Mg^{2+} 1.1, Cl^- 145.4, HCO_3^- 16.31, $H_2PO_4^-$ 1.38 mmol/L (temperature 25°C) [16].

After 7 days, fluorescence microscopy revealed that the silica surfaces were covered by a confluent cell monolayer (Fig. 2). For this, the cells were rinsed with PBS–Dulbecco solution, fixed in ethanol (first 70%, then 100%), and air-dried. The fixed cells were incubated overnight at 4°C in an IgG fraction of antiserum containing anti-mouse monoclonal LDL antibodies (PROGEN Biotechnik, Heidelberg, Germany). After being repeatedly rinsed with PBS–Dulbecco solution, the cells were treated with Cy2 goat anti-mouse IgG (111-225-045, dianova, Germany) as secondary antibody for 1 h at 37°C. Again, the cells were rinsed with PBS–Dulbecco solution, their nuclei stained by propidium iodide (0.05 mg/mL, Sigma, Germany) for 1 min, then rinsed with aqua bidest, and finally embedded in Aquatex (108562, Merck, Germany). Immunofluorescence microscopy of the human umbilical vein endothelial cells was carried out on an Axiophot microscope (Carl Zeiss, Germany) in vertical illumination version.

2.5 Determination of biomarkers and clinical investigations

Ellipsometry, photometric methods, commercial ELISAs and EIAs, all described previously, were applied [1,3,5,10,17]. In the ellipsometry measurements, the adsorbed amount was determined according to de Feijter using a refractive index increment of $dn/dc = 0.16$ cm^3/g (Fig. 2) resp. 0.18 cm^3/g (Fig. 3) [18]. Oscillometric systolic (BP_{sys}) and diastolic (BP_{dias}) blood pressure (mm Hg) and pulse rate (bpm) were measured after 10 min recumbent rest and after 1 min relaxed standing. Measurements were made from the upper arm according to Riva-Rocci using an automated oscillometric device (Omron M5 Professional, Mannheim, Germany). Care was taken a) that the cuff size was adjusted to the upper arm circumference, and b) that the arm was fully extended with the cuff at the level of the heart.

2.6 Statistical analysis

Statistical significance of the blood parameter and blood pressure changes was calculated by the paired, two-sided Student's *t*-test and the Wilcoxon signed-rank test. Changes were considered statistically significant at $p < 0.05$. For linear correlation analysis we calculated Pearson's correlation coefficient *r*. Statistical significance was obtained from Student's *t*-

distribution using $t = r$ sqrt $[(n - 2)/(1 - r^2)]$, where n is the number of data points and $n - 2$ the degree of freedom. A correlation between two data sets is significant if the two-sided probability $p < 0.05$ [5,10,17].

3 Results

The metabolic syndrome is not a simple disease entity, but, as the term syndrome says, a complex of multiple symptoms. This cluster of cardiovascular risk factors that frequently appear together, includes abdominally accentuated obesity, impaired glucose tolerance, hypertension, and dislipidemia. Operational definitions of metabolic syndrome have been proposed by the World Health Organization (WHO) [19], the National Cholesterol Education Program ($NCEP_{III}ATP$) [20,21] and the International Diabetes Federation (IDF) [22]. Fig. 1 illustrates the study design of our clinical trial as well as the interconnective network and historical development of pathophysiologic pathways culminating in cardio-cerebro-vascular events [cf. 23]. Oxidative stress (ROS, reactive oxygen species) with its lipid peroxidation plays the central role in this atherogenic process. Therefore, besides nanoplaque formation and size, a wide spectrum of biomarkers of oxidative stress, plaque stability and progression, inflammation, lipid composition including Lp(a) and second messenger nucleotides were measured. The markers selected are suited to provide a comprehensive risk profile for the prevention of arteriosclerosis and point-of-care theranostics [1,5,7].

In a number of investigations [1,3-10], a good correlation has been observed between *in vivo* biomarkers and risk factors, on one hand, and lipoprotein risk factor deposition to HS-PG under accelerated *in vitro* conditions. While the latter could most realistically be performed with endothelial cells adhered to methylated silica surfaces for ellipsometry and microscopy, analogous results were found with simpler and more robust endothelial model surfaces, in which HS-PG was simply adsorbed to methylated silica surfaces [13]. As seen in Fig. 2, application of 1.25 mmol/L oxLDL to the cell-based substrate and following addition of Krebs solution to a final Ca^{2+} concentration of 10.08 mmol/L entails the expected nanoplaque formation with the native syndecan receptor (HS/CS-PG) of the endothelial cells as reflected by an increase in Ψ and decrease in Δ (Fig. 2C). In this case, the arteriosclerotic nanoplaques consist of the ternary aggregational complex HS/CS-PG – oxLDL – Ca^{2+}. Furthermore, we succeeded in proving the oxLDL binding to the native syndecan and perlecan receptor

molecules of human endothelial cells and vascular matrices with a secondary antibody (Fig. 2A,B). For this purpose, the silica surfaces were overgrown – as in the ellipsometry experiment – with a confluent monolayer of primary cultured endothelial cells.

