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Biological effect of cigarette smoking in endothelial dysfunction: Study of biomarkers of endothelial function, oxidative stress, inflammation, and lipids

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ABSTRACT

Aims: Tobacco use is involved in endothelial dysfunction, a key marker of cardiovascular diseases. The contribution of tobacco use in their development is assessed by endothelial dysfunction-related biomarkers in smokers and non-smokers.

Methods: 138 smokers and 83 non-smokers were recruited. Parameters reflecting the endothelial function, lipid profile and oxidative and inflammatory status, were quantified. Data were used to determine their ability to differentiate smokers and non-smokers.

Results: Elevation of inflammation and oxidative stress as well as alteration of endothelial function and lipids profile in smokers were observed. Two biomarkers combinations, including one implying only routine parameters, were identified and allowed to correctly classify >84% of cases.

Conclusions: Oxidative status, inflammatory status, and lipids profile were shown altered in smokers, leading to endothelial dysfunction. Endothelial dysfunction-related biomarkers were assessed in terms of their ability to discriminate smokers from non-smokers. The possibility of discrimination based only on classic parameters of blood test appeared conceivable.

Keywords: smoking, endothelial dysfunction, biomarkers, oxidative stress, inflammation, lipids

INTRODUCTION

Tobacco use kills more than 8 million people each year worldwide and remains the most preventable cause of death. It is associated with different pathological conditions and is, especially, the major preventable risk factor for the development and progression of cardiovascular diseases (CVDs), which are the main cause of death globally [1, 2]. Tobacco consumption is responsible for oxidative stress, inflammation, lipid profile and hemodynamic alterations, and hypercoagulability, all being involved in endothelial dysfunction, the first step of CVDs development [3-5].

The effect of tobacco on endothelial dysfunction can be assessed by several techniques more or less invasive: intra-arterial/venous infusion of vasoactive substances, flow mediated dilation (FMD), endothelial cell culture, etc. [6]. Another possibility is the evaluation of different biomarkers related to the endothelial function and associated processes such as oxidative stress, inflammation, and lipid profile modifications [3, 4, 6].

A multitude of biomarkers can be used to reflect the endothelial function, oxidative status, inflammatory status, and lipids [6-9]. Among them, some are commonly used to assess endothelial dysfunction and are already shown altered by tobacco smoking. Different studies have reported an increased level of inflammatory biomarkers, including C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor alpha (TNF-α) in smokers [4, 10, 11]. On the other hand, level of IL-10, an anti-inflammatory cytokine, is shown lower in smokers [4, 11]. This elevation of inflammation is associated with the increase of leukocyte-endothelial cell interactions mediated by the soluble vascular cell adhesion molecule (sVCAM) and the soluble intercellular adhesion molecule

Table 1. Baseline characteristics

	Smokers (n=138)	Non-smokers (n=83)
Age, years	55 (44-64)	55 (38-64)
Male, n (%)	64 (46.38)	40 (48.19)
BMI (kg/m²)	25.0 (21.2-28.8)	26.5 (24.6-28.9)
COPD, n (%)	10 (7.25)	0 (0)

Note. Data are expressed as median (interquartile range) or n (%); BMI: Body mass index; & COPD: Chronic obstructive pulmonary disease

(sICAM). Both are shown at higher concentration in smokers than in non-smokers [10]. Although the involved mechanisms remain not clear, tobacco smoking seems to lead to dyslipidemia: research have indicated that smokers have higher cholesterol, triglycerides, and low-density lipoprotein (LDL) levels as well as lower high-density lipoprotein (HDL) level [10]. Oxidative modification, including oxidation of LDL, is also increased in smokers. Products of lipid peroxidation, such as malondialdehyde (MDA), and anti-oxidized LDL (oxLDL) antibodies are reported at higher concentrations in smokers [10, 12]. Oxidative stress in smokers is also associated with weaker antioxidant defenses: vitamins C and E levels are found lower than in non-smokers [4, 12].

Although some parameters have been addressed, the influence of smoking on other ones remains unknown. The aim of this study is to propose a more extensive study of endothelial dysfunction-related biomarkers in smokers versus non-smokers. Oxidative stress is evaluated through the quantification of some antioxidants (vitamins A/E/C, β-carotene, coenzyme Q10, uric acid), pro-oxidants or indicators of oxidation (iron, MDA, anti-oxLDL antibodies), and regulatory enzymes (ferritin, transferrin, glutathione peroxidase (GPX), superoxide dismutase [SOD]).

