Smoking-induced long-lasting modifications of human platelet serotonin catabolism through a MAO epigenetic regulation

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<u>Short Title</u>: Platelet serotonin and smoking status

<u>Non standard abbreviations</u>: S: current smokers, FS: former smokers, NS: non (never) smokers, 5-HT: serotonin, 5-HIAA: 5-hydroxyindole acetic acid, MAO: monoamine oxidase, PRP: platelet rich plasma, PPP: platelet poor plasma.

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Abstract

Postulating that serotonin, secreted from smoking-activated platelets, could be involved in smoking-induced vascular modifications, we studied 115 men distributed in smokers (S), former smokers (FS) and never smokers (NS). The platelet serotonin content was similar in S and NS but lower in FS. This was unexpected because the monoamine oxidase (MAO) activity, which catabolizes serotonin, was inhibited during smoking. However, the amount of platelet MAO was higher in S and FS than in NS. The persistent elevated MAO amount in FS prompted us to study the methylation of its gene promoter in an additional series of patients: it was markedly lower for S and FS *vs.* NS due to cigarette smoke-induced increase of nucleic acid demethylase activity. This smoking-induced demethylation of the MAO gene promoter, resulting in high MAO amount persisting long after quitting smoking, has cardiovascular consequences and could impact fields such as behavior, mental health, and cancer.

Serotonin (5-hydroxytryptamine, 5-HT) is a potent biogenic amine, first described as the vasoconstrictor compound contained in the serum and later identified as a neurotransmitter[1]. Once synthesized in the gastrointestinal tract, peripheral serotonin is actively incorporated by platelets which store the amine within their dense granules. As a result, serotonin is widely distributed in the body through the blood flow. Under normal physiological conditions, platelet granule-stored 5-HT can be either delivered into blood flow through the open canalicular system or exposed to the platelet mitochondrial monoamine oxidase (MAO). In the latter case, it is degraded into 5-hydroxyindole acetic acid (5-HIAA) which also passes through the open canalicular system towards blood. However, the platelet storage of serotonin occurs against such a high concentration gradient that it protects the organism from serotonin-induced vascular tone abrupt modifications, maintains the serotonin plasma level in the low nM range and prevents the amine of being degraded.

Degradation of serotonin mainly occurs through monoamine oxidases (MAOs). There are two MAO isoforms: MAO-A preferentially degrades endogenous bioamines such as serotonin and norepinephrine, and MAO-B preferentially degrades exogenous bioamines such as phenylethylamine and benzylamine[2]. These specificities are relative however. Human platelets and lymphocytes contain only the MAO-B isoform. Human *MAOA* and *MAOB* genes are both located on the short arm of the X chromosome (Xp.11.4-11.3). The two genes are arranged in a tail-to-tail orientation, and both span at least 60 kb, consist of 15 exons, and exhibit an identical exon-intron organization[2].

Serotonin has been proposed to be predictive of coronary artery diseases especially in young people[3]. In addition, inhibitors of 5-HT incorporation, the so-called specific serotonin reuptake inhibitors (SSRIs) used as major depression therapy[4], reduce the cardiovascular complications of these patients by inhibiting platelet activation and aggregation[5]. We therefore decided to measure the platelet serotonin level and to study its catabolism in a series

of untreated men at low risk for cardiovascular disease. Unexpectedly, the greatest modifications in serotonin catabolism were found in former smokers (FS) rather than in current smokers (S) as compared to patients who had never smoked (NS). We found that smoking induced an epigenetic regulation of *MAOB*, *i.e.* a reduction of its gene promoter methylation, resulting in high MAO amounts which persist long after (over 10 years) quitting smoking.

RESULTS

Characteristics of patients

With the aim to study the impact of smoking on platelet activation, independently of other cardiovascular risk factors such as diabetes and hormonal factors, the men population was carefully selected as untreated and free of any major cardiovascular risk. The clinical profile of the studied population in relation with the smoking status is shown in **Table 1** and **Supplementary Table 1**. The smoking group was typical[6], *i.e.* with more femoral plaques and higher number of atherosclerotic sites. Smoking duration was higher in S than in FS group ($P < 0.05$) but lifelong cumulative consumption, as reflected by pack.years, was not different between the two groups. The number of patients having one or more plaque(s) at any site, and more specifically one or more femoral plaque(s) was significantly higher in the S group than in the FS and NS groups ($P < 0.01$).

Platelet aggregation, platelet 5-HT and plasma 5-hydroxyindole acetic acid (5-HIAA)

Because, so far, most studies dealing with smoking have compared S and NS, we initially considered only two groups, *i.e.* current smokers [S] and presently-non-smokers who had not smoked for at least one year on the day of blood sampling (including never smokers [NS] and former smokers [FS]). In response to 2 µg/ml collagen, platelet aggregation amplitude in whole blood was similar in the two groups (16 ± 5 *vs.* 16 ± 4 ohms), whereas the aggregation velocity (ohms.min^{-1}) was significantly lower ($P < 0.01$) in smokers (7.9 ± 0.5) than in presently-non-smokers (10.3 ± 0.6). The platelet 5-HT content was almost identical in the two groups (S and NS + FS), as was plasma 5-HIAA, the main 5-HT catabolite (**Fig. 1a,b**).

This surprising lack of differences between current smokers (S) and presently-non-smokers (NS + FS) prompted us to further analyze NS + FS in order to differentiate those who had never smoked (NS) from those who had smoked but had quitted one year ago or more, *i.e.*

former smokers (FS). The less rapid aggregation of smokers' platelets was confirmed (velocity 7.9 ± 0.5 ohms.min^{-1} for S *vs.* 10 ± 0.6 and 11 ± 1 for FS and NS respectively, $P < 0.01$), indicating that platelet aggregation velocity was back to normal after quitting smoking. The discrimination of FS in the non-smoking group allowed us to evidence that FS had a significantly reduced platelet amount of 5-HT as compared to NS and S (**Fig. 1a**). We thus hypothesized that 5-HT was released from the storage granules of FS platelets and, once released, was degraded into 5-HIAA. Indeed, the 5-HIAA amounts measured in FS plasmas were significantly higher than in NS and S (**Fig. 1b**).

Platelet monoamine oxidase

The fact that platelet 5-HT was more degraded into 5-HIAA suggested that the enzyme responsible for this degradation, MAO, was either more active or more abundant in FS. We thus measured both MAO activity and MAO amount in platelets, the most easily accessible source of MAO. As previously reported[2,7,8], we confirmed that MAO-B (the only isoenzyme present in human platelets) activity was significantly weaker in current smokers than in presently-non-smokers (**Fig. 1c**). But when discriminating FS in the non smoking group, MAO-B activity was found at a similar level in S and NS, whereas it was significantly greater in FS (**Fig. 1c**).