With the present study, we wanted to evaluate the applicability of the molecular model for nanoplaque formation in the clinical situation. Although there was no change in bulk lipid concentrations after ginkgo medication in the patients, it is demonstrated in Fig. 3 how nanoplaque generation can be reduced. The lipid docking mechanism to HS-PG changed after drug treatment, and the figure shows how the first gradual and then steep increase in nanoplaque formation and size was largely blocked by treatment with ginkgo. Since nanoplaque formation is a Ca^{2+} driven process, a complete Ca^{2+} titration curve was measured. As one can easily see, the stepwise increase in adsorbed amount and layer thickness upon Ca^{2+} additions of 10.08 and 17.64 mmol/L in the baseline measurement is strongly reduced after ginkgo treatment. Calculating the percent reduction in nanoplaque formation and size at each Ca^{2+} concentration used, the mean value of all experimental points during the Ca^{2+} period in question was related to the mean value of all experimental points during the VLDL/IDL/LDL binding period ($[Ca^{2+}]$ = 0 mmol/L). The extent of average inhibition in nanoplaque formation (nanoplaque size) was for the different Ca^{2+} concentrations [mmol/L]: *2.52* -14.3%; $p < 0.004$ (-23.4%; $p < 0.001$); *5.04* -16.2%; $p < 0.001$ (-30.4%; $p < 0.001$); *7.56* -20.0%; $p < 0.001$ (-34.0%; $p < 0.001$); *10.08* -26.7%; $p < 0.001$ (-43.2%; $p < 0.001$); *17.64* -54.1%; $p < 0.001$ (-71.1%; $p < 0.001$).

In cardiovascular high-risk patients it became clear that the reduction of oxLDL/LDL was decisively involved in nanoplaque diminution [cf. 10,24]. In the present metabolic syndrome study, changes in nanoplaque formation were well correlated to changes in oxLDL/LDL ratio ($r = 0.83$; $p < 0.0210$). That is why we searched for further biomarkers and actuators of oxidative stress and inflammation which could evoke changes in oxLDL/LDL ratio and nanoplaque formation. Before discussing specific correlations between the biomarkers, their averaged changes after ginkgo therapy shall be provided: SOD +17.7% ($p < 0.009$), GPx +11.6% ($p < 0.001$), oxLDL/LDL -21.0% ($p < 0.002$), MPO -29.6% ($p < 0.013$), 8-*iso*-$PGF_{2\alpha}$ -39.8% ($p < 0.002$), IL-6 -12.9% ($p < 0.041$), hs-CRP -39.3% ($p < 0.004$), ALP -14.8% ($p < 0.003$), CREA -11.3% ($p < 0.001$), URAC -10.6% ($p < 0.004$), insulin -9.4% ($p < 0.042$), HOMA-IR -14.0% ($p < 0.024$). Table 1 demonstrates a selection of such biomarkers and clinical parameters from the interwoven network illustrated in Fig. 1 in their correlation to

ΔoxLDL/LDL and Δnanoplaque formation. It is striking that the markers which directly influence the formation of oxLDL had the highest correlation coefficients.

Since ΔoxLDL/LDL is strongly correlated with Δ8-*iso*-$PGF_{2\alpha}$ (Table 1), which is a potent vasoconstrictor besides MPO (Fig. 1) [25] and which is likewise tightly correlated to ΔMPO ($r = 0.90$; $p < 0.0003$), an association between blood pressure and oxLDL/LDL was to be expected. In fact, Fig. 4 shows that changes of the diastolic and systolic blood pressure are closely coupled with changes of the oxLDL/LDL quotient.

On the whole, the results show that the interdependencies between the biomarkers in Fig. 1 and additional clinical routine values are insufficiently represented. Apart from the correlations to changes in oxLDL/LDL and nanoplaque formation summarized in Table 1, the following interorgan associations shall be mentioned for the characterization of the complicated network: ΔGPx vs. ΔSOD ($r = 0.55$, $p < 0.0630$); ΔCREA vs. ΔSOD ($r = -0.61$, $p < 0.0723$); ΔIL-6 vs. ΔALP ($r = 0.79$, $p < 0.0068$); Δhs-CRP vs. ΔALP ($r = 0.63$, $p < 0.0492$); Δinsulin vs. ΔALP ($r = 0.69$, $p < 0.0389$); ΔHOMA-IR vs. ΔALP ($r = 0.72$, $p < 0.0447$). Including as one additional example the relationship between insulin resistance (ΔHOMA-IR) and uric acid (ΔURAC) with $r = 0.73$ and $p < 0.03$, it becomes clear that also renal diseases have to be included into diseases elicited by oxidative stress. Therefore, in the integrative scheme of Fig. 5 the multifaceted symptoms of metabolic syndrome targeting polyorganic impairment in their causal relationship to oxidative stress and inflammation are itemized in an organ-specific characteristic.