Inflammatory status is estimated by the concentration measurement of high-sensitivity CRP (hsCRP), monocyte chemoattractant protein 1 (MCP-1), IL 6/8/10, and TNF-α. Asymmetric dimethylarginine (ADMA)–interferent in the production of the vasodilator nitric oxide (NO), sVCAM, and sICAM levels are quantified to reflect the endothelial function. Lipid profile is monitored by the concentration quantification of cholesterol, HDL, LDL, triglycerides, lipoprotein (a) (Lp[a]), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), and small dense LDL cholesterol (sdLDL). For the first time to our knowledge, these biomarkers are also addressed in terms of their ability in discriminating between smokers and non-smokers. Their discrimination features are compared with the ones of five tobacco exposure biomarkers: nicotine and its metabolites–cotinine and trans-3'-hydroxycotinine (3HC)–anatabine and anabasine. Finally, the combination of several biological markers–other than tobacco–to distinguish the two groups is evaluated.

MATERIALS AND METHODS

Participants

As shown in **Table 1**, 138 active cigarette smokers, 64 men and 74 women between 26 to 80 years of age, were recruited at the tobaccology unit of the Centre Hospitalier Universitaire of Catholic University of Louvain (Namur, Belgium). 10 of them suffered from chronic obstructive pulmonary disease (COPD) without acute pathology.

The average consumption was 19 cigarettes per day (range: three-60) and the time since last cigarette varied from 0.5 to two hours. 83 healthy long-term non-smokers, 40 men and 43 women between 21 to 83 years of age, were recruited on a voluntary basis through advertisement in the same hospital. The potential presence of clinical pathology that may influence the endothelial function was addressed based on participants' health database and the analysis of confounding factors for the two groups. Informed consent was provided from all participants. The project was approved by the hospital ethics committee and was led according to the Declaration of Helsinki and the good clinical practice guidelines [13].

Clinical Protocol

Blood samples and urine from smokers and non-smokers were collected to measure biological parameters of interest. Several aliquots (urine, heparin whole blood, and EDTA whole blood) were taken and immediately frozen at -20°C for later batch analyses. Blood tubes were then centrifuged (15 minutes, 5±3°C, 5950 g, SL16R, Thermo Scientific, USA) to collect serum, sodium fluoride plasma and heparinized plasma. Analyses were performed on this fresh material or on serum/plasma aliquots stored at -20°C for chromatographic assays and enzyme linked immunosorbent assays (ELISA).

Instruments for Quantitative Analyses

VITROS® 5600 System and VITROS® 3600 System from Ortho Clinical Diagnostics (Raritan, USA) were used for measurement of some parameters directly after blood collection and centrifugation.

The chromatographic assays were performed using a high-performance liquid chromatography (HPLC) system coupled with an ultraviolet (UV) detector (Alliance 2695, detector 2489, Waters, Milford, USA) and an ultra-high performance liquid chromatography (UHPLC) system (Acquity UPLC® H-Class), coupled with a photodiode array detector (Acquity PDA), fluorescence detector (Acquity FLR), or a mass detector (Acquity QDa). Acquisition and process software was Empower 3 (Waters). Runs were carried out in batch and systems were calibrated each day of analysis.

ELISA assays were also performed in batch and required a microplate strip washer (ELx50, Agilent, Santa Clara, USA) for washing steps and an absorbance microplate reader (800TS, Agilent) for readings.

Other batch analyses were performed on BN ProSpec® System (Siemens Healthcare, Saint-Denis Cedex, France).

Biological Parameters Evaluation

Smoking status

The exhaled carbon monoxide (CO) amount was measured for smokers by electrochemistry with a Smokerlyzer® monitor (Bedfont Scientific Ltd, Harrietsham, UK). Five tobacco biomarkers (nicotine, cotinine, 3HC, anatabine, and anabasine) were quantified in urine by a previously described method (UHPLC-mass detection) [14]. These parameters were quantified for smokers but also for non-smokers to validate the absence of tobacco biomarkers.