This high platelet MAO activity found in FS was explained by a higher MAO-B amount than in NS as assessed by both binding and western blot (**Fig. 1d**). Unexpectedly however, the platelet MAO amount was also high in S (**Fig. 1d**). This prompted us to calculate for each donor the enzyme catalytic activity, *i.e.* the ratio of MAO-B activity to its amount. This evidenced that the lowest mean MAO-B catalytic activity was that of S platelets where one enzyme molecule hydrolyzed 9,000 molecules of substrate per hour as compared to NS

(13,000) and FS (11,000). Platelet 5-HT and plasma 5-HIAA levels were highly correlated (*P*<0.001, not shown) with both platelet MAO activities and amounts.

The present results reassert that MAO-B activity is inhibited by smoking and reveal for the first time that (**i**) platelet MAO-B amount increased during smoking and (**ii**) this increase lasted long after quitting smoking (13 yrs average in our group of FS).

Methylation of the MAO gene promoter

The platelet MAO-B amount remaining elevated several years after quitting smoking suggested a smoking-induced gene modification. A detailed analysis reported that the human *MAOB* core promoter contains 22 CpG sites that can be methylated, with methylation reducing the transcription of *MAOB*[9]. We therefore analyzed the methylation patterns of the *MAOB* core promoter in a new series (5 S, 4 FS, 4 NS) clinically identical to the initial cohort (**Supplementary Table 2**). As for the previous population, the MAO-B amount was the lowest in NS patients, whereas the MAO-B activity was higher in FS (**Fig. 2 insert**).

DNA analysis was performed on peripheral blood mononuclear cells (PBMC) of S, FS and NS subjects, which exhibited similar MAO profiles as platelets (**Supplementary Fig. 1**). The methylation frequency was notably higher for NS, both at each of the 22 sites (**Fig. 2a, left**) or as the mean of the 22 sites (**Fig. 2a, right**). According to the repressing effect of DNA methylation upon general gene transcription[10] and upon specific transcription of the human *MAOB*[9], the individual methylation frequencies were negatively correlated with the platelet MAO-B amounts of the 13 studied patients (**Fig. 2b**). These findings bring an explanation for the high MAO-B amount long-lasting after quitting smoking.

In addition, in order to verify that tobacco smoke is truly responsible for this methylation change, DNA methyltransferase and nucleic acid demethylase activities were measured in lung nuclear extracts of mice exposed to cigarette smoke. Interestingly, these mice exhibited

lung MAO profiles (MAO-B and MAO-A, **Fig. 3a**) comparable to those found in human S

patient's platelets, *i.e.* higher amounts and lower activity in Sm than in "non-smoking" mice

(NSm). Noteworthy, the MAO profile of mice platelets (MAO-B, **Fig. 3 insert**) was also

comparable. The lung DNA methyltransferase activity appeared not significantly different

(**Fig. 3b**), whereas the nucleic acid demethylase activity was significantly higher in

"smoking" mice (Sm, **Fig. 3c**). Moreover, the *in vitro* nucleic acid demethylase activity of

mouse lung nuclear extracts was increased by harman and norharman, two known inhibitors

of MAO activities present in tobacco smoke[11,12]. This demethylase activity increase was of

12.3 ± 0.4 and 9.6 ± 0.5 % for harman and norharman (100 nM), respectively (n = 3).

DISCUSSION

The present report is one of the very few to differentiate former smokers (FS) from those who have never smoked in terms of platelet 5-HT catabolism. We showed that the 5-HT content was significantly reduced in platelets of FS who had quitted smoking for a mean of 13 years. This smoking-induced long-lasting effect was due to demethylation of the *MAOB* gene promoter which resulted in a persisting high platelet MAO-B amount. Moreover, such results obtained in two species (human and mice), with three cell types (platelets, PBMC, lung cells) and concerning two MAO isoenzymes, indicate that this smoking-induced *MAO* gene deregulation could have a more general impact than vascular modifications.

Platelets from smokers were reported to show increased aggregability[13] and to be more prone to spontaneous aggregation[14]. Platelet aggregation was, however, studied with platelet rich plasma or isolated platelets. Under similar conditions, we could not find any difference (not shown), the sensitivity of the turbidimetric method being too low. Platelet aggregation has also often been measured after acute smoking of 1-3 cigarettes, accounting for some discrepancies in the literature. Here, we measured platelet aggregation in whole blood, *i.e.* in the physiological platelet environment which might differ from one group to the other. Fitting with a trend for a higher fibrinogen plasma level (**Supplementary Table 1**) and thus a higher blood viscosity in S[15], the collagen-induced aggregation velocity was significantly lower for S than for NS and FS. This result emphasizes the importance of studying platelet functions in whole blood.

Platelet activation was reported for both acute and chronic smokers with more activation markers exposed on platelet surface[16,17]. The present results suggest that this smoking-induced platelet activation may not be sufficient to release the stored 5-HT from dense granules since it was found similar in platelets from S or NS. Only FS platelets had lower levels of 5-HT, a finding fitting with the deficiency of the vesicular monoamine transporter reported in platelets

of smokers[18] leading to a platelet dense granule 5-HT storage defect. Accordingly, we found a higher 5-HIAA level in FS plasma. The untransformed 5-HT dispersed from platelet to plasma is largely diluted, and plasma 5-HT concentrations were not significantly different between the three groups (not shown).

To account for the higher level of 5-HIAA in FS plasma, platelet MAO-B could be either more active or in higher quantity, inasmuch that we did not find any difference in other possible 5-HT metabolites (sulfo- and nitro(so)-conjugates, not shown). So far, MAO-B activity was repeatedly reported to be lower in current smokers than in non smokers[2,7,8,19]. Here, we report that the FS group accounts for the significantly higher platelet MAO-B activity found in "presently-non-smokers". More noticeably, the platelet MAO amount was elevated in FS and also in S. One could thus expect that more MAO-B protein would result in high MAO-B activity in the two groups. Yet, this was only true for FS but not for S. By calculating the MAO-B catalytic activity, we found that the lowest was that of S platelets, reasserting the low MAO-B activity of current smokers[2,7,8]. This is consistent with the fact that MAOs are oxygen-dependent and carbon monoxide-inhibited enzymes[2]. This inhibition of MAO-B catalytic activity during smoking is also in agreement with PET-scan data obtained with brain[19] and peripheral organs[20] of smokers, non-smokers and ex-smokers using a radiotracer which binds irreversibly to MAO-B: on average MAO-B is inhibited by over 40% in smokers *vs.* non-smokers with no significant difference between brains of non- and ex-smokers. Two reports assessing whether MAO inhibition could recover after an overnight abstinence[21] or after 10-31 days of smoking abstinence[22] led to the conclusions that (**i**) the enzyme is irreversibly inhibited by compounds in cigarette smoke, and (**ii**) after smoking cessation, MAO activity probably returns to normal through *de novo* synthesis of the enzyme. Our present report not only confirms but enlarges and promotes these two deductions with the elucidation of the mechanism regulating MAO-B inhibition and slow recovery.