4 Discussion

As markers of oxidative stress, 8-*iso*-$PGF_{2\alpha}$, MPO, the ratio oxLDL/LDL, and the activity of SOD and GPx were determined (Fig. 1). The inflammatory status was characterized by hs-CRP, MPO, IL-6 and Lp(a). Furthermore, MMP-9 was measured as a relatively new marker to assess plaque stability. Lp(a) is placed at a prominent site in the midst of the network of oxidative stress and inflammation parameters. These markers were deliberately chosen because various correlations and mutual inductions during atherogenesis became apparent and contribute to the general view of a risk assessment. MPO catalyzes hydrogen peroxide being formed upon ‘detoxification’ of superoxide anions by SOD, with chloride ions to

hypochlorous acid (HOCl), which is a potent antibacterial agent [26]. Thus, MPO is part of the physiologic defence mechanism. Moreover, MPO contributes to the enzymatic modification of LDL particles in macrophages [27]. Coincidently, it is correlated to the formation of MMP-9. MMP-9 in patients suffering from an acute coronary syndrome seems to be directly correlated to hs-CRP [28,29]. This is an indication towards a close connection between inflammatory status and plaque stability [30].

As mentioned, MPO and hs-CRP constitute important inflammation markers; MPO also modulates intravascular signalling cascades. It oxidizes NO after its endothelial transcytosis and has a leukocyte-activating and cytokinic effect. That is why MPO has recently been designated as a key protein for the establishment of a pro-inflammatory environment [31]. Moreover, MPO is implicated in plaque stability via a direct correlation to MMP-9. Thus, the circle is completed between ROS status and plaque stability [32].

In the present clinical trial investigating the effectiveness of ginkgo extract on lipoprotein subfractions in patients with metabolic syndrome was examined. The blood of the patients was taken before and after 2 months therapy with 2 × 120 mg ginkgo daily, and the *in vivo* concentration of the VLDL/IDL/LDL-fraction applied in our assay [cf. 33,34]. The arteriosclerotic nanoplaque as represented by the ternary complex of the HS-PG receptor, lipoprotein particles, and calcium ions, was then pursued by increasing the Ca^{2+} concentration (Fig. 3). This ternary aggregational formation on endothelial cell membranes and vascular matrices may mimic the arteriosclerotic "primary lesion" by causing endothelial dysfunction with its restricted NO release [35], the very earliest stage of atherogenesis on a molecular level before any cellular reactivity [4]. As already pointed out, nanoplaque formation and size were substantially diminished. On the one side, these effects were clearly due to a significantly reduced lipoprotein oxidation and, on the other side, the latter was strongly associated with a reduction in nanoplaque formation (cf. Table 1).

Patients with metabolic syndrome have already *per definitionem* a diverse symptom complex which can ultimately lead to massive cardio-cerebro-vascular events. In the center, there is an abundant ROS production (Fig. 1). In order to approach the aim of a personalized medicine, a spectrum of more than 20 cytokinic, inflammatory, lipidic, diabetic, arteriosclerotic and oxidative stress biomarkers and clinical parameters, all interconnected more or less with each other, served for a detailed diagnosis and therapy monitoring. Nor should it be forgotten that

the biomarkers are in part simultaneously actuators as well. Through the ginkgo therapy, which reduces lipid peroxidation by one fifth, the balance between the biomarkers was newly adjusted, and the correlation between the parameters became visible (Table 1). As can be seen, some biomarkers are negatively correlated to both ΔoxLDL/LDL and Δnanoplaque formation. In the case of SOD and GPx this is plausible: the higher their radical scavenging activities are, the larger is the reduction of oxLDL/LDL and thus of nanoplaque formation. MPO and Lp(a) are obviously counterregulated in the sense of a cytokine balance [36,37]. Changes in one biomarker can be expected to be most effective with changes in the other for their middle ranges. In general, eliminating the dependent variable and then correlating ΔoxLDL/LDL with Δnanoplaque formation, the association is positive, as measured by us. Several of these biomarkers and actuators characterize and participate in polyorganic impairment (Fig. 5), as, e.g., ALP which can be increased in liver, gastrointestinal and cerebral (blood-brain barrier [38]) diseases. Furthermore, as we have seen, ΔALP is directly correlated with ΔoxLDL/LDL, ΔIL-6, Δhs-CRP, Δinsulin and ΔHOMA-IR. Thus, the circle is completed also to pancreatic diseases (Fig. 5).

Previous clinical trials reported a blood pressure lowering effect of statins in hyperlipidemic hypertensive patients who were not taking any antihypertensive drugs [39] and who had already received antihypertensive therapy [40-42]. The mechanisms of the reduction of blood pressure by statins in these patients included the activation of endothelial nitric oxide synthase [43], the downregulation of angiotensin II type 1 receptors [44], and a reduction in the vascular production of ROS [45]. As already pointed out, ROS generation does not only play a decisive role in hypertension, but also in lipid peroxidation [46], inflammation [47], atherogenesis [48] and cardio-cerebro-vascular events [11,49-51]. Therefore, we found that changes in BP_{dias} and BP_{sys} were closely linked to changes in oxLDL/LDL ratio (Fig. 4) [52]. Moreover, BP_{dias} was lowered significantly by 5.2 ± 2.3 mm Hg ($p < 0.0479$), while BP_{sys} did not change. The correlations depicted in Fig. 4 show significant positive linearities allowing for 17 mm Hg regulation breadth. This means that in the oxLDL/LDL range concerned here, the metabolic syndrome patients could in principle have varied their oxLDL/LDL quotient and thus their blood pressure by 17 mm Hg in total through simple measures like lifestyle changes.