Table 2. Median concentrations of tobacco biomarkers in smokers vs. non-smokers

	Smokers: Median (interquartile range) (n=116)	Non-smokers: Median (interquartile range) (n=47)	p
CO (ppm)	18 (13-23)*	-	-
Cotinine (µg/ml)	1.21 (0.85-1.71)*	0.01 (0.01-0.01)	<0.0001
Nicotine (µg/ml)	0.581 (0.227-1.114)	0.055 (0.055-0.055)	<0.0001
3HC (µg/ml)	3.72 (1.58-6.07)	0.02 (0.02-0.02)	<0.0001
Anatabine (ng/ml)	5.595 (2.013-11.286)	0.029 (0.029-0.029)	<0.0001
Anabasine (ng/ml)	1.785 (0.559-7.440)	0.029 (0.029-0.137)	<0.0001

Note. CO: Carbon monoxide; 3HC: *Trans*-3'-hydroxycotinine; & *n=137

Oxidative stress

Serum iron and uric acid concentrations were assessed by colorimetry on VITROS 5600. Serum ferritin was measured by immunoassay on VITROS 3600, and serum transferrin was quantified on VITROS 5600 by turbidimetry (reagent pack TFTUR-C00, DiAgam, Ghislenghien, Belgium).

GPX and SOD were both analyzed on VITROS 5600 in batch, through an enzymatic method (kit RANSEL, Randox, Crumlin, UK) for GPX in heparin whole blood and through a colorimetric activity kit (kit RANSOD, Randox, Crumlin, UK) for SOD in EDTA whole blood.

HPLC connected with an UV detector allowed to detect vitamin C in plasma, coenzyme Q10 and β-carotene in serum. Respective chromsystems kits (Munich, Germany) were used. Chromsystems kits also allowed the extraction of vitamin A, vitamin E, and malondialdehyde from plasma. Detection was performed with an UHPLC system, using an UHPLC reverse phase column (Acquity UPLC HSS T3 1.8 µm 2.1 X 50 mm, Waters) with a flow rate of 0.6 ml/min and photodiode array detection (scan 210-400 nm) for vitamins and an UHPLC reverse phase column (Luna Omega 1,6 µm C18 100Å 100X2,1 mm, Phenomenex, Torrance, USA) with a flow rate of 0.3 ml/min and fluorescence detection (excitation wavelength: 515 nm; emission wavelength: 553 nm) for malondialdehyde.

Anti-oxLDL antibodies in serum were assessed by ELISA kit (Biomedica Medizinprodukte GmbH, Wien, Austria).

Inflammatory status

MCP-1, IL-6, 8, and 10 were measured in serum through ELISA kits from Abcam B.V. (Amsterdam, Netherlands). TNF-α was assessed in serum by an ELISA kit from Tecan (IBL International GmbH, Hamburg, Germany). High-sensitivity (hs) CRP was quantified in serum by nephelometry using the BN ProSpec.

Endothelial function

ADMA, sVCAM, and sICAM were measured in serum by ELISA kits (MT-Diagnostics B.V., Etten-Leur, Netherlands).

Lipid profile

Cholesterol, HDL, LDL, and triglyceride were assessed in serum by enzymatic methods on VITROS 5600. Lp(a), ApoA1, ApoB were quantified by the BN ProSpec by nephelometry. sdLDL was measured with an ELISA kit (Cubasio, Houston, USA).

Confounding factors

Glycemia, glutamic-pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), γ -glutamyl transferase (GGT), creatinine, albumin, and magnesium tests were performed on VITROS 5600. Matrix was fresh serum for all tests, except sodium fluoride plasma for glycemia. Glycohemoglobin (HbA1c) was quantified on fresh EDTA whole blood by an automated analyzer based on HPLC (ADAMS A1c HA-8180V, Arkray, Amstelveen, Netherlands).

Statistical Analyses

Normality of the data was rejected by Shapiro-Wilk test. Therefore, non-parametric tests were used, and results were expressed by median with interquartile range into bracket. The biomarkers comparison in smokers versus non-smokers was performed by Wilcoxon rank sum test. Differences were considered significant when $p<0.05$. Logistic regressions were carried out with backward selection of variables. The diagnostic value of significant variables was assessed by sensitivity (Se), specificity (Sp), positive (PPV) and negative predictive values (NPV), Youden index [15], and area under (AUC) the receiver operating characteristic (ROC) curves. PPV and NPV were calculated based on a prevalence of smoking of 20% [16]. All analyses were performed using MedCalc® (version 20.109, MedCalc Software Ltd, Ostend, Belgium).

RESULTS

Evaluation of the Smoking Status

The smoking status of enrolled participants was self-reported and confirmed by the CO measurement and the quantification of five tobacco biomarkers. Median concentrations are shown in **Table 2**. As expected, all biomarkers were significantly higher in smokers when compared with non-smokers.

Confounding Factors

Parameters that may indicate clinical pathology (diabetes, liver disease, renal failure) were studied. Although levels of GPT, creatinine, and magnesium were significantly lower in smokers than in non-smokers, there was no biological difference between the two groups (**Table 3**). No other statistical differences were observed in confounding factors.