We showed here that MAO activity was inhibited during smoking and restored after quitting, whereas MAO amount was increased during smoking and remained high many years after quitting. From a mechanistic point of view, the persistence of increased amounts of MAO-B was unexpected, even though some haematological and inflammation characteristics remain modified for several years after quitting smoking[6,23,24]. Modifications regarding the gene encoding MAO-B might bring a new explanation. Three recent studies identify an association between genetic variation on chromosome 15, in a region containing the gene encoding the nicotinic acetylcholine receptor, and risk of lung cancer[25-27]. A SNP is clearly linked to smoking quantity level[26]. Here we demonstrated how epigenetics may also participate to tobacco addiction and its long-lasting effects after quiting. A higher methylation of *MAOA* was found associated with nicotine- and alcohol-dependence[29] in women whereas a down-regulation of *MAOA* transcription was reported in several cancers including lung cancer[28].

Here we found that both S and FS exhibited a reduced methylation of the *MAOB* promoter, leading to a more active transcription of the gene and hence a higher MAO-B amount. Moreover, our *ex vivo* animal experiments showing that tobacco smoke induced an increase of nucleic acid demethylase activity give support to the fact that reduction in the *MAOB* promoter methylation is actually due to smoking. This is the first report showing that smoking has a long-lasting effect in modifying *MAOB* transcription. So far, the latter had only been shown during the differentiation of a human colon adenocarcinoma cell line[9]. DNA methylation is a covalent modification associated with long-term gene silencing and potential links to tumorigenesis[10] and suicide[30]. Thus, the epigenetic process found in the present report, which regulate gene activity without altering the DNA code, might explain at least some of the reported long-lasting effects and health problems generated by smoking[6,23,24]. Now, the nucleic acid demethylase activity, which we found increased by tobacco smoke, generates aldehydes[31,32] (**Fig. 4**). The latter are able to further cyclize bioamines, and from 5-

HT to generate, among others, β-carbolines such as harman or norharman[2,11,12]. Smoking periods might, thus, keep the MAO inhibited, contributing to a sustained 5-HT neurotransmission, and therefore mimicking an antidepressant effect (**Fig. 4**). In addition, being inverse agonists of GABA-A receptors, β-carbolines are involved in mood regulation and more precisely in anxious states. Accordingly, several data indicate that tobacco smoke is an anti-depressive addict[2,8,33]. About half of the patients studied here were submitted to an anxiety-depression questionnaire (not shown). All of these were underscored, *i.e.* below the characteristic threshold scores for depression and anxiety, indicating that none of the evaluated patients was presenting with any patent anxio-depressive disease. As previously reported[22,33], we observed that the S group was the most proned to depression. One can therefore speculate that smokers, instead of using a MAO inhibitor or another antidepressant, need to inhibit their MAO by smoking. In this context, epigenetic modifications have been reported for psychiatric disorders in animal models[34]. Our present finding is the first report of an epigenetic modification in human smokers.

In conclusion, as it is increasingly evident that 5-HT is at the crossroads of cardiovascular and depressive diseases[35], we suggest that MAO could be a determinant player at this cross-section (**Supplementary data** including **Supplementary Fig. 2**) as it is already for alcoholic addiction[36] and for predisposition to lung cancer[26,28]. A best understanding of the epigenome may open new fields of investigation in elucidating phyiopathology and developing new therapeutic approaches in tobacco addiction.

METHODS

Subjects. The studied population was selected as 115 consecutive men aged from 35 to 56 years free from cardiovascular disease including stroke, transient ischemia, coronary heart disease, congestive heart failure and intermittent claudication. Patients referred to the "Centre de Médecine Préventive Cardio-Vasculaire" between January 2003 and January 2007 for cardiovascular risk assessment were included in the study (i) if they had one or more mild cardiovascular risk factors among hypertension (defined as systolic blood pressure (BP) between 140 and 160 mm Hg and/or diastolic BP between 90 and 95 mm Hg), hypercholesterolemia (defined as total cholesterol measured by usual enzymatic methods after subjects had fasted for 14 hours between 5.2 and 7.2 mmol/L) and smoking; (ii) if they were free from diabetes (fasting blood glucose level < 7 mmol/L), from obesity (body mass index < 30 kg/m²) and from antihypertensive, lipid-lowering, antidiabetic and platelet antiaggregant therapies. Usual biological variables were measured on an LX20 automate (Beckman-Coulter) using various kits (creatinine, CRP, triglycerides, total, HDL and LDL cholesterol, lipoprotein a, folates, homocysteine, glucose) and procedures according to the manufacturer's instructions. Ten-year risk of coronary event was calculated by the Framingham equations on the basis of age, gender, systolic BP, total to HDL cholesterol ratio, and smoking[37]. Relative risk (RR) was calculated as the actual divided by the ideal (normotensive, normocholesterolemic, non smoker subject for each age category) 10-year coronary risk. A genetic approach had not been considered when patients were recruited. We thus undertook a new study on a small additional series of patients with consents for a genetic study but without authorization for human lung biopsies. All clinical investigations were conducted according to the Declaration of Helsinki principles and informed consents were obtained from

all subjects prior to inclusion in the study. The local Research Ethics Board approved the study protocols (CPP AP-HP 06-223).

Smoking status and classification. Smoking status, duration of smoking, number of years since quitting and lifelong smoking dose (calculated by multiplying the mean number of cigarettes smoked daily by the number of years of smoking and expressed in pack-year) were carefully assessed by questioning the subjects. The latter were classified into three groups on the basis of their smoking status: (i) never smokers (NS); (ii) current smokers (S) – those who currently smoke daily or have smoked for the previous year, regardless of the amount smoked; and (iii) former smokers (FS) - those who have smoked, but have quitted one year ago or more. Occasional smokers who had not quitted for at least one year were not included in the study.