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Figure legends

Fig. 1. Starting from the metabolic syndrome as a complex of multiple symptoms, the pathophysiologic development of cardiovascular events is represented via atherogenic processes in their chronology (➡). Furthermore, the essential biomarkers (→) are depicted as resultant products (→) and target quantities (→) of these pathologic processes in their cross-linkage and correlation (↔).● Biomarker and measurable variable; ■ Clinical pictures and sequelae; ● Tissue factors and radical status; CAD, coronary artery disease; ACS, acute coronary syndrome; AMI, acute myocardial infarction; TIA, transitory ischemic attack; AD, Alzheimer dementia; ROS, radical oxygen species; MIF, macrophage migration inhibiting factor; TF, tissue factor (modified from [23]).

Fig. 2. Hydrophobized silica surface overgrown with a confluent monolayer of human umbilical vein endothelial cells. Immunofluorescence microscopy was executed utilizing anti-human mouse monoclonal LDL antibody as primary antibody against the cytoplasm of the human endothelial cells. *A:* After addition of oxLDL (1.25 mmol/L), Cy2 goat anti-mouse IgG served as secondary antibody to visualize oxLDL and oxLDL clusters. The cell surfaces are more or less homogeneously covered with oxLDL, but some oxLDL aggregates (⇐) can be clearly seen. The magnification was 40×. *B:* Same as in *A*, but without addition of oxLDL (control). *C:* Ellipsometric response (Ψ ○ and Δ □) on addition of oxLDL to the cell-based substrate. The first and second arrows indicate addition of oxLDL to a final concentration of 1.25 mmol/L and addition of $CaCl_2$ to a final concentration of 10.08 mmol/L, respectively (modified from [16]).

Fig. 3. Total adsorbed amount (A) and layer thickness (B) versus time. At time zero, HS-PG (0.1 mg/mL) was adsorbed on hydrophobic silica from a Ca^{2+}-free Krebs solution. The first arrow indicates the addition of the plasma VLDL/IDL/LDL fraction at its *in vivo* concentration from a metabolic syndrome patient either untreated (○, black curve) or after 2 months treatment with daily 2 × 120 mg Ginkgo biloba extract (○, red curve). Total Ca^{2+} concentrations in solution are indicated at the arrows. The thick, solid lines were computed by an iterative parameter fit of the nonlinear allosteric-cooperative, simple saturative or exponential kinetics to the experimental points using an algorithm for least-squares estimates. The pH was 7.38 (○) and 7.20 (○), respectively.

Fig. 4. Significant correlations between changes in BP_{dias} (A) as well as BP_{sys} (B) and changes in oxLDL/LDL ratio from metabolic syndrome patients treated with ginkgo extract for two months.

Fig. 5. Multifacet symptoms of metabolic syndrome targeting polyorganic impairment. Significant origin is oxidative stress (ROS) with its inflammation.

Table 1. Correlations between changes in various biomarkers and changes in oxLDL/LDL as well as nanoplaque formation from metabolic syndrome patients treated with ginkgo extract for two months. Pearson's correlation coefficient r and statistical significance p were calculated.

	ΔoxLDL/LDL [U/g]		Δnanoplaque formation [mg·m^{-2}]	
	r	p	r	p
ΔSOD [U/mL]	-0.45	n.s.	-0.86	<0.0121
ΔGPx [U/mL]	-0.61	<0.0358	-0.68	<0.0458
ΔMPO [ng/mL]	-0.84	<0.0094	-0.77	<0.0444
Δ8-*iso*-$PGF_{2\alpha}$ [nmol/L]	0.99	<0.0001	0.72	<0.0299
ΔLp(a) [mg/dL]	-0.84	<0.0176	-0.64	<0.0451
Lp(a) (before) [mg/dL]	0.70	<0.0399	0.78	<0.0223
Δhs-CRP [mg/L]	0.78	<0.0390	0.69	<0.0561
ΔALP [U/L]	0.62	<0.0399	0.19	n.s.
ΔHOMA-IR [mU/L×mg/dL]	0.77	<0.0368	0.75	<0.0325
ΔGGT [U/L]	0.53	n.s.	0.45	n.s.
ΔCREA [µmol/L]	0.33	n.s.	0.80	<0.0296

Table 1

Graphical abstract

Highlights

- In a clinical trial, metabolic syndrome (MS) patients were treated with *Ginkgo biloba*.
- Ellipsometry, photometric methods and ELISAs were applied for biosensor profiling of MS risk, status and treatment outcome.
- After medication, biomarkers of oxidative stress, inflammation and arteriosclerosis were significantly diminished.
- Multiple correlations unravelled the network of biomarker interactions and demonstrated its usefulness in personalized medicine.