Evaluation of the Effect of Cigarette Smoking on Biomarkers Involved in the Endothelial Function

Inflammatory biomarkers

Concentrations of inflammatory biomarkers in smokers and non-smokers are given in **Table 4**. Increased inflammation was observed in smokers by a significant elevation in the chemokine IL-8 and hsCRP levels when compared with non-smokers.

Table 3. Median concentrations of confounding factors in smokers vs. non-smokers

	Smokers: Median (interquartile range) (n=138)	Non-smokers: Median (interquartile range) (n=83)	p
Glycemia (mg/dl)	92 (85-100)	93 (86-101)	0.8500
GPT (U/l)	30 (21-38)	35 (27-46)	0.0030
GOT (U/l)	25 (21-30)	26 (22-31)	0.1477
GGT (U/l)	30 (19-50)	25 (19-37)	0.0915
Creatinine (mg/dl)	0.73 (0.66-0.87)	0.83 (0.72-0.96)	0.0002
Albumin (g/l)	42.3 (40.6-44.6)	41.7 (39.2-45.0)	0.1780
Mg (mmol/l)	0.83 (0.77-0.88)	0.85 (0.80-0.89)	0.0121
HbA1c (%)	5.4 (5.2-5.7)	5.4 (5.1-5.6)	0.0913

Note. GPT: Glutamic-pyruvic transaminase; GOT: Glutamic oxaloacetic transaminase; GGT: γ -glutamyl transferase; Mg: Magnesium; & HbA1c: Glycohemoglobin

Table 4. Median concentrations of inflammatory biomarkers in smokers vs. non-smokers

	Smokers: Median (interquartile range) (n=138)	Non-smokers: Median (interquartile range) (n=83)	p
IL-6 (pg/ml)	3.5 (2.0-5.3)	3.1 (1.8-5.8)	0.4134
IL-8 (pg/ml)	9.8 (6.2-15.4)	6.1 (3.2-10.3)	<0.0001
IL-10 (pg/ml)	0.8 (0.4-2.2)	0.5 (0.4-1.3)	0.0317
TNF-α (pg/ml)	0.15 (0.15-0.35)	0.15 (0.15-0.15)	0.0055
MCP-1 (pg/ml)	76.0 (55.4-127.8)	84.9 (60.0-106.2)	0.7315
hsCRP (mg/l)	1.6 (0.8-3.4)	1.2 (0.6-2.1)	0.0078

Note. IL: Interleukine; TNF: Tumor necrosis factor; MCP: Monocyte chemoattractant protein; & hsCRP: High-sensitivity C-reactive protein

Table 5. Median concentrations of biomarkers of endothelial function in smokers vs. non-smokers

	Smokers: Median (interquartile range) (n=138)	Non-smokers: Median (interquartile range) (n=80)	p
ADMA (µmol/l)	0.6 (0.5-0.8)	0.6 (0.5-0.7)	0.5654
sVCAM (ng/ml)	815.5 (695.6-1026.0)	783.7 (624.6-956.0)	0.0603
sICAM (ng/ml)	384.6 (302.8-504.8)	328.1 (254.3-405.1)	0.0003

Note. ADMA: Asymmetric dimethylarginine; sVCAM: Soluble vascular cell adhesion molecule; & sICAM: Soluble intercellular adhesion molecule

Table 6. Median concentrations of biomarkers of oxidative status in smokers vs. non-smokers

	Smokers: Median (interquartile range) (n=138)	Non-smokers: Median (interquartile range) (n=83)	p
SOD (U/ml)	242.0 (213.9-282.0)	238.0 (191.5-279.5)	0.3075
GPX (U/l)	12,206 (9876-14814)*	13,166 (9875-14814)**	0.9464
MDA (µg/l)	7.4 (5.7-11.3)*	5.7 (4.9-6.5)**	<0.0001
Uric acid (mg/dl)	4.8 (3.8-5.9)	5.4 (4.3-6.3)	0.0032
Iron (µg/dl)	98 (73-121)	92 (78-123)	0.7519
Ferritin (ng/ml)	72.4 (37.6-117.0)	48.9 (20.8-113.5)	0.0242
Transferrin (g/l)	2.56 (2.29-2.81)*	2.64 (2.43-17.7)**	0.0094
Vitamin A (mg/l)	0.55 (0.43-0.69)	0.57 (0.48-0.71)	0.1661
Vitamin E (mg/l)	13.4 (11.1-16.6)	14.5 (11.6-17.0)	0.4425
Vitamin C (mg/l)	8.8 (4.9-11.7)	11.0 (8.7-13.9)	<0.0001
Coenzyme Q10 (µg/l)	794 (607-1033)	742 (582-952)	0.1766
β-carotene (ng/ml)	157 (104-247)	379 (217-542)	<0.0001
Anti-oxLDL (U/l)	155.6 (80.2-339.5)	328.5 (143.5-939.5)	0.0001