Materials and reagents. The high-resolution B-mode Ultramark 5000 echograph was from Philips (Les Ulis, F). The aggregometer was from Coulter (Villepinte, F). HPLC-grade acetonitrile was obtained from VWR, Strasbourg, F); 5-HT binoxalate, ascorbic acid, and potassium dihydrogen phosphate were from Fluka (Coger, Paris, F), EDTA and sodium metabisulfite ($Na_2S_2O_5$) were from British Drug House (Poole, UK). All other reagents were at least of analytical reagent grade from Sigma-Aldrich (St Quentin-Fallavier, F). Reagent-grade water, obtained from a Millipore MilliQ-system (St-Quentin-Fallavier, F), was used throughout.

Ultrasound Investigation. Ultrasonography was performed by experienced sonographer physicians using a 7.5 MHz probe for the extracranial carotid and femoral arteries and a 3-MHz probe for the abdominal aorta, as previously described[38]. At each of the three sites, data were classified into two categories: absence of any atherosclerotic plaque and presence of one

or more arterial plaque(s), regardless of the precise location and number. The intima media thickness (IMT) was measured on the common carotid artery on both sides in the far wall of at least 1 cm of longitudinal length, and calculated by a program as previously described[39].

Platelet, PBMC and plasma preparation, platelet aggregation. Blood was drawn from antecubital veins of fasting subjects in 3.8 % sodium citrate-anticoagulated tubes (1:10 v/v). Whole blood aggregometry was measured using 500 µL aliquots of blood diluted 1:1 (v/v) in saline under constant stirring by the impedance technique, after addition of 3 µg/mL of collagen. Platelet rich plasma (PRP) was obtained by 10 min centrifugation at 100g (20°C) and platelet poor plasma (PPP) by 10 min centrifugation at 20°C and at 1500g. PRP and PPP samples were stored at - 80°C until further analysis (within 2 weeks). PBMC were obtained from each patient of the additional series using the standard Ficoll-Hypaque procedure.

Serotonin and 5-HIAA measurements. Serotonin and its deaminated metabolite 5-HIAA were measured using HPLC, as described by Kema *et al.*[40] in PRP and PPP samples, respectively.

MAO activities and amounts. MAO (EC.1.4.3.4.) enzymatic activity was determined as previously reported[8] on human PRP or PBMC samples by a radioenzymatic assay using [^{14}C]-β-phenylethylamine (2.07 GBq/mmol, Amersham GE Healthcare, Saclay, F, final concentration 20 µM, Michaelis constant 1.25 µM) as substrate. The MAO activity measured by this method is fully accounted for by MAO-B (the only isoform present in human platelets and PBMC)[41].Platelet MAO-B amount was assessed for each patient by measuring the binding of [^{3}H]Ro 19-6327 [*N*-(2-aminoethyl)-5-chloropicolinamide HCl, lazabemide] (0.96 TBq/mmol, Amersham GE Healthcare), a reversible inhibitor of MAO-B, to human platelet

membranes, as described by Cesura *et al.*[42] for [³H]Ro 16-6491. Platelets in PRP and PBMC (only on the additional series of patients) samples were lysed by an SH-dependent toxin as previously described[43] in Tris buffer (50 mM Tris, 130 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 1 mM $MgCl_2$, pH 7.40; 10 mg protein/ml) before incubation for 1 h at 20°C in the presence of 50 nM of [³H]Ro 19-6327. Incubation was terminated by addition of cold Tris buffer and 2 min centrifugation at 12,000 g at 4°C. The pellets were rinsed and solubilized in 200 µL of SDS (20%, wt/vol) for radioactivity counting. The amount of not specifically bound [³H]Ro 19-6327 was determined in the presence of 10 µM *l*-deprenyl. The non specific binding due to plasma was also deduced. The same protocol was applied to human PBMC, mouse platelets and mouse lungs. MAO-A activities and amounts in mouse lungs were determined exactly as for MAO-B except that [¹⁴C]-5-HT creatinine sulfate (1.96 GBq/mmol, Amersham GE Healthcare, Saclay, F,) and [³H]Ro 41-1049 (0.31 TBq/mmol, Amersham GE Healthcare) were used as substrate and radioligand respectively. Platelet MAO-B amount was also assessed by western blot. Briefly, samples were collected from S, NS, and FS patients in order to obtain a total of 10 µg protein (protein concentration was determined by the bicinchoninic acid method, Pierce, Chichester, UK). Proteins were resolved by 4-12% Bis-Tris NuPAGE. Separated proteins were electrotransferred to polyvinylidene difluoride membrane (Novex, San Diego, CA) and incubated with specific antibody to MAO-B (C-17, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and MAO-B detected by the ECL system (Amersham GE Healthcare).

Sodium bisulfite genomic sequencing of the MAO-B gene promoter. Genomic DNA was obtained from PBMC of each patient of the additional series using standard procedures. Bisulfite treatments were performed as described[44] with minor modifications. Briefly, genomic DNA (1 µg) was denatured in 0.2 M NaOH at 42°C for 20 min and then mixed with 10 mM hydroquinone and 3 M sodium bisulfite (pH 5.0). Reactants were incubated at

55°C for 18 h, and bisulfite-modified DNA was purified using the Wizard purification resin (Promega, Madison, WI, USA) and eluted into water. A fragment of the genomic DNA of the *MAOB* gene promoter (Genbank M89637 5'flanking sequence -55 to -752 bp, +1 assigned to the start of translation) was amplified using the following primers: 5'-GCCTTCCTGACTTAATCAC-3' (forward -752) and 5'-CCTCGATCCCAGTCCTGCC-3' (reverse -55). PCR amplification was performed in 50 µL reaction mixture containing 5 µL of bisulfite-modified DNA, 0.5 µM primers, 100 µM dNTPs, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.30), and 2.5 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA). The PCR conditions were: 97°C for 4 min (1 cycle) and 95°C for 1 min, 56°C for 1 min, 72°C for 2 min (30 cycles). PCR products amplified from each blood sample were cloned into pCR II and sequenced.

