

ORIGINAL ARTICLE

Relationship between serum bilirubin and uric acid to oxidative stress markers in Italian and Czech populations

Libor Vitek^{1,2}, Ladislav Novotný³, Aleš Žák², Barbora Staňková², Tomáš Zima¹, Angela Polito⁴, Greco Cesare⁵, Chiara Zerbinati⁶, Luigi Iuliano⁶

¹Institute of Medical Biochemistry and Laboratory Diagnostics; ²4th Department of Internal Medicine; ³Institute of Hygienic Medicine and Medical Epidemiology, 1st Faculty of Medicine of the Charles University in Prague, Prague 2, Czech Republic

⁴Food and Nutrition National Research Institute, Rome, Italy

⁵Department “Attilio Reale”, Sapienza University of Rome, Rome, Italy

⁶Department of Medical Sciences & Biotechnology, Sapienza University of Rome, Latina, Italy

Received 31st October 2012.

Revised 14th December 2012.

Published online 19th December 2012.

Summary

Recently, a South-to-North oxidative stress marker gradient has been reported; consistent with known differences in the incidence of coronary heart disease between southern and northern European countries. The aim of the present study was to compare the plasma concentrations of 7-oxocholesterol (7OxCH) and 7 β -hydroxycholesterol (7BCH) with systemic antioxidants in healthy Italian and Czech subjects. The study was performed in healthy subjects of Italian (n=131) and Czech (n=84) origins. In all subjects routine biochemistry work-ups were performed; additionally, plasma oxysterols and the peroxy radicals scavenging activity (PERSA) of the sera were determined. Close relationship of serum bilirubin and uric acid to markers of oxidative stress was observed in both examined populations. Compared to the Czechs, the Italian population showed higher plasma concentrations of both oxysterols (7OxCH: 3.6 vs. 6.0 ng/ml, $p < 10^{-6}$; 7BCH: 5.3 vs. 8.6 ng/ml, $p < 10^{-6}$), lower PERSA ($p < 10^{-6}$), and lower serum concentrations of bilirubin and uric acid ($p < 10^{-6}$ in both cases). The dietary patterns of the Italian population did not match the Mediterranean style, but was more similar to the Continental type of diet, presumably due to non-adherence to a Mediterranean diet.

Key words: oxidative stress; oxysterols; bilirubin; uric acid; Mediterranean diet

Abbreviations: CVD, cardiovascular disease; oxLDL, oxidatively modified LDL; 7OxCH, 7-oxocholesterol; 7BCH, 7 β -hydroxycholesterol; FRAP, ferric reducing ability of plasma, PERSA, peroxy radicals scavenging activity; ROS, reactive oxygen species; TEAC, Trolox equivalent antioxidant capacity

✉ Libor Vitek, Institute of Medical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Na Bojišti 3, 120 00 Praha 2, Czech Republic

✉ vitek@cesnet.cz

☎ +420 224 964 203

☎ +420 224 964 203

INTRODUCTION

Oxidative stress largely contributes to atherogenesis, as evidenced by lipid and protein oxidation in the vascular wall of affected subjects (Stocker and Keaney 2004). Oxidatively modified LDL (oxLDL), which is a surrogate marker of upregulated oxidative stress, promotes diverse potentially pro-atherogenic events and is a hallmark of atherosclerosis and cardiovascular disease (CVD) development (Stocker

and Keaney 2004, Tsimikas et al. 2010). Recently, an increased South-to-North gradient in circulating oxLDL concentrations, which paralleled the differences in mortality and incidence rates of CVD, has been reported among the European countries (Menotti et al. 2000, Grau et al. 2007). However, unexpectedly high concentrations of oxLDL were reported in the Italian population, compared to the other European countries (Stocker and Keaney 2004).

The process of LDL oxidation results in the progressive oxidation of lipid moieties in lipoproteins, which are responsible for the oxidative modification of apolipoprotein B, with the associated atherogenicity (Iuliano 2001). Among the lipids transported within the LDL, cholesterol is prone to oxidative stress-dependent modification, leading to a number of oxidized products, which are named oxysterols. These products are known to exert a multitude of biological effects of potential pathophysiological relevance, which are dependent on the position of the additional oxygen in the cholesterol molecule. These effects are mediated by direct biophysical effects on the membranes and/or stereospecific interactions

with proteins. Oxysterols can be viewed as the end products of reactive oxygen species (ROS)-dependent reactions and as a part of the cellular machinery that governs the integrity and function of the cell by acting at the level of signaling and translational and post-translational gene expression (Iuliano 2011). Oxysterols exert a multitude of biological effects of potential pathophysiological relevance, in multiple disease settings, including atherosclerosis (Bjorkhem and Diczfalusy 2002). Among the oxysterols class, 7-oxocholesterol (7OxCH) and 7 β -hydroxycholesterol (7BCH) (Fig. 1), which are major components of oxLDL (Vejux and Lizard 2009), have received the greatest amount of attention as surrogate markers for non-invasive *in vivo* measurement of oxidative stress. Their measurement in biological matrices is made available by sensitive and specific mass spectrometric methods. In the vasculature, 7OxCH and 7BCH induce oxidative pathways, stimulate ROS generation; and do so in a loop that perpetuates lipid peroxidation, with potential deleterious effects on the formation and evolution of atherosclerotic lesions (Vejux and Lizard 2009).

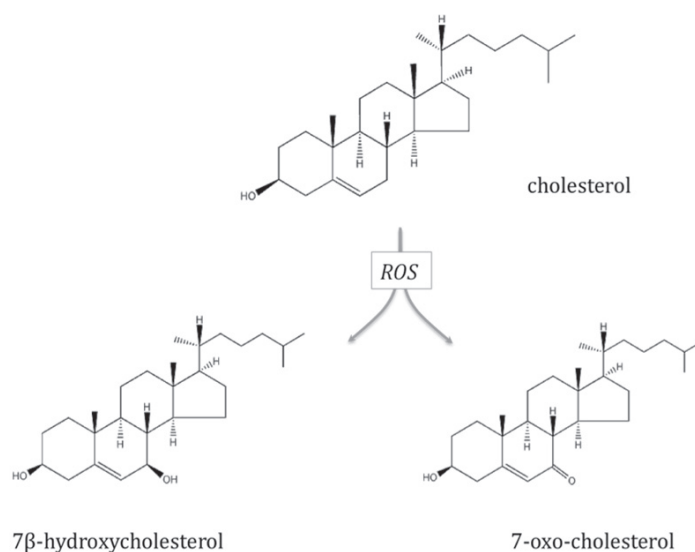


Fig. 1. Structure of 7-oxo-cholesterol and 7 β -hydroxycholesterol.