Note. SOD: Superoxide dismutase; GPX: Glutathione peroxidase; MDA: Malondialdehyde; anti-oxLDL: Anti-oxidized LDL antibodies; *n=70; & **n=41

An opposite result was found for IL-10 level: an increase was observed in smokers while it is an anti-inflammatory cytokine. The medians of TNF-α concentrations in smokers and non-smokers were found identical, despite a light but significant shift in both values distributions. There were no differences for other studied biomarkers.

Endothelial biomarkers

The cell adhesion molecules, sVCAM and sICAM, were both at increased concentration in smokers (almost significant for sVCAM) compared with non-smokers (**Table 5**). ADMA, interfering with the production of NO, was not found to be affected by the smoking status.

Oxidative stress biomarkers

The lipid peroxidation biomarker, MDA, was at significantly higher concentration in smokers versus non-smokers (**Table 6**). Levels of uric acid, vitamin C and β -carotene, all antioxidants, showed a decrease in smokers versus non-smokers. Levels of ferritin and transferrin, both involved in the regulation of the iron level, were respectively increased and decreased in smokers. Anti-oxLDL antibodies concentration was lower in smokers. No other significant differences were found in the studied biomarkers.

Table 7. Median concentrations of lipids in smokers vs. non-smokers

	Smokers: Median (interquartile range) (n=138)	Non-smokers: Median (interquartile range) (n=83)	p
Cholesterol (mg/dl)	192 (167-224)	197 (170-227)	0.4795
HDL (mg/dl)	56 (43-67)	54 (48-68)	0.7893
LDL (mg/dl)	119 (87-151)	120 (97-155)	0.4005
Triglycerides (mg/dl)	121 (93-169)	104 (82-162)	0.0117
Lp(a) (g/l)	0.09 (0.04-0.31)	0.08 (0.03-0.25)	0.3335
ApoA1 (g/l)	1.54 (1.38-1.80)	1.59 (1.42-1.79)	0.6273
ApoB (g/l)	0.95 (0.77-1.15)	0.93 (0.80-1.14)	0.7776
sdLDL (nmol/ml)	1464 (706-3143)	2355 (1456-3294)	0.0400

Note. HDL: High-density lipoprotein; LDL: Low-density lipoprotein; Lp(a): Lipoprotein (a); Apo: Apolipoprotein; & sdLDL: Small dense LDL

Table 8. Features of tobacco exposure biomarkers for discriminating between smokers and non-smokers

	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	Cut-off	Youden index (%)	p
Cotinine	100.0	100.0	100	100	1.000	>0.047 µg/ml	100.0	<0.0001
Nicotine	87.9	100.0	100	97.07	0.940	>0.055 µg/ml	87.9	<0.0001
3HC	99.1	100.0	100	99.79	0.995	>0.094 µg/ml	99.1	<0.0001
Anatabine	96.6	97.9	91.89	99.13	0.980	>0.097 ng/ml	94.4	<0.0001
Anabasine	88.8	97.9	91.24	97.22	0.954	>0.236 ng/ml	86.7	<0.0001

Note. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the receiver operating characteristic (ROC) curve; & 3HC: Trans-3'-hydroxycotinine

Table 9. Features of inflammatory biomarkers for discriminating between smokers and non-smokers

	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	Cut-off	Youden index (%)	p
IL-8	81.9	46.9	27.83	91.19	0.674	>5.43 pg/ml	28.8	<0.0001
IL-10	39.1	78.8	31.52	83.81	0.585	>1.39 pg/ml	17.9	0.0317
TNF-α	47.1	77.1	33.97	85.36	0.597	>0.15 pg/ml	24.2	0.0055
hsCRP	40.6	75.9	29.62	83.63	0.607	>2.15 mg/l	16.5	0.0078

Note. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the receiver operating characteristic (ROC) curve; IL: Interleukine; TNF: Tumor necrosis factor; & hsCRP: High-sensitivity C-reactive protein