DNA methyltransferase and nucleic acid demethylase activities of mouse lung. Male A/J mice, aged 6 to 8 weeks, were purchased from Charles River (Orléans, F) and allowed to acclimate for 2 weeks before the start of the study. Mice were exposed to filtered air or mainstream cigarette smoke (10 mice in each group) using the exposure regimen developed by Witschi *et al.*[45] at a concentration of 250 mg/m³ for 5.5 hours/day, 5 days/week for 5 months, and sacrificed. Nuclear extracts were prepared from lungs according to the protocol of Dignam *et al.*[46]. Their DNA methyltransferase activity was determined as described previously[47]. The nucleic acid demethylase activity of the mouse lung nuclear extracts was assayed as their ability to demethylate 3-methylcytosine in [^{14}C]-methylated poly(dC). The substrate was generated by treatment of poly(dC) with [^{14}C]-methyl iodide (1.91 GBq/mmol, Amersham GE Healthcare). Demethylation was assayed by measuring the release of [^{14}C]-formaldehyde as described previously[32]. Briefly, reaction mixtures contained 50 mM Tris-HCl pH 7.50, 1 mM 2-oxoglutarate, 2 mM ascorbate, 75 µM $(NH_4)_2Fe(SO_4)_2$, 50 µg/mL BSA

and the [^{14}C]-methylated poly(dC) (approximately 10,000 dpm) in a total volume of 100 µL, and were incubated at 37°C for 30 minutes. The reactions were stopped by the addition of EDTA to a final concentration of 10 mM and the ethanol-soluble radioactive material was monitored by scintillation counting. All animal experimentation was performed in accordance with institutional guidelines. Protocols were approved by the French Animal Care Committee in accordance with European regulations.

Statistical analysis. It was carried out with the use of JMP (SAS) and Excel (Microsoft) softwares. For all tests, statistical significance was set at $P < 0.02$ (α, two-sided type 1 error of < 2 %).

Overall study sample. Continuous parameters are expressed as means $\pm$ SD. Normality of the distribution was analyzed by the Shapiro-Wilk W test. Normally-distributed variables were compared between smoking status groups by ANOVA, pairwise comparisons being performed by using the Student's t-test. Non normally-distributed variables were compared between smoking status groups by non parametric (Wilcoxon and Kruskal-Wallis) tests. Qualitative parameters are expressed as percent of subjects and compared between smoking status groups by the chi-square test. Linear regressions were used to analyze the relationships between continuous parameters, after logarithmic transformation in the case of non normal distribution.

Patients selected for the genetic study and mice studies. Enzyme activities and amounts were compared between groups by Fisher's exact test.

AUTHOR CONTRIBUTIONS

FR designed the study, performed aggregation studies, analyzed data and wrote the paper; AS and J-LM selected patients after careful clinical and ultrasound investigations as well as GC who also contributed to statistical analysis; KP performed genetic studies and was in charge of the figures; JM brought conceptual insights; JC performed 5-HT and 5-HIAA measurements, assumed the mice study, and contributed to statistical analysis; MDP was in charge of routine biochemical determinations, data collection, and statistical analysis; J-ML performed MAO determinations, analyzed data and wrote the paper.

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Figure Legends

Figure 1: Platelet 5-HT catabolism according to smoking status.

PRP 5-HT (**1A**) and PPP 5-HIAA (**1B**) were measured by HPLC in samples of 34 NS, 44 S and 37 FS. Results are means of nmoles 5-HT/10^9 platelets and of nM 5-HIAA. Bars represent standard deviations. Significance: *P<0.001. All 5-HT values were within the normal range (0.5-5.0 nmoles/10^9 platelets), ensuring that none of the patients studied was presenting with a storage pool disease.

Platelet MAO-B activities (**1C**) were determined on PRP samples of 34 NS, 44 S and 37 FS by radioenzymology. Results are given in nmoles of substrate per mg protein and per hour. Platelet MAO-B amounts (**1D**) were assessed both by Western blot and by measuring the binding of a reversible inhibitor of MAO-B to platelet membranes of 34 NS, 44 S and 37 FS and expressed as pmoles of MAO-B per mg platelet protein. Results are mean values and bars represent standard deviations. Significance: *P<0.001; ‡P<0.00001.

Figure 2: Analysis of the MAO-B gene promoter methylation status.

A Left: methylation frequencies at each CpG site within the human MAO-B gene core promoter. Values (%) are means for each group (4 NS, 5 S, and 4 FS). **A Right**: mean methylation frequency of the 22 CpG sites of the MAO-B gene promoter according to the smoking status (4 NS, 5 S, 4 FS) (*P<0.0001).

B: correlation between the mean methylation frequencies of the MAO-B gene promoter 22 CpG sites and the platelet MAO amounts (n = 13, r = 0.70, P<0.001).

Insert: platelet MAO activities (nmoles.mg protein^{-1}.h^{-1}) and amounts (pmoles.mg protein^{-1}) according to the smoking status of the patients selected for the genetic study (4 NS, 5 S, 4 FS). Significance: *P<0.02

Figure 3: Effect of cigarette smoke on DNA methyltransferase and nucleic acid demethylase activities.

Mice (10 for each group) were exposed (Sm) or not (NSm) to cigarette smoke and enzyme activities (**A**: DNA methyltransferase, **B**: nucleic acid demethylase, and **C**: MAO-A and MAO-B) determined on lung extracts as described in methods. Results are expressed as means ± SD. **Insert**: platelet MAO activities (nmoles.mg protein^{-1}.h^{-1}) and amounts (pmoles.mg protein^{-1}) according to the smoking status of mice. Significance: *P<0.001

Figure 4: Effect of cigarette smoke on serotonin (5-HT) catabolism.

Cigarette smoke stimulates the nucleic acid demethylase activity (1.) which generates aldehydes (HCHO) and demethylates the MAO promoter gene (2.). The demethylated MAO promoter gene leads to increased MAO amount (4.). Aldehydes, together with 5-HT and upon the action of methylene tetrahydrofolate reductase (3. MTHFR), lead to the formation of β-carbolines, such as harman and norharman. The latter are known inhibitors of MAO activity (5.) and, being present in cigarette smoke*, keep the sequence 1-5 switched on in smokers. The β-carbolines also act on GABA-A receptors, thus contributing to mood regulation as do MAO and 5-HT. Noteworthy, the MTHFR activity does not appear to be the limiting step in this process since folates were not significantly different between smokers and non smokers (cf. supplementary Table 1).

*The *in vivo* levels of plasma harman (6.8 nM) and norharman (20.02 nM) reported in smokers [48] might, however, be high enough to affect nucleic acid demethylase activity, as it is the case for platelet MAO-B inhibition[48].

Supplementary Information Titles

| Nature Medicine	
Article title	Smoking-induced long-lasting modifications of human platelet serotonin catabolism through a MAO epigenetic regulation
Authors	Jean-Marie Launay, Muriel Del Pino, Gilles Chironi, Jacques Callebert, Katell Peoc'h, Jean-Louis Mégnien, Jacques Mallet, Alain Simon, Francine Rendu
Supplementary Table 1	Baseline characteristics of the 115 patients according to smoking status.
Supplementary Table 2	Baseline characteristics of patients selected for the genetic study.
Supplementary Figure S1	PBMC MAO activities and amounts.
Supplementary Figure S2	MAO, cardiovascular risk and smoking status.