To counteract ROS produced in the human body, several antioxidant defense systems have evolved, including enzymatic pathways as well as potent antioxidant substrates. Among these, bilirubin, the major intravascular product of heme catabolic pathway, is among the most potent of the circulating endogenous antioxidants (Frei et al. 1988). Consistently, a direct correlation between serum bilirubin and total antioxidant capacity has

consistently been reported in subjects with Gilbert syndrome (Vitek et al. 2002), as well as in newborns exhibiting neonatal jaundice (Gopinathan et al. 1994, Hammerman et al. 1998). Further evidence on the contribution of bilirubin to total antioxidant capacity was provided in a recent study by Bulmer et al. (2008), who demonstrated close correlation between serum bilirubin and Trolox equivalent antioxidant capacity (TEAC), as well as ferric reducing ability of plasma

(FRAP), and provided the first mechanistic evidence in humans for protection from lipid oxidation in serum. Finally, a negative association between serum bilirubin and CVD has been reported in diverse studies (Vitek et al. 2002, Novotny and Vitek 2003, Vitek and Schwertner 2007, Lin et al. 2010); and low serum bilirubin concentrations (<10 µmol/l) have been recognized as a negative prognostic factor for CVD (Novotny and Vitek 2003).

The aim of the present study was to compare plasma concentrations of 7OxCH and 7BCH, as well as other systemic antioxidants in healthy Czech and Italian subjects; additionally, to assess whether these parameters are associated with one another.

MATERIAL AND METHODS

Subjects

The clinical study was performed in 215 healthy Caucasian subjects of Italian and Czech origins. The oxidative stress status was evaluated in 131 clinically healthy Italian consecutive subjects presenting to their general practitioners, and in 84 Czech individuals recruited between 2006 and 2007. The Italian participants were enrolled in two towns of

Calabria Province (Rosarno and Bova). These are non-metropolitan rural towns in the south of Italy, in which the inhabitants are presumed to be adherent to the Mediterranean-style diet. General practitioners used a random recruitment from their databases of healthy individuals. The Czech individuals were clinically healthy subjects, representative of the general population, recruited from employees of the University General Hospital as well as from employees of a large administrative institution in Prague.

All subjects in both cohorts were of Caucasian ancestry. The other eligibility criteria included being in the age range of 20–70 years, no chronic medication, and/or no known chronic underlying diseases (such as: CVD, hypertension, diabetes, cancer, inflammatory and neurodegenerative diseases, psychiatric illnesses requiring chronic drug treatment, endocrine, kidney and liver diseases, any chronic drug use, and nutritional and/or antioxidant supplements in the 90 days prior to enrollment). The basic characteristics of both studied populations are given in Table 1.

Peripheral venous blood was obtained from all participants after overnight fasting. The serum and plasma samples were then removed and stored at –80 °C until analysis.

Table 1. Basic characteristics and major biochemical risk markers for atherosclerosis of both studied populations.

	Italian (n=138)	Czech (n=84)	P-value
Age (years)	47 38.8–60	44 38–51	0.11
M:F ratio	0.48	0.60	1.0
Body mass index (kg/m ²)	26.2 23.4–29.6	26.4 25.1–29.4	0.521
Smoking (%)	30	19	1.0
Total cholesterol (mmol/l)	4.88 4.10–5.48	5.46 4.82–5.94	<10 ⁻⁶
LDL cholesterol (mmol/l)	2.98±0.79	3.16±0.83	0.126
HDL cholesterol (mmol/l)	1.29 1.11–1.47	1.59 1.34–1.85	<10 ⁻⁶
Triglycerides (mmol/l)	1.10 0.84–1.69	1.19 0.85–1.57	0.738

Data was expressed as mean±SD, or median and 25–75%, depending on data normality.

The nutritional intake of the main dietary components, based on 1 normal week's self-monitoring, was assessed in all subjects. The nutritional data in the Czech subjects were analyzed by Nutrimaster SE, version 1.0. The nutritional data in the Italian subjects were analyzed as previously reported (Polito et al. 2005).

The study's protocol conformed to all ethical guidelines of the 1975 Declaration of Helsinki, reflected in the *a priori* approval by the institution's Ethics Committee. Additionally, all subjects signed informed consent forms before participation in the study.

Methods

All standard serum biochemical markers were determined on an automatic analyzer (Modular analyzer, Roche Diagnostics GmbH, Germany), using routine laboratory assays. The blood samples were immediately processed and analyzed accordingly.

The serum peroxy radical scavenging capacity (PERSA) was determined, using a fluorimetric method, as the relative proportion of chain breaking antioxidant consumption present in the serum compared to that of Trolox (a reference and calibration antioxidant compound) (Iuliano et al. 2000). The plasma concentrations of oxysterols and of α -tocopherol (as the major vitamin E isotopomer) were analyzed by mass spectrometry, using isotope dilution methods as previously reported (Iuliano et al. 2003). The samples were immediately stored at -80°C and analyzed within 12 months; in separate analyses the samples were found sufficiently stable for this period of time. Due to the association between oxysterols and vitamin E with cholesterol, the results are also presented as relative values corrected for the cholesterol concentration (Traber and Jialal 2000).

Statistical analyses

The data are presented as the mean \pm SD, or the median and 25–75% interquartile range. Data were evaluated by t-test or Rank Sum test depending on their normality. For some of the parametric analyses, the serum bilirubin and oxysterol values were transformed, in order to comply with requirements for both normality and equal variance. A chi-square test was used to evaluate the sex distribution and smoking status in both cohorts. To assess the relationship between serum bilirubin and uric acid concentrations, as well as other variables related to oxidative stress, the subjects were divided into subgroups according to the quartiles of serum bilirubin and uric acid concentrations. These associations were tested by

ANOVA on Ranks with Dunn's post hoc analysis. Linear regression analyses were used to assess the association between serum bilirubin, plasma oxysterol concentrations, and PERSA, nonlinear regression analysis was used for assessment of the relationship between PERSA and uric acid concentrations. Spearman correlation analysis was used to analyze the association of PERSA with selected variables. The contribution of serum bilirubin concentrations on PERSA was analyzed by logistic regression, with multiple adjustments for possible confounding factors. All tests were made at the significance level $2\alpha=0.05$. All statistical analyses were performed using SigmaPlot software, version 11.0 (Systat Software, Inc., USA).

RESULTS

Basic characteristics of the studied populations

The basic characteristics of the studied populations are described in Table 1. Both cohorts were of the same distributions for age and sex, as well as having comparable body mass indexes (BMI) and smoking prevalence.

Serum blood lipids in Italian and Czech subjects

Compared to the Czech subjects, the Italians had substantially lower total cholesterol concentrations. This difference was mainly due to substantially lower HDL cholesterol concentrations ($p<10^{-6}$, Table 1); while LDL cholesterol concentrations were comparable between both cohorts (Table 1).