*Graphical Abstract (for review)

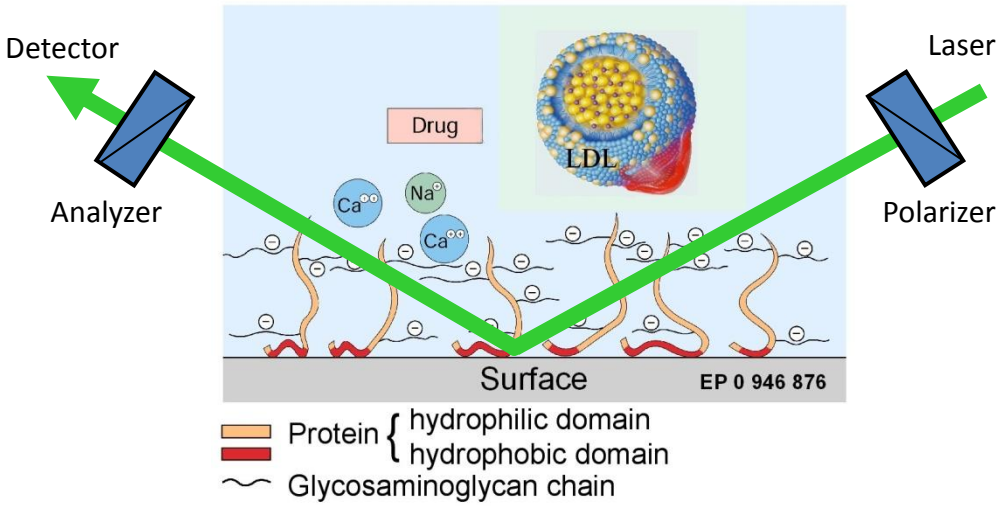


Figure 1