**a** Platelet 5-HT

b Plasma 5-HIAA

c Platelet MAO activity

d Platelet MAO amount
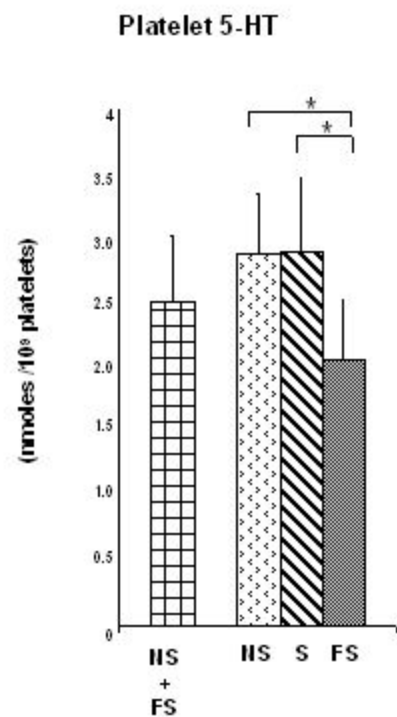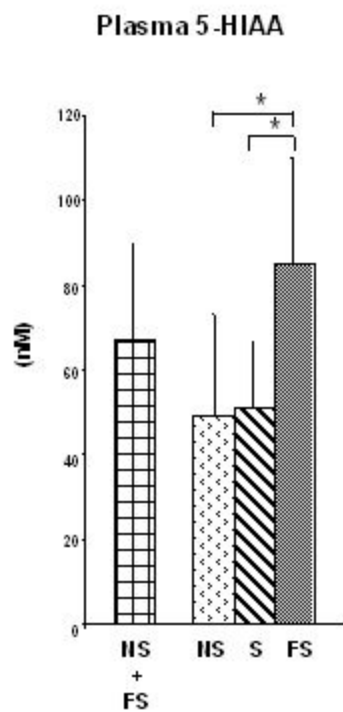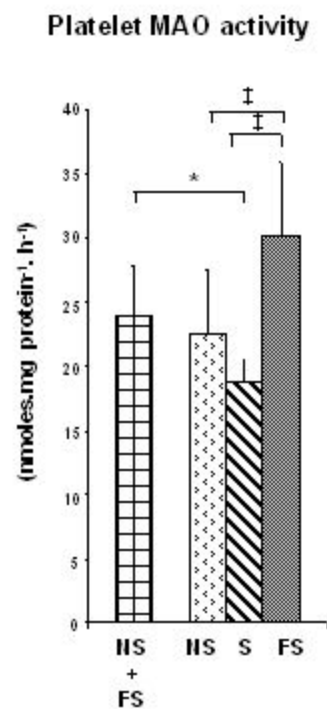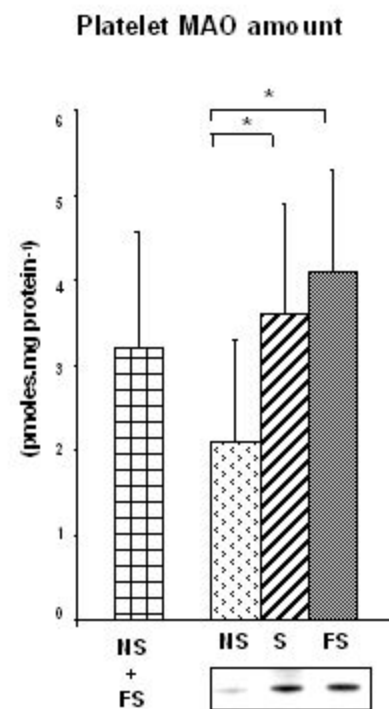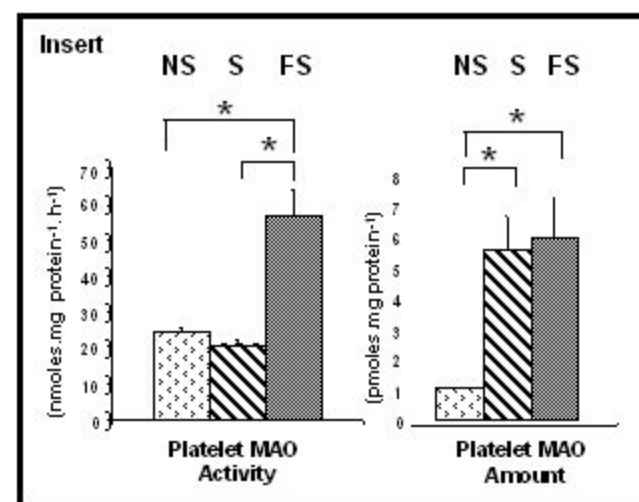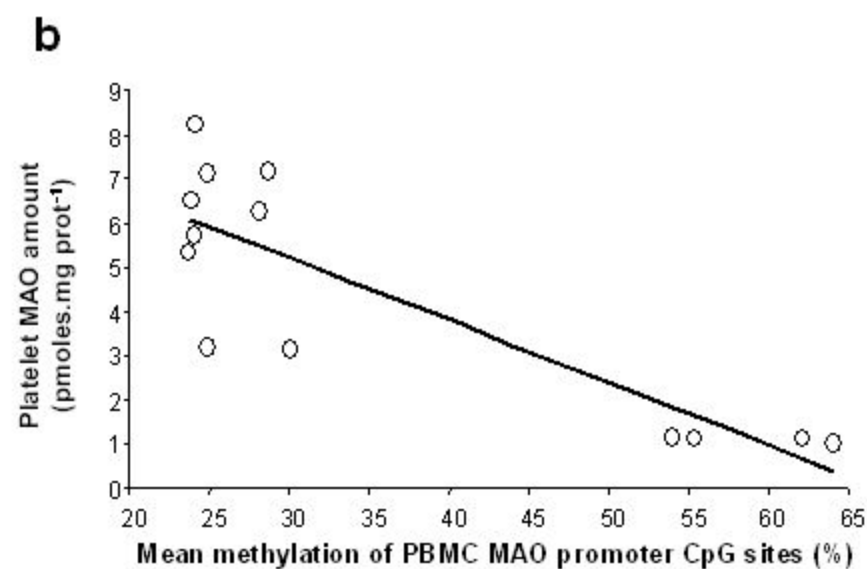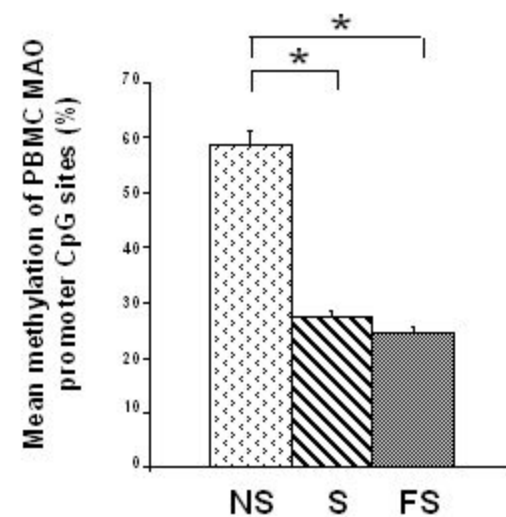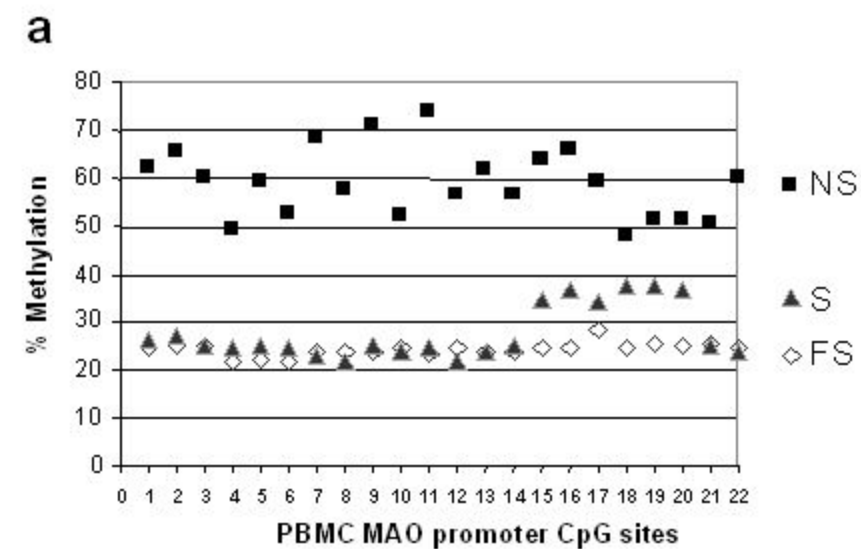