Oxidative stress status in Italian and Czech subjects

Compared to the Czech cohort, significantly higher plasma concentrations of both 7OxCH and 7BCH were found in the Italian population ($p<10^{-6}$, Table 2). To eliminate possible confounding effect of total cholesterol levels, both plasma oxysterols were adjusted for this initial oxysterol substrate. Similarly, the ratios of both 7OxCH and 7BCH to total cholesterol were much higher in the Italian population ($p<10^{-6}$, Table 2). In accordance, the Italian subjects showed a lower plasma PERSA activity ($p<10^{-6}$, Table 2), and substantially lower concentrations of the major endogenous serum antioxidants bilirubin and uric acid compared to the Czech subjects.

In contrast, no differences in plasma vitamin E concentrations (adjusted to total cholesterol concentrations) were detected between the two populations (Table 2).

Table 2. Plasma oxysterols and some serum antioxidants in the healthy Italian and Czech subjects.

	Italian (n=138)	Czech (n=84)	P-value
7OxCH (ng/ml)	7.3 5.3–11	4.0 3.3–5.2	<10 ⁻⁶
7OxCH/total cholesterol	1.59 1.3–2.6	0.76 0.59–0.94	<10 ⁻⁶
7BCH (ng/ml)	10.5 7.7–19.7	6.0 5.3–7.4	<10 ⁻⁶
7BCH/total cholesterol	2.28 1.6–4.3	1.15 0.99–1.3	<10 ⁻⁶
PERSA*	5.75 4.8–6.90	6.95 5.7–8.5	<10 ⁻⁶
Bilirubin (μmol/l)	7.2 5.1–10.2	11.3 8.6–15.6	<10 ⁻⁶
Uric acid (μmol/l)	271 215–329	324 252–364	<10 ⁻⁶
Vitamin E/total cholesterol	0.13 0.11–0.17	0.14 0.1–0.19	0.612

7OxCH: 7-oxo-cholesterol; 7BCH: 7β-hydroxycholesterol; PERSA: peroxy radical scavenging activity. Bilirubin means total bilirubin concentration. Data expressed as median and 25–75%; * values represent micromoles of Trolox equivalents.

Association of total antioxidant capacity with antioxidant substrates and oxysterols

To substantiate the contribution of bilirubin and uric acid to the antioxidant defense system, we tested the correlation of these variables with PERSA, which is based on a dynamic *in vitro* fluorescence test for measuring global antioxidant activity. The association of total antioxidant capacity with selected laboratory markers was analyzed for both studied cohorts, as

well as for the entire population (Table 3). PERSA correlated positively with uric acid and bilirubin; whereas, only a weak correlation was found with the plasma α-tocopherol/total cholesterol ratio (Table 3). The lack of a stronger correlation between PERSA and α-tocopherol was expected because α-tocopherol is not an efficient reductant in the aqueous system used in the PERSA test (Iuliano et al. 2000).

Table 3. Association of total antioxidant capacity with antioxidant substrates.

PERSA vs.	Italian (n=138)	Czech (n=84)	Total
Bilirubin	0.330 (9×10 ⁻⁵)	0.207 (0.059)	0.355 (7×10 ⁻⁸)
Uric acid	0.595 (2×10 ⁻⁷)	0.625 (2×10 ⁻⁷)	0.623 (2×10 ⁻⁷)
Vitamin E/total cholesterol	0.10 (NS)	0.1 (NS)	0.183 (0.01)

Data analyzed by Spearman correlation analysis; results are expressed as a correlation coefficient and p-value (in parentheses). Bilirubin means total bilirubin concentration. NS, not significant.

Based on logistic regression analysis, it was found that with each increased micromole of serum bilirubin there was an almost 10% associated increase in PERSA; even after multiple adjustments for possible confounding factors, including age, sex, smoking, and alcohol intake (OR 1.096, statistically significant). Among the others, only uric acid was found to be another factor independently affecting PERSA. Each micromole increase in serum uric acid concentration

was associated with an almost 2% increase of PERSA ($p < 10^{-6}$).

The positive relationship between PERSA and serum bilirubin concentrations was also corroborated by linear regression analysis, which demonstrated a clear positive association in all subjects, as well as in the separate Czech and Italian populations (Fig. 2a–c). The same relationship was also true for uric acid concentrations, again for both studied cohorts,