Table 10. Features of biomarkers of endothelial function for discriminating between smokers and non-smokers

	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	Cut-off	Youden index (%)	p
sVCAM	47.8	71.6	29.63	84.59	0.576	>856.6 ng/ml	19.4	0.0603
sICAM	62.3	65.0	30.80	87.34	0.647	>351 ng/ml	27.3	0.0003

Note. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the receiver operating characteristic (ROC) curve; sVCAM: Soluble vascular cell adhesion molecule; & sICAM: Soluble intercellular adhesion molecule

Lipid profile

No differences were observed when comparing the lipid profile in smokers or in non-smokers, except the triglyceride level that was increased in smokers (**Table 7**). sdLDL level showed a significant decrease in smokers.

Discrimination Between Smokers and Non-Smokers Based on Biomarkers of Tobacco, Inflammation, Endothelial Function, Oxidative Stress, and Lipids

Biomarkers significantly affected by the smoking status ($p<0.05$ in **Table 4**, **Table 5**, **Table 6**, and **Table 7**) were evaluated in terms of their ability to differentiate smokers and non-smokers.

Features of each biomarker are given in **Table 8**, **Table 9**, **Table 10**, **Table 11**, and **Table 12**. All tobacco exposure biomarkers shown the best features of discrimination with excellent sensitivity (>96%, except for nicotine and anabasine >87%), specificity (>97%), positive predictive value (>91%), negative predictive value (>97%), and area under the ROC curve (>0.940). The ability of discriminating was weaker for other biomarkers, however all presented negative predictive values >83%. IL-8, ferritin and transferrin allowed good sensitivity (>81%) while MDA and vitamin C allowed good specificity (>80%).

None of the studied biomarkers, other than tobacco biomarkers, showed both good sensitivity and specificity. Therefore, logistic regressions were performed to find a combination of biomarkers, including confounding factors, allowing better discrimination between smokers and non-smokers. Preliminary regressions were performed in each category of parameters. Significant variables in these selections were regrouped into a subsequent analysis to obtain the final model (**Table 13**, model 1).

It was associated with a good area under the ROC curve (AUC=0.877) and 88.5% of cases correctly classified. Another model was carried out according to the same principle but based only on parameters–routine parameters–easily and commonly measurable in medical laboratories (**Table 13**, model 2). This one was also associated with a good area under the ROC curve (AUC=0.928) and 84.3% of cases correctly classified.

Table 11. Features of biomarkers of oxidative status for discriminating between smokers and non-smokers

	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	Cut-off	Youden index (%)	p
MDA	57.9	85.4	49.82	89.03	0.730	>6.7 µg/l	43.3	<0.0001
Uric acid	61.6	59.0	27.32	86.01	0.619	<5.19 mg/dl	20.6	0.0032
Ferritin	85.5	33.7	24.39	90.30	0.591	>27.1 ng/ml	19.2	0.0242
Transferrin	94.9	39.6	28.20	96.90	0.638	<3 g/l	34.5	0.0094
Vitamin C	48.2	80.7	38.44	86.16	0.670	<8.3 mg/l	28.9	<0.0001
β-carotene	79.7	69.9	39.82	93.23	0.769	<277.5 ng/ml	49.6	<0.0001
Anti-oxLDL	71.0	59.3	30.35	83.10	0.663	<268.1 U/l	30.3	<0.0001

Note. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the receiver operating characteristic (ROC) curve; MDA: Malondialdehyde; & anti-oxLDL: Anti-oxidized LDL antibodies

Table 12. Features of lipid parameters for discriminating between smokers and non-smokers

	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	Cut-off	Youden index (%)	p
Triglycerides	64.5	57.8	27.66	86.69	0.601	>108 mg/dl	22.3	0.0117
sdLDL	48.1	79.3	36.68	85.93	0.597	<1327 nmol/ml	27.3	0.0400

Note. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the receiver operating characteristic (ROC) curve; & sdLDL: Small dense low-density lipoprotein

Table 13. Final models obtain by logistic regressions based on all studied parameters (model 1) or on routine parameters (model 2) to discriminate between smokers and non-smokers

Variable	Coefficient	p
Model 1		
Vitamin C	-0.237	0.0197
ApoB	3.844	0.0269
HbA1c	2.470	0.0360
GPT	-0.099	0.0101
GGT	0.076	0.0159
Constant	-12.320	0.0613
Model 2		
hsCRP	0.594	0.0058
Uric acid	-0.658	0.0036
Transferrin	-0.774	0.0092
HDL	-0.048	0.0131
Glycemia	-0.041	0.0109
GOT	-0.168	0.0009
GGT	0.036	0.0404
Albumin	0.494	0.0001
Constant	-5.120	0.2762