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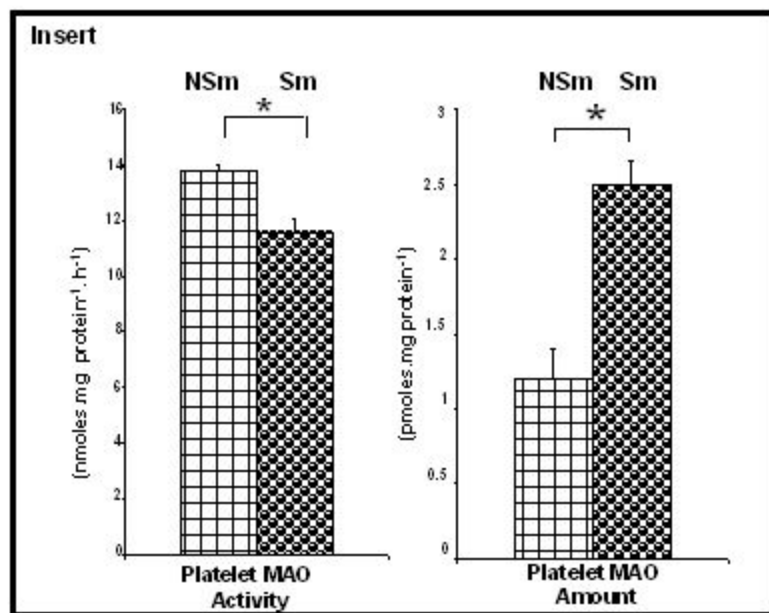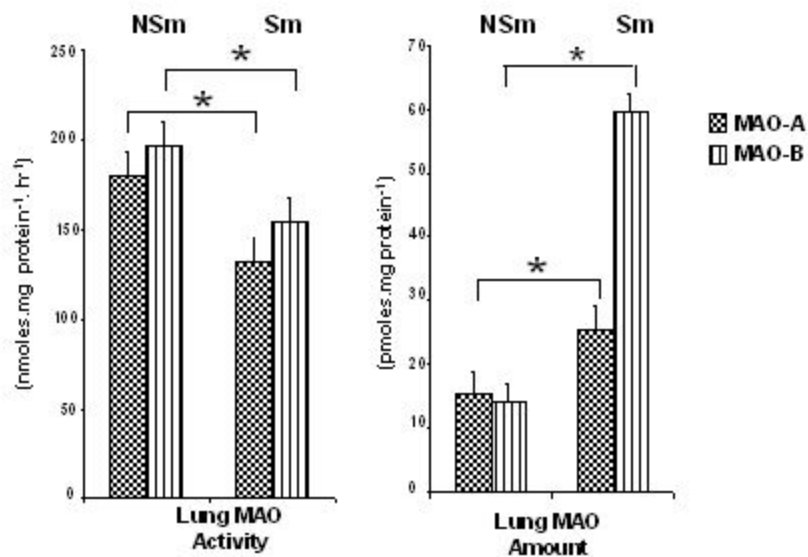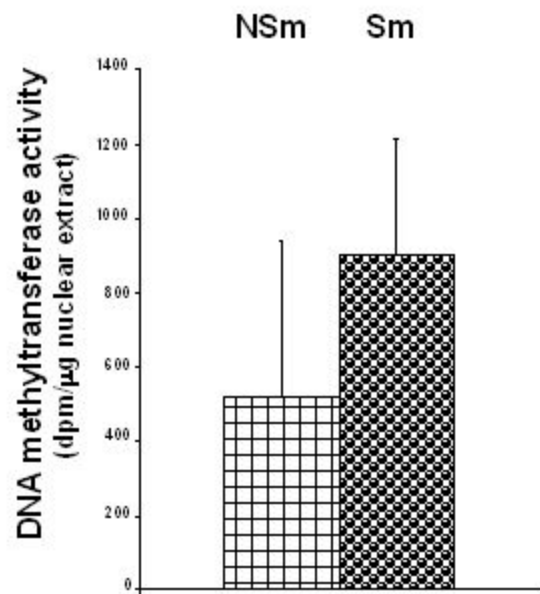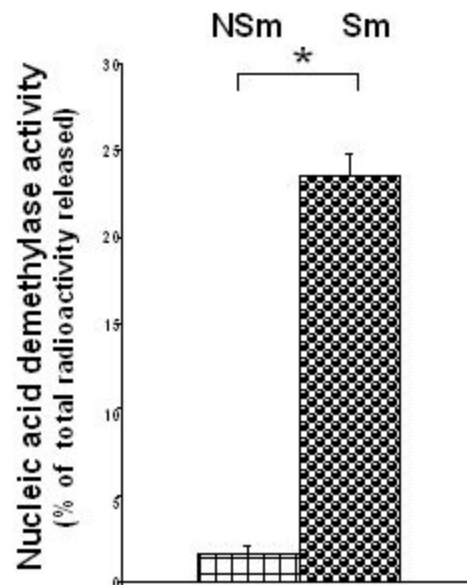
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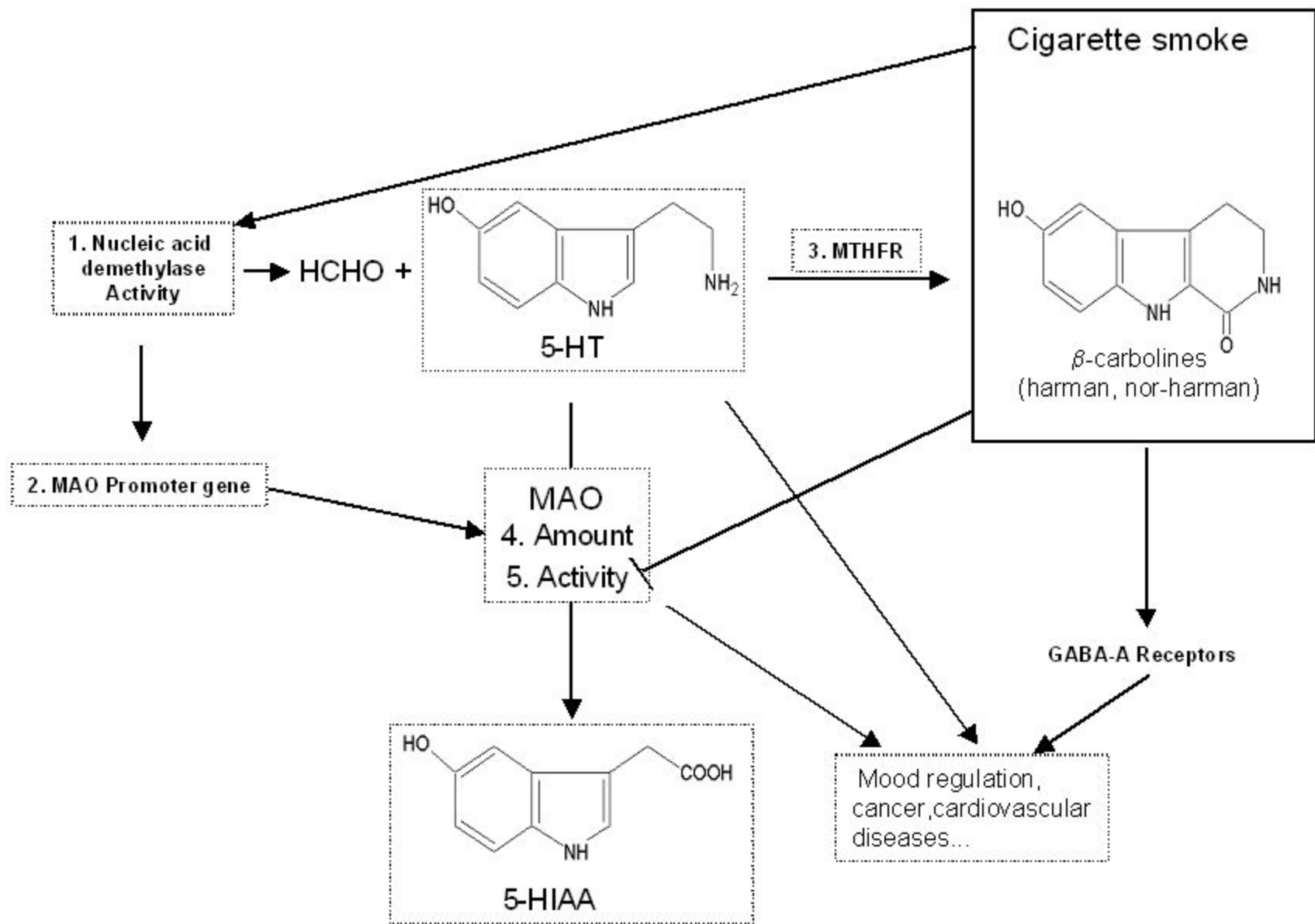
Figure 3