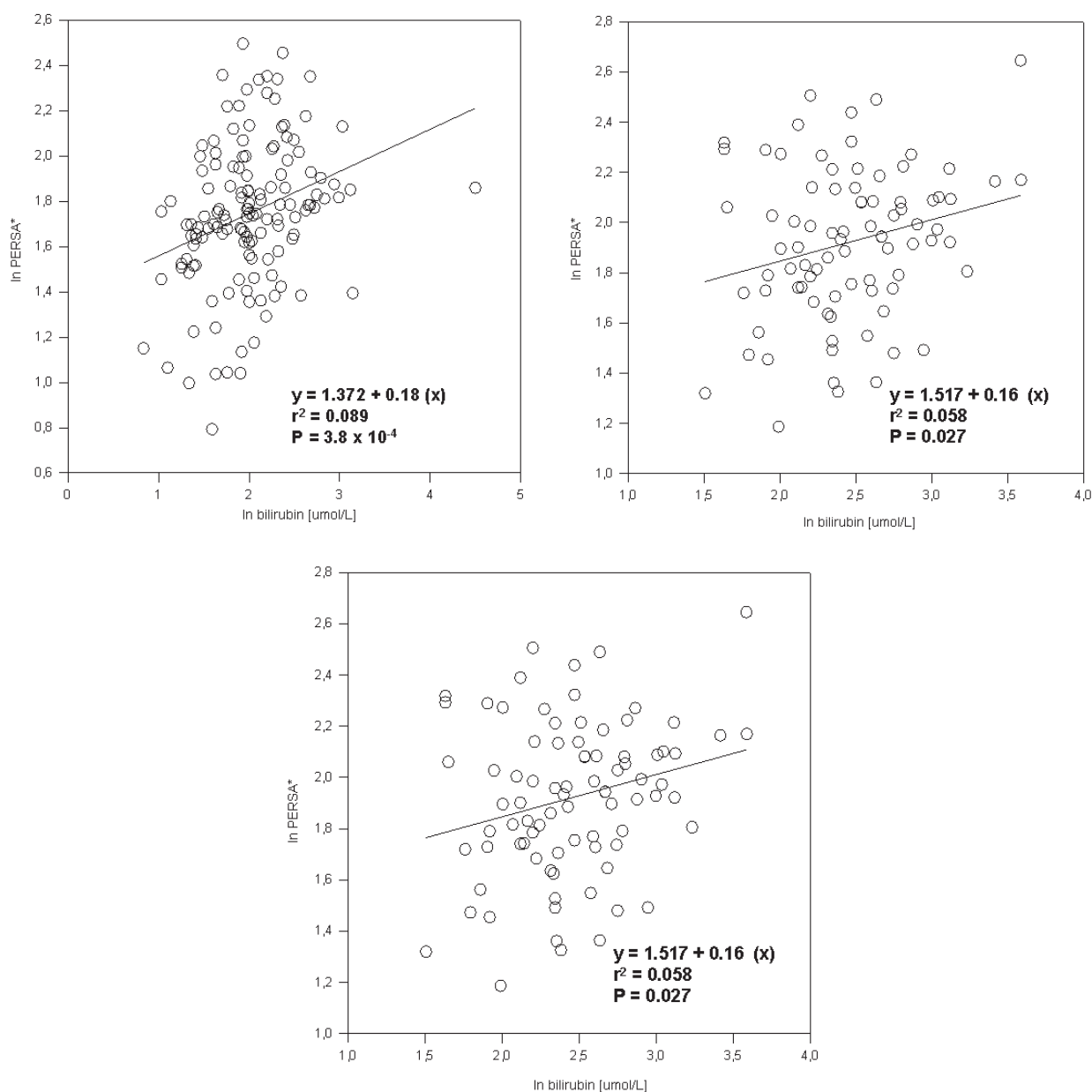


Fig. 2. Relationship between PERSA and serum bilirubin in the Italian subjects (a), Czech subjects (b), and in the whole population (c). Linear regression analysis of log-transformed data was used; * values represent micromoles of Trolox equivalents; PERSA = peroxy radicals scavenging activity.

as well as for the combined populations (Fig. 3a–c). On the other hand, a negative linear relationship was observed between serum bilirubin and both 7OxCH and 7BCH (Fig. 4), and the same trend (although reaching only borderline significance) was seen also for association of uric acid and plasma levels

of both oxysterols (Fig. 5a, b). Similar negative relationships were observed when ratios of plasma oxysterols to total cholesterol were plotted against both antioxidants, indicating that these effects were independent of circulating cholesterol concentrations (data not shown).

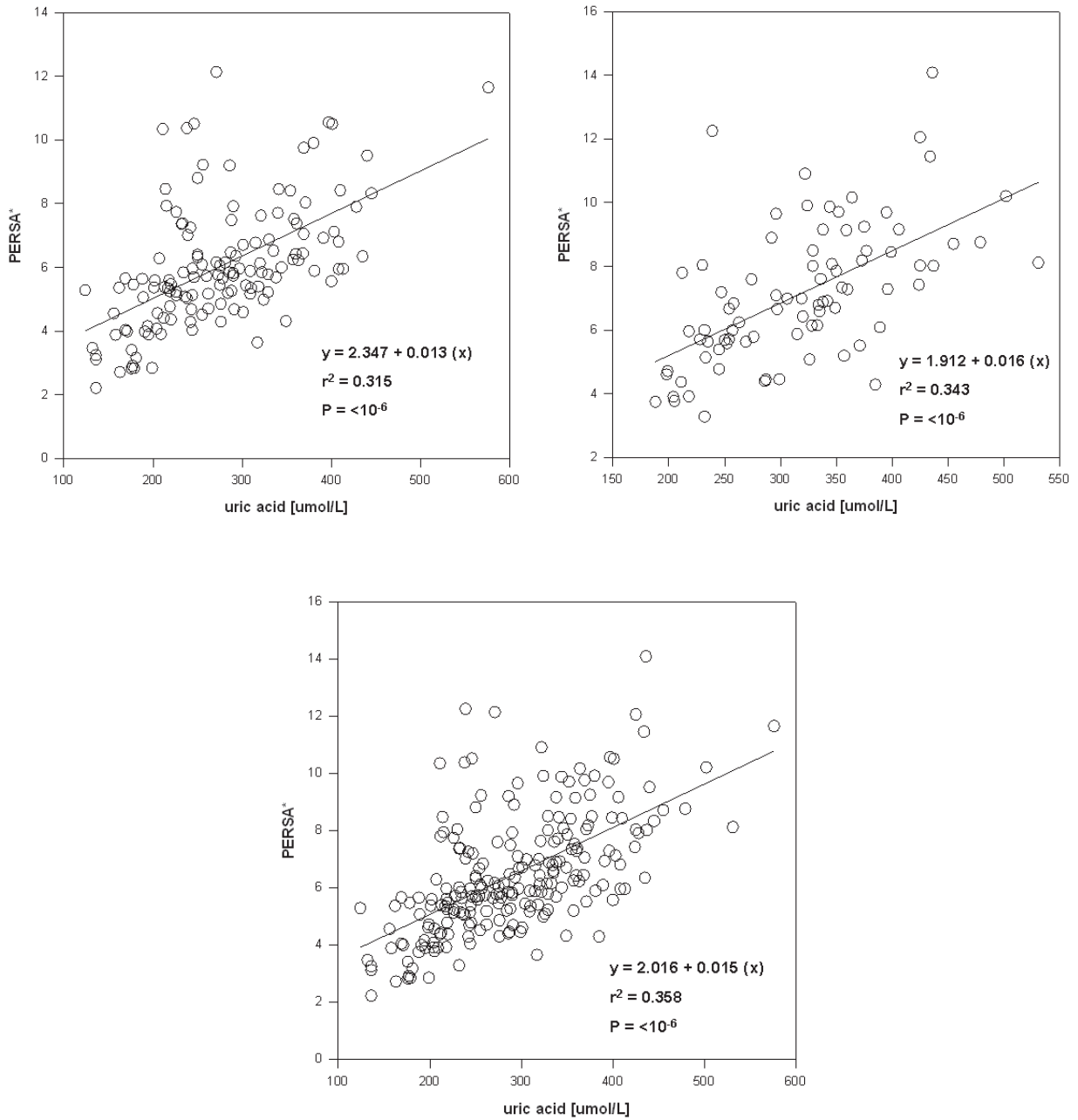


Fig. 3. Relationship between PERSA and serum uric acid in the Italian subjects (a), Czech subjects (b), and in the whole population (c). Nonlinear regression analysis of native data was used. Others symbols as in Fig. 2.