Note. Apo: Apolipoprotein; HbA1c: Glycated hemoglobin; GPT: Glutamic-pyruvic transaminase; GGT: Gamma-glutamyltransferase; hsCRP: High-sensitivity C-reactive protein; HDL: High-density lipoprotein; GOT: Glutamic oxaloacetic transaminase; & GGT: γ-glutamyl transferase

DISCUSSION

This research proposes an extensive study of endothelial dysfunction-related biomarkers depending on the smoking status. It is known that tobacco affects the endothelial function through its effect in associated processes, such as oxidative stress, inflammation, and changes in lipid profile, but implied mechanisms are still not clear. Since endothelial dysfunction is considered the early step in the development of CVDs, the main cause of death worldwide, it is crucial to assess the effect of tobacco in this process [2, 3, 17].

The self-reported participants' smoking status was objectified by the measurement of five urinary tobacco biomarkers–nicotine, cotinine, 3HC, anatabine and anabasine. Some cutoff values are proposed in the literature to differentiate smokers from non-smokers with these biomarkers: >0.1 µg/ml for nicotine, >0.073 µg/ml for cotinine, >0.4 µg/ml for 3HC, and >2 ng/ml for anatabine and anabasine [18]. Non-smoking was proven for the non-smokers group given that concentrations were lower than these cutoff values.

Cigarette smoke has pro-inflammatory effects by inducing the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-8 [19]. It is associated with increased circulating levels of CRP, an acute phase protein [20]. An elevation in IL-8 and hsCRP levels were indeed observed in smokers, but no difference was observed for TNF-α and IL-6 concentrations. It could be due to low sensitivity of assay kits, especially for TNF-α: 53% of recruited smokers and 77% of non-smokers had concentrations under the limit of quantification (LOQ) of 0.15 pg/ml. However, TNF-α is a primary pro-inflammatory cytokine that induces the production of chemokines, including IL-8, and the generation of IL-6, a secondary pro-inflammatory cytokine, which subsequently induces the production of acute phase proteins by the liver, such as CRP [21]. Consequently, the effects of TNF-α and IL-6 are visible through the increase of IL-8 and hsCRP levels. On the other hand, an unexpected result was obtained for IL-10, an anti-inflammatory cytokine which prevents the expression of inflammatory mediators [22]: the level was higher in smokers than in non-smokers while the opposite was shown in [11]. The medians difference between the two groups (0.8 for smokers versus 0.5 pg/ml for non-smokers) was, however, quite slight and probably not significant biologically. Moreover, the accurate value was not available for many participants due to the LOQ of 0.39 pg/ml (33% in the smokers' group and 43% in the non-smokers' group below the LOQ). However, another hypothesis could explain the observed results: an upregulation of the anti-inflammatory cytokine IL-10 could occur in response to the increased inflammation observed in smokers.

In endothelial dysfunction, inflammation is associated with an increased ability of endothelial cells to adhere to immunity cells, such as monocytes, macrophages, T lymphocytes, and platelets [10, 23]. It leads to higher concentrations of adhesion molecules in plasma, as in smokers' plasma [4, 10]. This study confirms this by the

elevation of sICAM and sVCAM (almost significant) concentrations in smokers.

The pro-inflammatory status is also associated with oxidative stress [3, 4]. It was demonstrated that cigarette smoke induces oxidative stress due to increased reactive oxygen species (ROS) production and antioxidants defenses depletion [4]. The latter was visible through the diminution of levels of vitamin C, β-carotene and uric acid in smokers versus non-smokers. These results support the hypothesis that uric acid acts as an antioxidant in plasma. Actually, some evidence indicates that uric acid may function either as an antioxidant (mainly in plasma) or pro-oxidant (mainly within the cell) [24]. Oxidative degradation of lipids (lipid peroxidation) has been shown to be higher in smokers compared with non-smokers [12, 25]. It was here demonstrated by the measurement of MDA level that was higher in smokers. Oxidative modification of LDL can also be evaluated through the measurement of anti-oxLDL antibodies concentration, reported increased in smokers [10]. However, the results obtained in this study shown reduced levels in smokers. It could be explained by a protective role of anti-oxLDL antibodies against oxLDL. Indeed, the role and the interpretation of these antibodies are still controversial in the literature. On one hand, titers of anti-oxLDL antibodies were found to be correlated with the extent of CVDs and, on the other hand, experimental data have indicated that they may neutralize oxLDL, thereby reducing the incidence of CVDs [26]. Although the concentration of iron, a prooxidant, was not shown different when comparing smokers and non-smokers, levels of ferritin and transferrin, two key proteins in its homeostasis were found impacted. As in another study [27], ferritin concentration was shown higher in smokers. It was suggested that expression of this protein was increased in case of iron-catalyzed oxidative stress in order to prevent the generation of ROS and free radicals via iron sequestration [27].