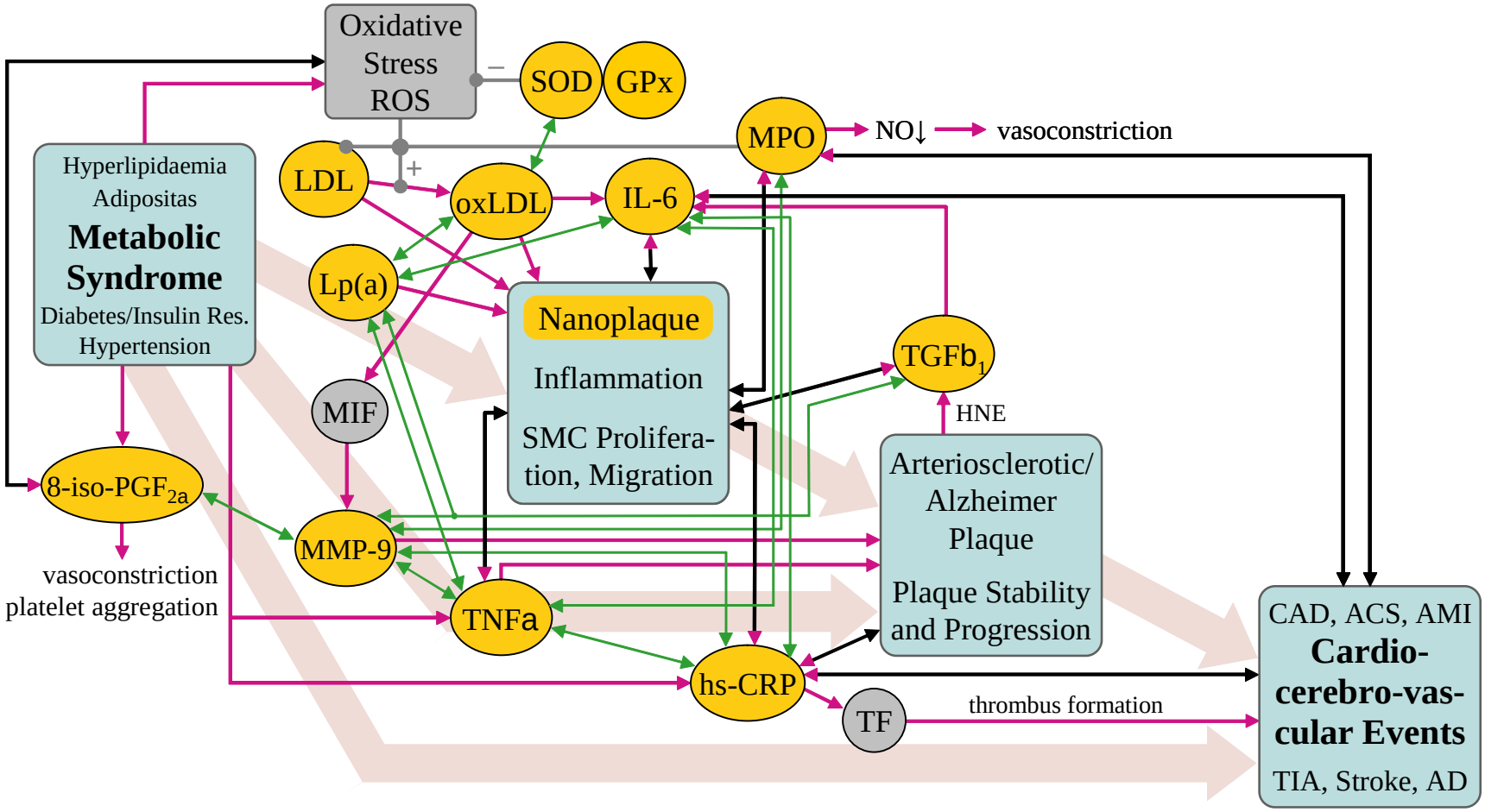


Figure 1

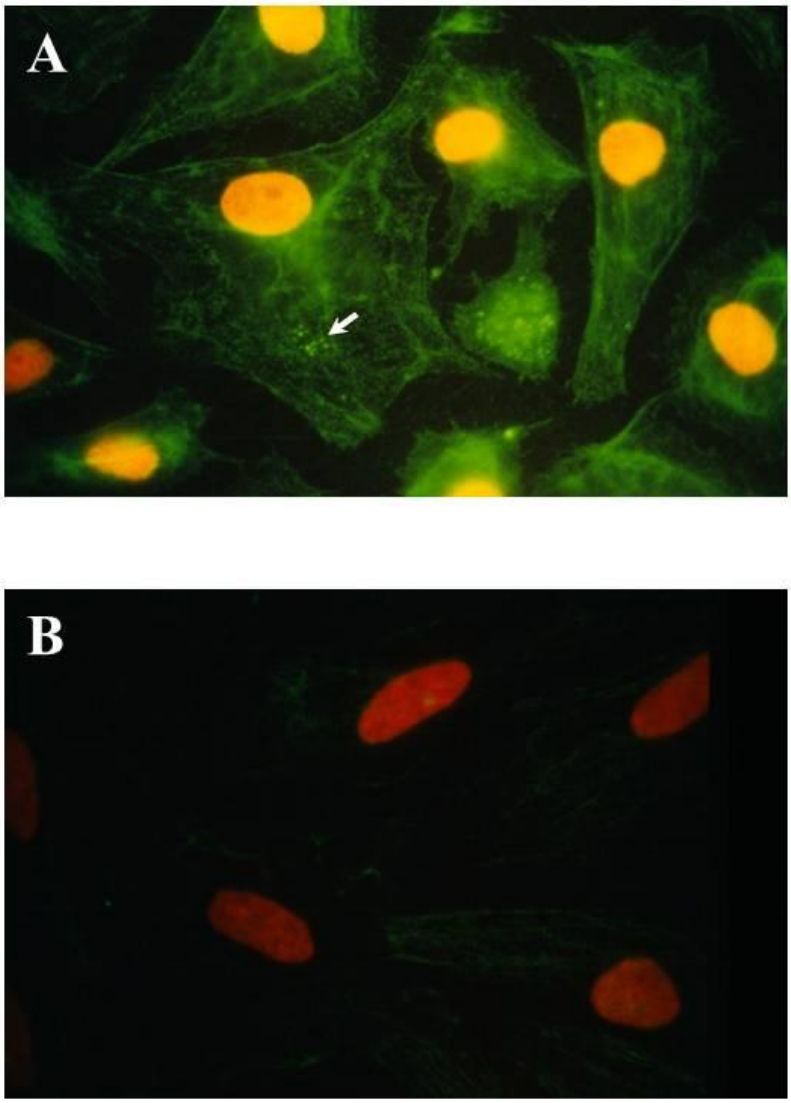