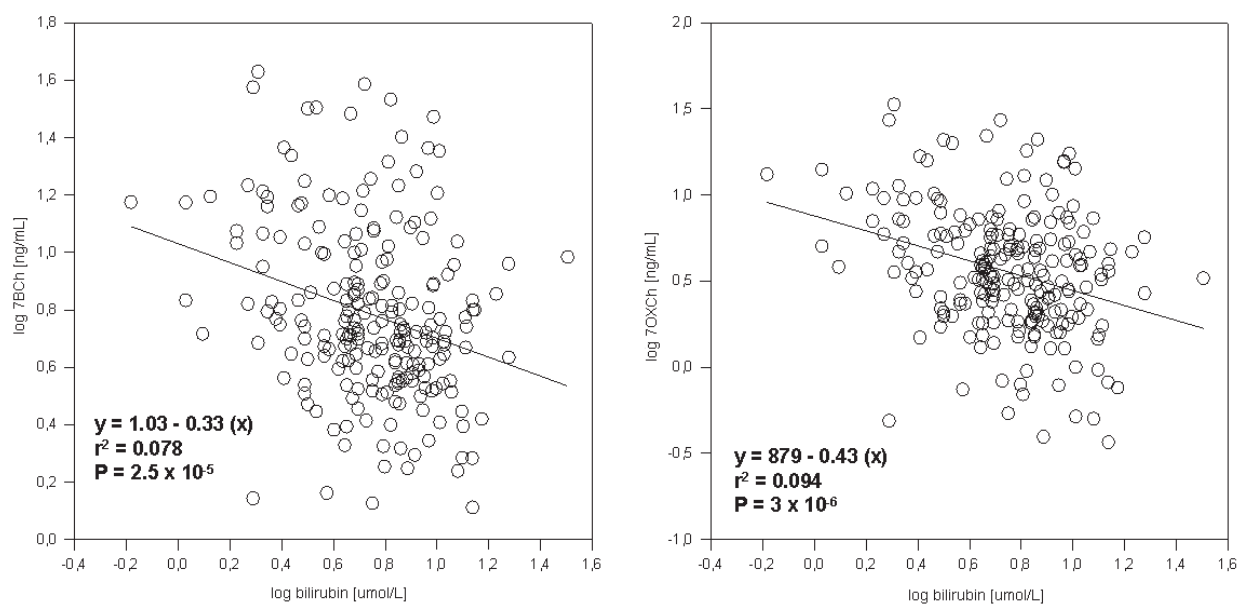


Fig. 4. Relationship between plasma levels of (a) 7β-hydroxycholesterol (b) 7-oxo-cholesterol, and serum bilirubin in both studied populations. Linear regression analysis of log-log transformed data was used.

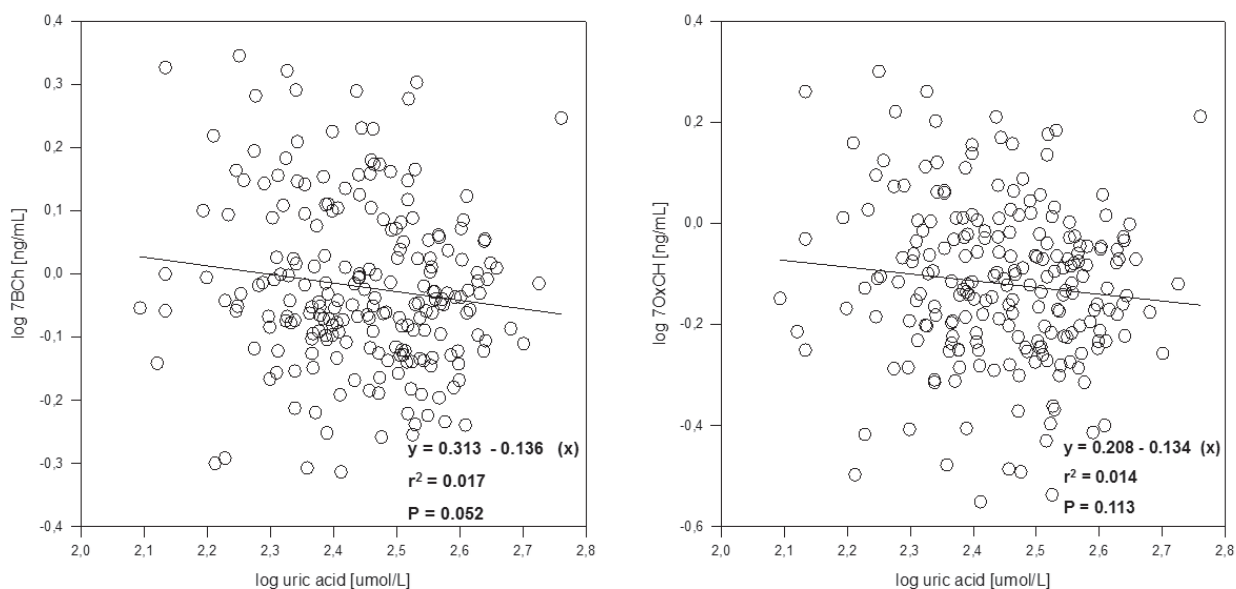


Fig. 5. Relationship between plasma levels of (a) 7β-hydroxycholesterol (b) 7-oxo-cholesterol, and serum uric acid in both studied populations. Linear regression analysis of log-log transformed data was used.

Similarly, when selected antioxidants and oxysterols were analyzed in subgroups, according to serum bilirubin quartiles, it was found that the higher was the serum bilirubin, the higher were both the serum PERSA and uric acid concentrations (Table 4). On the contrary, the concentrations of both oxysterols were negatively associated with serum bilirubin, while no correlation was observed with the vitamin E/total cholesterol ratio (Table 4). When the same analysis was performed in the subgroups according to their serum uric acid concentrations, highly significant positive associations were observed for serum PERSA and bilirubin concentrations; however, this was not so for the plasma oxysterol and vitamin E concentrations (Table 4).

Spearman correlation analysis confirmed a close association between serum bilirubin and uric acid in both the Italian and Czech populations (respective correlation coefficients: 0.275, statistically significant; and 0.299, statistically significant).

Dietary habits

The intake of dietary macronutrients is shown in the Table 5. Apart from the intake of fiber, which was higher in Italians, the two populations did not differ in terms of the total amounts of calories, proteins, lipids, or carbohydrates consumed in their diets. The two populations also had the same mean daily alcohol consumption.

Table 4. Relationship between serum bilirubin and uric acid levels with selected markers of oxidative stress.