Cigarette smoking is classically associated with lipids modifications. Triglycerides level was significantly higher in smokers and thus confirms the literature [28, 29]. Interestingly, level of sdLDL which have been associated with an increased risk of CVDs were found lower in smokers than in non-smokers. This observation supports the study [30] that also related this surprising result. The causal mechanism is, however, not yet understood.

Overall, results described above agreed with the endothelial dysfunction-related processes reported in the literature. Therefore, the data could be trusted and used to evaluate the possibility of discriminating smokers from non-smokers. For the first time to our knowledge, the features of discrimination of biomarkers other than tobacco were evaluated. As expected, tobacco biomarkers allowed the best discrimination between the two groups with sensitivity and specificity above 88%. Anyway, a sensitivity >81% was achieved for IL-8, ferritin, and transferrin and a specificity >80% was observed for MDA and vitamin C. Given that none of the studied biomarkers allowed both good sensitivity and specificity, combination by logistic regressions was evaluated. The model including vitamin C, ApoB, HbA1c, GPT, and GGT was associated with a high area under the ROC curve (0.877) and allowed 88.5% of correct classifications. Another model including only routine parameters, hsCRP, uric acid, transferrin, HDL, glycemia, GOT, GGT, and albumin, was identified. It was also associated with a good area under the ROC curve (0.928) and allowed 84.3% of correct classifications. This result may open the prospect of assessing patients' tobacco use based on classic parameters of blood test, without the need of specific equipment for medical laboratories. Further work is needed to validate these two models.

A first limitation of this study was that the biomarkers concentrations were measured only once at baseline. Therefore, observed differences between smokers and non-smokers could be due to biological variation, even though statistically significant. Data about biological variation are not available for each parameter, but the differences were still analyzed considering the variability of analytical tests. The differences were higher than the assays imprecision. A second limitation was the inclusion of COPD patients among smokers, which could affect the visible differences between smokers and non-smokers. Indeed, COPD has been linked to endothelial dysfunction and increased oxidative stress [31]. Since the major environmental risk factor for COPD is inhalation of cigarette smoke [31, 32], the recruitment of smokers in real conditions implied the presence of COPD patients. However, participants with acute exacerbations of COPD were excluded. A second comparison between smokers and non-smokers was still carried out excluding COPD patients (128 smokers versus 83 non-smokers). No difference in significance was found. Finally, a third limitation concerns the models obtained from logistic regressions that have to be validated before considering their use.

CONCLUSION

Cigarette smoking is associated with endothelial dysfunction, the first step in the development of CVDs. This research evaluated the effect of tobacco smoke in endothelial dysfunction through the study of 138 smokers and 83 non-smokers for which an extensive panel of biomarkers of endothelial function and related processes, such as inflammation, oxidative stress, and lipid profile, were measured. Inflammation was shown increased in smokers through the elevation of IL-8 and hsCRP levels. Concentrations of adhesion molecules, sVCAM and sICAM, were also increased, indicating an increase of the adherence of endothelial cells to immunity cells. Oxidative stress in smokers were reflecting by the depletion of antioxidants (vitamin C, β-carotene, uric acid), the increase of lipid peroxidation (MDA, anti-oxLDL antibodies) and the disruptions of proteins involved in the homeostasis of the pro-oxidant iron (ferritin, transferrin). Lipid profile was also impacted given that elevation of triglycerides and diminution of sdLDL concentrations were observed in smokers. The data were used to determine for the first time to our knowledge the possibility of evaluating the individuals' tobacco status with biomarkers different from the usual tobacco biomarkers. A relation including vitamin C, ApoB, HbA1c, GPT, and GGT allowed to classify correctly 88.5% of cases. Another one including hsCRP, uric acid, transferrin, HDL, glycemia, GOT, GGT, and albumin (only routine parameters) allowed to classify correctly

84.3% of cases. Further work is needed to validate these two models.

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Data sharing statement: Data supporting the findings and conclusions are available upon request from corresponding author.

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