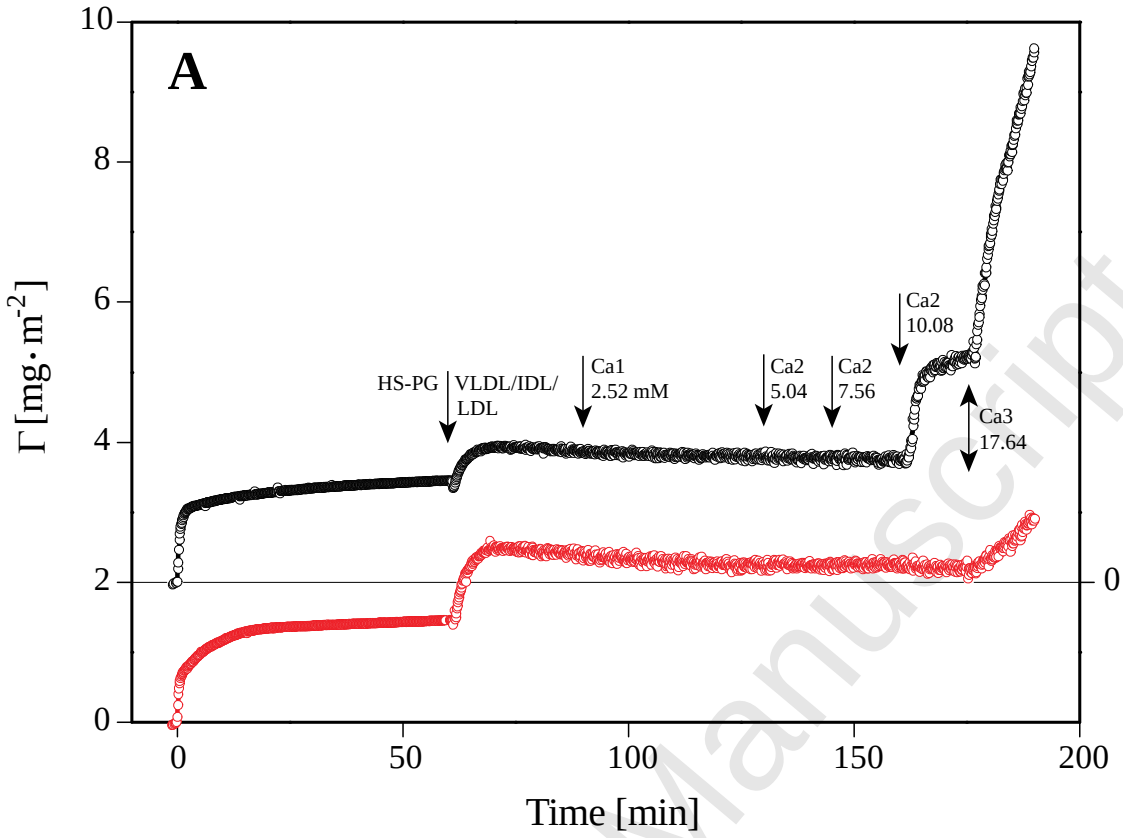
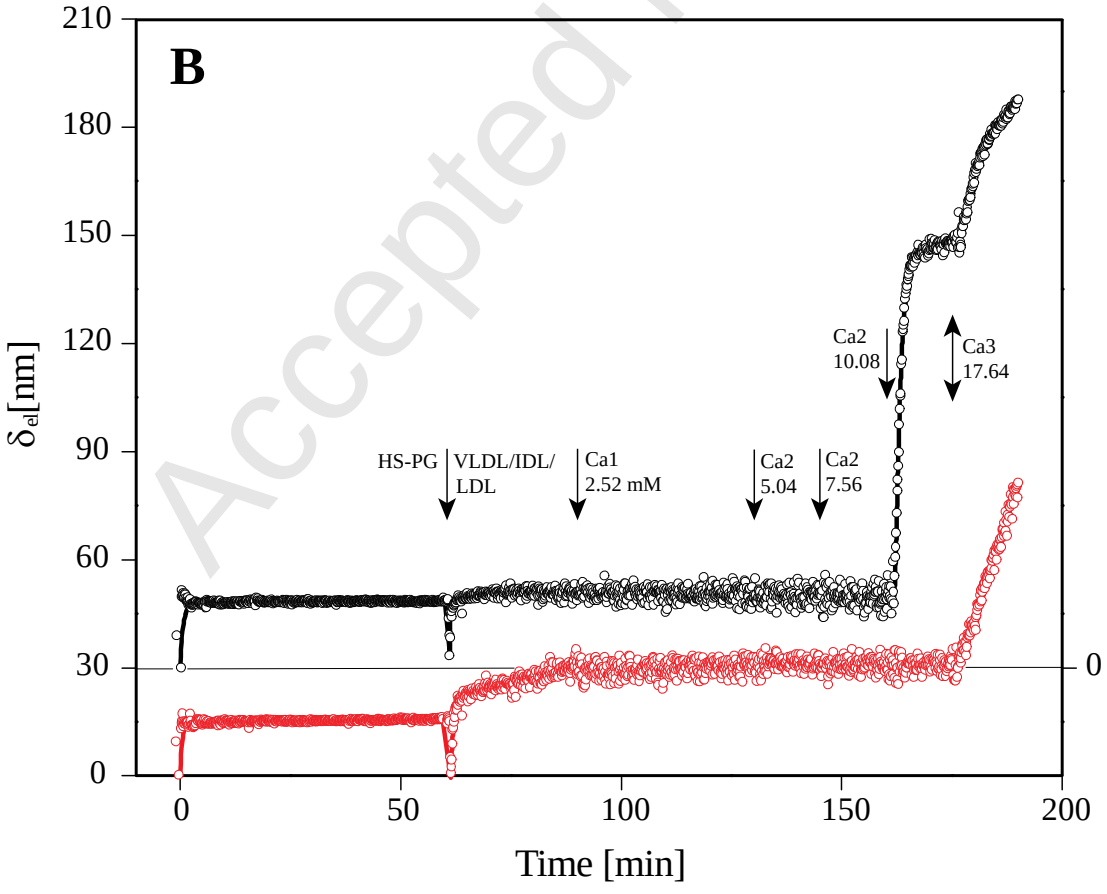