Figure 4

Parameter	SMOKING STATUS			*P* Value
	Non smokers (NS) n = 34 (29.6%)	Smokers (S) n = 44 (38.2%)	Former Smokers (FS) n = 37 (32.2%)	
Smoking parameters				(S *vs* FS)
cigarettes per day	0	19 ± 3	0	-
yrs of smoking	0	23 ± 1	18 ± 2	0.02
yrs since quitting	0	0	13 ± 8	-
packs.yrs	0	23 ± 17	18 ± 11	0.15
Inflammation and blood profile				(overall)
C reactive protein, mg/L	1.3 ± 1.2	2 ± 2	2 ± 3	0.10
fibrinogen, g/L	2.8 ± 0,5	3.1 ± 0.5	3 ± 0.4	0.06
hematocrit, %	42 ± 2	44 ± 3	43 ± 2	0.06
hemoglobin, g/100 mL	14.4 ± 0.7	14.8 ± 0.8	15 ± 1	0.10
leukocytes, G/L	5 ± 1	7 ± 2	6 ± 1	0.0001
platelets, G/L	224 ± 41	224 ± 55	242 ± 46	0.23
Atheromatous profile				(overall)
presence of plaque at any site, n (%)	14 (41)	31 (70.5)	22 (59.5)	0.05
presence of carotid plaque, n (%)	9 (26.5)	19 (43)	10 (27)	0.21
presence of femoral plaque, n (%)	9 (26.5)	29 (66)	16 (43)	0.002
presence of aortic plaque, n (%)	7 (21)	16 (36)	8 (22)	0.21
Intima media thickness, mm	0.56 ± 0.07	0.60 ± 0.10	0.60 ± 0.10	0.55

Table 1. Smoking, inflammation, blood parameters and atheromatous profile of 115 patients according to their smoking status. Data are means ± SD, or number and percent of subjects n (%).