	Bilirubin Quartile 1 (<6.4)	Bilirubin Quartile 2 (6.4–8.5)	Bilirubin Quartile 3 (8.5–12.3)	Bilirubin Quartile 4 (>12.3)	P-value
PERSA*	5.4 4.4–6.5	5.8 5–7	6.4** 5.2–8.4	6.9** 5.9–8.1	3×10 ⁻⁵ ***
Uric acid (µmol/l)	255 199–318	257 219–322	297** 239–359	330** 269–389	3.7×10 ⁻⁵ ***
7BCH (ng/ml)	10.7 7.3–35.4	8.5 6.5–11.5	7.2** 5.7–9.7	7.4** 5.4–11.4	10 ⁻³ ***
7OxCH (ng/ml)	8.5 4.7–14.4	5.6** 4.6–7.6	4.7** 3.7–7.3	5.3** 3.6–7.8	<10 ⁻⁶ ***
Vitamin E/total cholesterol	0.13 0.1–0.16	0.13 0.11–0.17	0.15 0.09–0.18	0.16 0.12–0.20	0.774

	Uric acid Quartile 1 (<231)	Uric acid Quartile 2 (231–287)	Uric acid Quartile 3 (287–348)	Uric acid Quartile 4 (>348)	P-value
PERSA*	4.6 3.9–5.5	5.8** 5.1–6.8	6.5** 5.7–7.6	8** 6.4–9.2	<10 ⁻⁶ ***
Bilirubin (µmol/l)	6.8 4.7–9.8	7.4 6.6–11.3	9.7** 7.3–14.4	10.7** 7.4–14.5	10 ⁻⁵ ***
7BCH (ng/mL)	9.5 7–24.6	7.7 6.3–11.2	7.4 5.5–16.3	8.3 6–10.6	0.148
7OxCH (ng/mL)	6.4 4.3–14.3	5.5 4–8.7	5.4 3.5–9.1	5.5 4–7.7	0.291
Vitamin E/total cholesterol	0.13 0.1–0.16	0.15 0.1–0.2	0.14 0.11–0.19	0.14 0.11–0.17	0.454

Data (expressed as median and IQ range) were analyzed by ANOVA on Ranks with Dunn's post hoc analysis vs. quartile 1 data as the reference. Bilirubin and uric acid concentrations are given in µmol/l; * values represent micromoles of Trolox equivalents; ** statistically significant as compared against Q1 in post hoc analysis; *** statistically significant before post hoc analysis.

Table 5. Caloric and macronutrient intake.

	Italian	Czech	P-value
Energy (kJ)	9112±1511	9247±2537	0.158
Protein (g)	79.6±18	74.7±20	0.098
(% of energy)	14.8	13.7	
Total fats (g)	79.6±18	81.6±24	0.63
(% of energy)	32.3	32.6	
Carbohydrates (g)	252.6±54	257.7±79	0.904
(% of energy)	47.1	47.4	
Alcohol intake (g/day)	13.4±14	17±23	0.653
(% of energy)	4.3	5.3	
Dietary fibre (g)	16.4±4.9	10.5±4.7	<10 ⁻⁶
(% of energy)	1.5	11.0	

The data (consumption per day) are given as the mean±SD.

DISCUSSION

In our study, we sought to examine the oxidative stress status in healthy Italian and Czech populations, using plasma levels of 7OxCH and 7BCH, two validated markers of lipid peroxidation *in vivo* (Iuliano et al. 2003), which have been found to be elevated in several clinical conditions characterized by a high systemic oxidative stress status (Iuliano et al. 2003, Larsson et al. 2007). Simultaneously, we assessed the PERSA, a global index of antioxidant defense (Iuliano et al. 2000), as well as the concentrations of α -tocopherol, bilirubin, and uric acid, and assessed the relationships between these antioxidants and oxidative stress markers.

Compared to the Czech cohort, the Italian population showed an upregulation of oxidative stress, reflected by significantly higher concentrations of oxysterols. Consistent with this, the higher oxysterol concentrations paralleled a substantially lower antioxidant status. In fact, both PERSA (which is a marker of global antioxidant activity of plasma), as well as bilirubin and uric acid (two major antioxidant substrates), were reduced in Italians compared to Czechs. Although the relative proportion of males (known to have higher both bilirubin and uric acid levels) was slightly higher in the Czech compared to Italian cohort, this fact cannot account for large differences observed in our study.

Considering the dietary habits, except for fiber intake (which was higher in Italians), the two populations did not differ in terms of the total amount of calories or the absolute amounts of proteins, lipids, carbohydrates, and alcohol consumed in the diet. This picture reveals an Italian dietary habit that is not consistent with the

Mediterranean diet, in which the relative contribution of proteins and lipids should be around 12% and 27%, respectively (Keys et al. 1986). Our data agrees with the opinion that the traditional Mediterranean diet is now progressively eroding due to the widespread dissemination of the Western-type dietary habits (Bach-Faig et al. 2011).

Oxidative stress can be defined as the condition where ROS formation is in an imbalance with the antioxidant defenses. The upregulation of oxidative stress may be due to an unbalanced increase in ROS and/or a decrease in antioxidants. Oxidative stress plays a major role in the pathophysiology of atherosclerosis (Stocker and Keaney 2004), which is the major source of morbidity and mortality in the developed world. 7OxCH and 7BCH are the main bioactive class of oxidized lipids produced during LDL oxidation, and they have several biological activities relevant to atherogenesis, including impairment of endothelial function, cytotoxicity and apoptosis, monocyte differentiation, and foam cell formation, and as well as inhibition of cellular cholesterol deloading (Gelissen et al. 1996). Moreover, increased plasma concentrations of oxysterols have been associated with dyslipidemia and hyperinsulinemia (Alkazemi et al. 2008), and are to be found in patients with severe carotid stenosis (Micheletta et al. 2004).

The upregulation of oxidative stress in healthy Italian subjects are consistent with recent findings demonstrating high oxLDL concentrations in the Italian population, compared to other European countries (Stocker and Keaney 2004). The Czech and Italian populations have different trends in their CVD mortality

rates. A decline in CVD mortality in the East European countries, including the Czech Republic (Cifkova et al. 2010), has been reported during the last 20 years. In contrast, a potential deleterious shift in CVD risk factors in the Seven Country Study populations has been reported (Lanti et al. 2005). These differences are most likely due to the substantial changes in nutritional habits. In Italy, there is an increasing prevalence of diabetes and childhood obesity (Binkin et al. 2010), both conditions being even higher in the south of Italy, from which the subjects of the present study were enrolled.

In this regard, increased markers of oxidative stress observed in the healthy Italian subjects might be due to changes from a Mediterranean to Continental dietary habits. Dietary patterns in the Mediterranean countries are changing rapidly. A dietary shift towards the unhealthy Western-style diet, with increased consumption of saturated fats and refined carbohydrates has already been reported (Kafatos et al. 1997). Non-adherence to a Mediterranean diet correlates well with the total, as well as cardiovascular mortality in these populations (Trichopoulos et al. 2003).