Figure 2





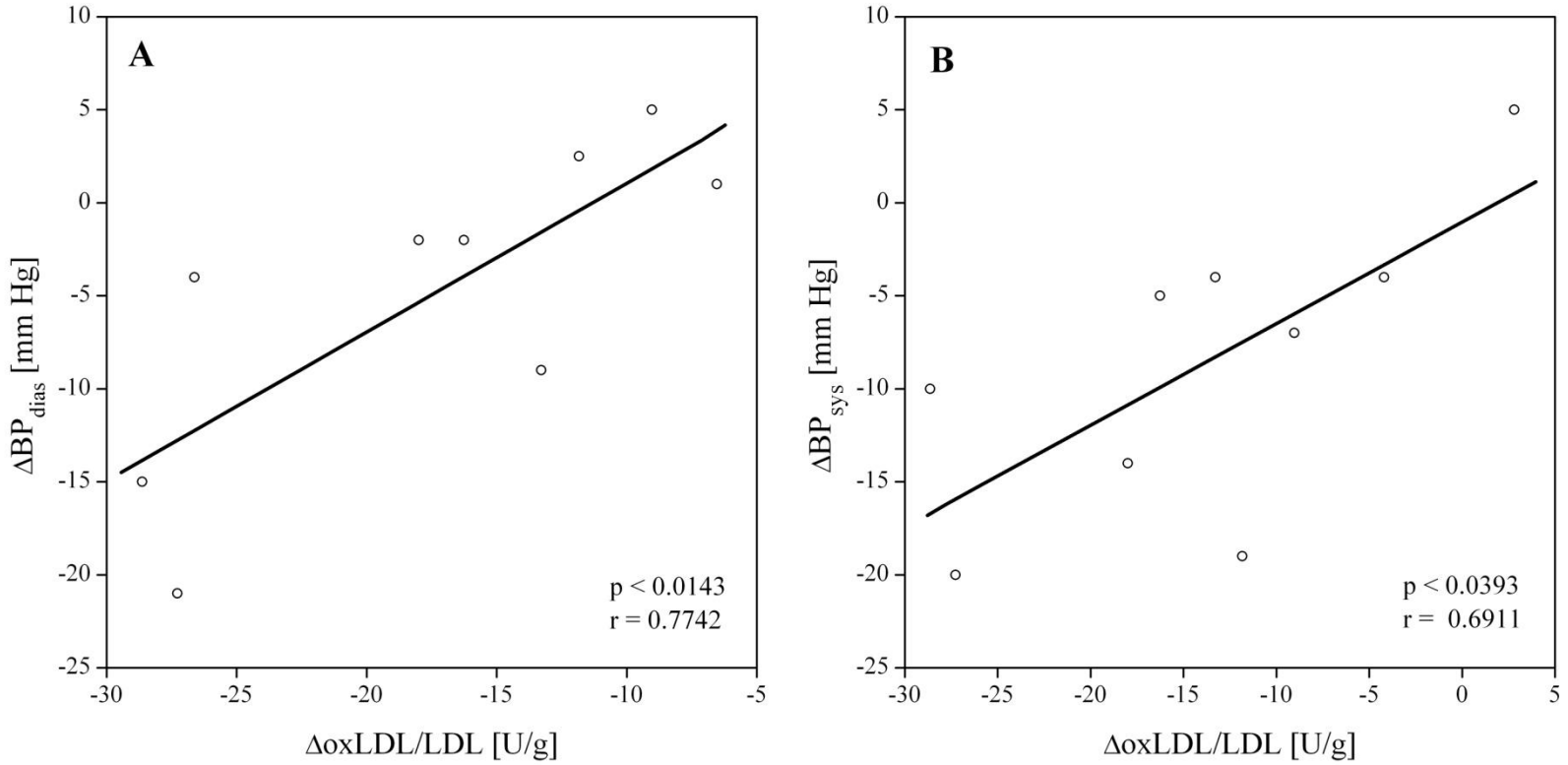


Figure 4

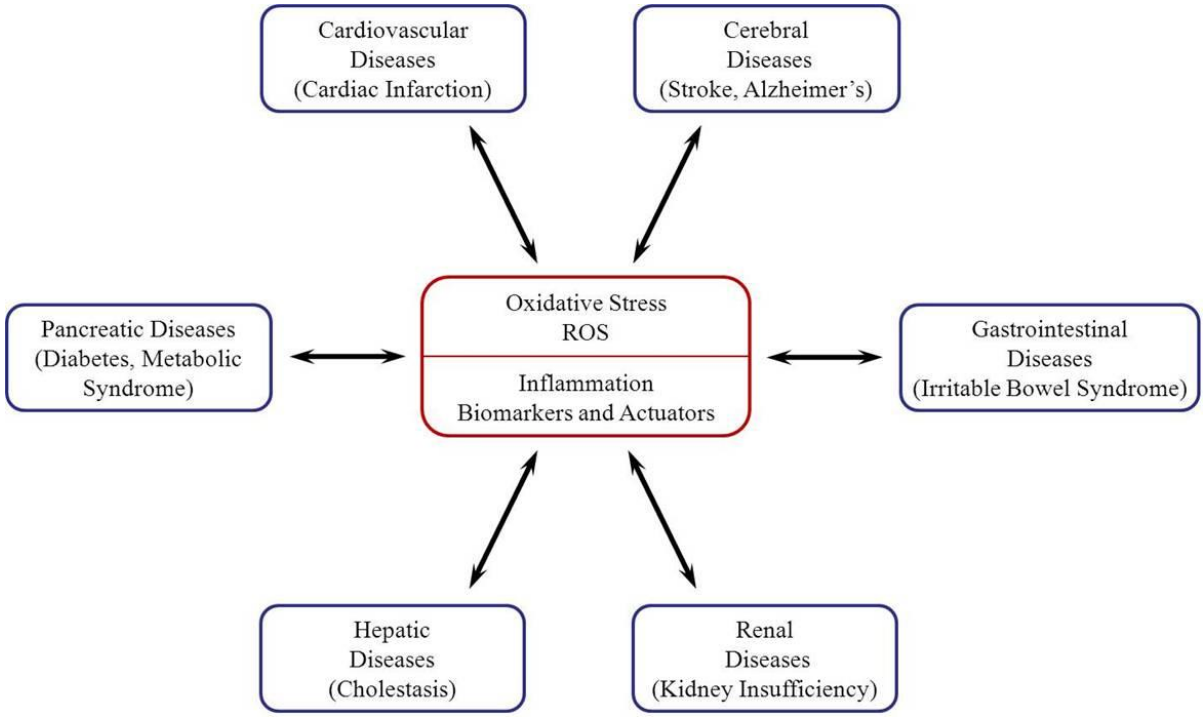


Figure 5