In our study, we have also proved strong negative association between serum bilirubin and oxidative stress markers, in particular plasma oxysterols. It is important to note that lower plasma oxysterols were found in the Czech subjects despite their higher total cholesterol levels suggesting that antioxidant capacity is more important than the concentration of the substrate needed for oxidation. Observed negative relationship of serum bilirubin to plasma concentrations of oxysterols is in accord with recent findings demonstrating lower levels of oxidatively modified LDL (Tapan et al. 2011, Boon et al. 2012, Maruhashi et al. 2012), as well as increased systemic antioxidant capacity (Vitek et al. 2002, Bulmer et al. 2008) in subjects with Gilbert syndrome.

Based on our data, the serum bilirubin and uric acid concentrations seem to be key oxidative stress defense factors, and our findings are in line with recent clinical data (Gopinathan et al. 1994, Novotny and Vitek 2003, Vitek and Schwertner 2007). In fact, mildly elevated serum bilirubin concentrations have consistently been associated with protection from CVD (Vitek et al. 2002, Novotny and Vitek 2003, Vitek and Schwertner 2007, Lin et al. 2010), but also cancer morbidity (Zucker et al. 2004, Jirásková et al. 2012), as well as all-cause mortality (Horsfall et al. 2011).

On the other hand, previous reports on the predictive value of uric acid in the relationship of CVD morbidity were less clear. While many studies concluded that uric acid is an independent risk factor for CVD (for review see (Gagliardi et al. 2009), others have suggested that uric acid is more accurately a marker for other risk factors such as hypertension, insulin resistance, hyperlipidemia, obesity, alcohol abuse, and

metabolic syndrome (Yano et al. 1984, Iribarren et al. 1996); or even dispute such relationships (Beever and Lip 1998). It should also be noted, that uric acid is an important evolutionary antioxidant substitute for the loss of the ability to synthesize ascorbate in higher primates. The strong positive association of serum uric acid concentrations and PERSA, which has been consistently reported by previous studies (Nieto et al. 2000), underpins the large contribution of uric acid to the global antioxidant activity of human plasma (Yeum et al. 2004).

In conclusion, our study demonstrates increased concentrations of oxidative stress markers in healthy Italian subjects, which may well be due to the changes in dietary habits, that have been recorded in recent decades. It is still unsettled whether to attribute this paradox to environmental factors, including a dietary shift from a Mediterranean to a Western-style lifestyle and food habits.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

LV, LL, AZ and TZ designed research; LV, LL, LN, BS, AP, CG and CZ conducted research; LV, LL, BS, AZ, TZ and LN analyzed data; LV and LL wrote the paper; LV had primary responsibility for final content. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from Charles University in Prague (PRVOUK-P25/LF1/2 to LV), Czech Ministry of Health (RVO-VFN64165/2013 to LV), and the Italian Ministry of Environment (to LI). The funding sources have had no role in study design; in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the article for publication.

We also acknowledge the fruitful collaboration of F. Greco (coordinator of subject recruitment), G. Ingegnere and V. Rombolà (blood sample collection). The following general practitioners collaborated by recruiting subjects in Rosarno: F. Barbieri, F. Brosio, M. Crubo, P. Cutri, P. D'Agostino, P. Fabiano, D. Greco,

L. Loiacono, P. Fabiano, G. Pangallo, G. Piromalli, D. Stelitano, and V. Varrà. S. Scordo recruited subjects in Bova. We also wish to thank P. Schreib for providing the Nutrimaster SE software, used for the dietary analysis of the Czech subjects.

REFERENCES

- Alkazemi D, Egeland G, Vaya J, Meltzer S, Kubow S. Oxysterol as a marker of atherogenic dyslipidemia in adolescence. *J Clin Endocrinol Metab.* 93: 4282–4289, 2008.
- Bach-Faig A, Berry EM, Lairon D, Reguant J, Trichopoulou A, Dernini S, Medina FX, Battino M, Belahsen R, Miranda G, Serra-Majem L; Mediterranean Diet Foundation Expert Group. Mediterranean diet pyramid today. Science and cultural updates. *Public Health Nutr.* 14: 2274–2284, 2011.
- Beevers DG, Lip GY. Is uric acid really an independent cardiovascular risk factor? *Lancet.* 352: 1556, 1998.
- Binkin N, Fontana G, Lamberti A, Cattaneo C, Baglio G, Perra A, Spinelli A. A national survey of the prevalence of childhood overweight and obesity in Italy. *Obes Rev.* 11: 2–10, 2010.
- Bjorkhem I, Diczfalusy U. Oxysterols: friends, foes, or just fellow passengers? *Arterioscler Thromb Vasc Biol.* 22: 734–742, 2002.
- Boon AC, Hawkins CL, Bisht K, Coombes JS, Bakrania B, Wagner KH, Bulmer AC. Reduced circulating oxidized LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert syndrome. *Free Rad Biol Med.* 52: 2120–2127, 2012.
- Bulmer AC, Blanchfield JT, Toth I, Fassett RG, Coombes JS. Improved resistance to serum oxidation in Gilbert's syndrome: A mechanism for cardiovascular protection. *Atherosclerosis.* 199: 390–396, 2008.
- Cifkova R, Skodova Z, Bruthans J, Adamkova V, Jozifova M, Galovcova M, Wohlfahrt P, Krajcoviechova A, Poledne R, Stavek P, Lanska V. Longitudinal trends in major cardiovascular risk factors in the Czech population between 1985 and 2007/8. *Czech MONICA and Czech post-MONICA. Atherosclerosis.* 211: 676–681, 2010.
- Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci USA.* 85: 9748–9752, 1988.
- Gagliardi AC, Miname MH, Santos RD. Uric acid: A marker of increased cardiovascular risk. *Atherosclerosis.* 202: 11–17, 2009.
- Gelissen IC, Brown AJ, Mander EL, Kritharides L, Dean RT, Jessup W. Sterol efflux is impaired from macrophage foam cells selectively enriched with 7-ketocholesterol. *J Biol Chem.* 271: 17852–17860, 1996.
- Gopinathan V, Miller NJ, Milner AD, Rice Evans CA. Bilirubin and ascorbate antioxidant activity in neonatal plasma. *FEBS Lett.* 349: 197–200, 1994.
- Grau M, Guxens M, Subirana I, Fito M, Covas MI, Jacquemin B, Sunyer J, Lanki T, Picciotto S, Bellander T, Katsouyanni K, Schneider A, Peters A, Marrugat J. South-to-North gradient in lipid peroxidation in men with stable coronary artery disease in Europe. *Eur Heart J.* 28: 2841–2849, 2007.
- Hammerman C, Goldstein R, Kaplan M, Eran M, Goldschmidt D, Eidelman AI, Gartner LM. Bilirubin in the premature: Toxic waste or natural defense? *Clin Chem.* 44: 2551–2553, 1998.
- Horsfall LJ, Rait G, Walters K, Swallow DM, Pereira SP, Nazareth I, Petersen I. Serum bilirubin and risk of respiratory disease and death. *J Am Med Assoc.* 305: 691–697, 2011.
- Iribarren C, Folsom AR, Eckfeldt JH, McGovern PG, Nieto FJ. Correlates of uric acid and its association with asymptomatic carotid atherosclerosis: the ARIC Study. *Atherosclerosis Risk in Communities. Ann Epidemiol.* 6: 331–340, 1996.
- Iuliano L. The oxidant stress hypothesis of atherogenesis. *Lipids.* 36 Suppl: S41–S44, 2001.
- Iuliano L. Pathways of cholesterol oxidation via non-enzymatic mechanisms. *Chem Phys Lipids.* 164: 457–468, 2011.
- Iuliano L, Piccheri C, Coppola I, Pratico D, Micheletta F, Violi F. Fluorescence quenching of dipyrindamole associated to peroxy radical scavenging: a versatile probe to measure the chain breaking antioxidant activity of biomolecules. *Biochim Biophys Acta.* 1474: 177–182, 2000.
- Iuliano L, Micheletta F, Natoli S, Ginanni C, Iappelli M, Elisei W, Giovannelli L, Violi F, Diczfalusy U. Measurement of oxysterols and alpha-tocopherol in plasma and tissue samples as indices of oxidant stress status. *Anal Biochem.* 312: 217–223, 2003.
- Jirásková A, Novotný J, Novotný L, Vodicka P, Pardini B, Naccarati A, Schwertner HA, Hubáček JA, Puncochářová L, Šmerhovský Z, Vítek L. Association of serum bilirubin and promoter variations in HMOX1 and UGT1A1 genes with sporadic colorectal cancer. *Int J Cancer.* 131: 1549–1555, 2012.
- Kafatos A, Diacatou A, Voukiklaris G, Nikolakakis N, Vlachonikolis J, Kounali D, Mamalakis G, Dontas AS. Heart disease risk-factor status and dietary changes in the Cretan population over the past 30 y: the Seven Countries Study. *Am J Clin Nutr.* 65: 1882–1886, 1997.

- Keys A, Menotti A, Karvonen MJ, Aravanis C, Blackburn H, Buzina R, Djordjevic BS, Dontas AS, Fidanza F, Keys MH et al. The diet and 15-year death rate in the seven countries study. *Am J Epidemiol*. 124: 903–915, 1986.
- Lanti M, Menotti A, Nedeljkovic S, Nissinen A, Kafatos A, Kromhout D. Long-term trends in major cardiovascular risk factors in cohorts of aging men in the European cohorts of the Seven Countries Study. *Aging Clin Exp Res*. 17: 306–315, 2005.
- Larsson H, Bottiger Y, Iuliano L, Diczfalusy U. *In vivo* interconversion of 7beta-hydroxycholesterol and 7-ketocholesterol, potential surrogate markers for oxidative stress. *Free Radic Biol Med*. 43: 695–701, 2007.
- Lin JP, Vitek L, Schwertner HA. Serum Bilirubin and Genes Controlling Bilirubin Concentrations as Biomarkers for Cardiovascular Disease. *Clin Chem*. 56: 1535–1543, 2010.
- Maruhashi T, Soga J, Fujimura N, Idei N, Mikami S, Iwamoto Y, Kajikawa M, Matsumoto T, Kihara Y, Chayama K, Noma K, Nakashima A et al. Hyperbilirubinemia, augmentation of endothelial function and decrease in oxidative stress in Gilbert syndrome. *Circulation*. 126: 598–603, 2012.
- Menotti A, Lanti M, Puddu PE, Kromhout D. Coronary heart disease incidence in northern and southern European populations: a reanalysis of the seven countries study for a European coronary risk chart. *Heart*. 84: 238–244, 2000.
- Micheletta F, Natoli S, Misuraca M, Sbarigia E, Diczfalusy U, Iuliano L. Vitamin e supplementation in patients with carotid atherosclerosis – Reversal of altered oxidative stress status in plasma but not in plaque. *Arterioscl Thromb Vasc Biol*. 24: 136–140, 2004.
- Nieto FJ, Iribarren C, Gross MD, Comstock GW, Cutler RG. Uric acid and serum antioxidant capacity: a reaction to atherosclerosis? *Atherosclerosis*. 148: 131–139, 2000.
- Novotny L, Vitek L. Inverse relationship between serum bilirubin and atherosclerosis in men: a meta-analysis of published studies. *Exp Biol Med (Maywood)*. 228: 568–571, 2003.
- Stocker R, Keaney JF. Role of oxidative modifications in atherosclerosis. *Physiol Rev*. 84: 1381–1478, 2004.
- Tapan S, Karadurmus N, Dogru T, Ercin CN, Tasci I, Bilgi C, Kurt I, Erbil MK. Decreased small dense LDL levels in Gilbert’s syndrome. *Clin Biochem*. 44: 300–303, 2011.
- Traber MG, Jialal I. Measurement of lipid-soluble vitamins – further adjustment needed? *Lancet*. 355: 2013–2014, 2000.
- Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med*. 348: 2599–2608, 2003.
- Tsimikas S, Mallat Z, Talmud PJ, Kastelein JJ, Wareham NJ, Sandhu MS, Miller ER, Benessiano J, Tedgui A, Witztum JL, Khaw KT, Boekholdt SM. Oxidation-specific biomarkers, lipoprotein(a), and risk of fatal and nonfatal coronary events. *J Am Coll Cardiol*. 56: 946–955, 2010.
- Vejux A, Lizard G. Cytotoxic effects of oxysterols associated with human diseases: Induction of cell death (apoptosis and/or oncosis), oxidative and inflammatory activities, and phospholipidosis. *Mol Aspects Med*. 30: 153–170, 2009.
- Vitek L, Schwertner HA. The heme catabolic pathway and its protective effects on oxidative stress-mediated diseases. *Adv Clin Chem*. 43: 1–57, 2007.
- Vitek L, Jirsa M, Brodanova M, Kalab M, Marecek Z, Danzig V, Novotny L, Kotal P. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. *Atherosclerosis*. 160: 449–456, 2002.
- Yano K, Reed DM, Mcgee DL. 10-Year Incidence of Coronary Heart-Disease in the Honolulu Heart Program – Relationship to Biologic and Lifestyle Characteristics. *Am J Epidemiol*. 119: 653–666, 1984.
- Yeum KJ, Russell RM, Krinsky NI, Aldini G. Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. *Arch Biochem Biophys*. 430: 97–103, 2004.
- Zucker SD, Horn PS, Sherman KE. Serum bilirubin levels in the US population: Gender effect and inverse correlation with colorectal cancer. *Hepatology*. 40: 827–835, 2004.