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Smoking-induced changes in leptin serum levels and c/EBPalpha-related methylation status of the leptin core promotor during smoking cessation

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ABSTRACT

Previous studies have provided evidence of an association between serum leptin levels and smoking as well as craving during smoking cessation. As promoter methylation also regulates leptin expression, we investigated the leptin gene promoter region of smokers before and after smoking cessation. Since leptin's core promoter region contains an essential c/EBPalpha transcription binding site, we narrowed our investigation to C-300 (-300 base pairs from the transcription start site) of that binding site. Female smokers showed hypermethylation of C-300 compared to non-smokers. Global methylation status is associated with higher craving and the degree of dependence in female smokers. Serum leptin levels in female smokers were significantly higher than in non-smokers. These findings support previous results and, for the first time, point to a pathophysiological role of c/EBPalpha-related C-300 methylation in tobacco dependence.

1. Introduction

Cigarette smoking is a severe public health hazard responsible for approximately 3–6 million premature deaths annually worldwide (Peto et al., 1996). It is the leading preventable cause of significant health costs, related chronic diseases and deaths worldwide (Jha, 2009; Neubauer et al., 2006). Although efforts have been made to develop effective smoking cessation strategies, long-term success rates remain disappointing (Fiore, 2000). Important factors contributing towards a reluctance to quit as well as for relapsing after cessation are weight gain (Spring et al., 1992) and, most critically, craving (Alsene et al., 2003; Zuo et al., 2016). The latter is an often cited phenomenon preceding relapse (Aguiar-Nemer et al., 2013).

Nicotine, the psychoactive component of tobacco, has been shown to modulate the mesolimbic system. It binds to nicotinic acetylcholine receptors located on neurons facilitating GABA and dopamine transmission within different brain regions (Kiefer et al., 2001; Palmiter, 2007). One of the key systems that are dysregulated in psychiatric disorders is the stress system known as the hypothalamic pituitary adrenal (HPA) axis (Tapinc et al., 2017). Concerning tobacco dependence, Gomes et al. showed that this dysregulation might even be linked to withdrawal symptoms during smoking cessation (Gomes Ada et al., 2015).

Although it is known that global methylation status is changed not only in alcohol addiction but also in smoking (Hillemacher et al., 2008), most of the research focusing on the effect of leptin on craving was based on alcohol-dependent patients (Aguiar-Nemer et al., 2013; Hillemacher et al., 2010, 2015; Kiefer et al., 2005).

Recent research into the effect of craving on serum leptin levels in smokers shows heterogeneous results (al'Absi et al., 2011; Gomes Ada et al., 2015; Koopmann et al., 2011, 2016; Potretzke et al., 2014; von der Goltz et al., 2010). Gomes and colleagues showed that the regulation of dopamine transmission by leptin during withdrawal appears to alter the intensity of craving: the highest leptin concentrations were associated with greater craving and greater difficulty to remain abstinent (Gomes Ada et al., 2015). In contrast, Wei and colleagues showed that chronic smokers had lower leptin levels than nonsmokers (Wei et al., 1997) while Eliasson and Smith found that cigarette smokers had higher leptin levels than people that had never smoked before (Eliasson and Smith, 1999). Moreover, Donahue et al. found no difference in leptin levels compared to nonsmokers (Donahue et al., 1999). The prospective effect of smoking on leptin levels is still

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Table 1
QSU: Questionnaire of Smoking Urges, FTND: Fagerström Test for Nicotine Dependence, BMI: Body Mass Index.

		Control		Smoker T0		Smoker T7		Smoker T14	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sex	male (N)	16		19					
	female (N)	25		17					
Age		27.88	7.23	31.33	7.97				
Education (in counts)	general qualification for university entrance (N)	39		31					
	General Certificate of Secondary Education(N)	2		5					
BMI		23.22	3.29	24.52	3.72	24.64	3.69	24.76	3.73
FTND				3	3				
QSUtotal				67	24	68	31	68	35
Packyears				7.04	8.23				
mg Nicotine/day				8.09	6.03				
cigarettes/day	1–7/d			11					
	8–14/d			10					
	15–25/d			8					

uncharacterized. In light of these results, further clinical research is necessary to elucidate the role of promoter methylation in regulating leptin expression. Addition of methyl groups to the 5′-position of the cytosine pyrimidine ring by DNA methyltransferases masks binding sites within gene promoter sequences. As a result, transcription factors can no longer bind. Methylation primarily takes place at cytosine-phosphatidyl-guanine (CpG) dinucleotides. In general, DNA-methylation exhibits a repressive function on gene expression (Bird, 1986).

In recent years, altered epigenetic patterns of DNA methylation have been discovered in alcohol dependence (Glahn et al., 2014; Glahn et al., 2017a,b; Hillemacher et al., 2010, 2015; Muschler et al., 2010). In contrast, there are only a few studies on epigenetic changes during smoking cessation in tobacco-dependent patients (Ehrlich et al., 2012; Glahn et al., 2017a,b; Hillemacher et al., 2008; Koopmann et al., 2016; Muschler et al., 2018). This is the first study investigating mean and individual methylation of the leptin gene promoter in tobacco-dependent patients and their association with craving on days 1, 7 and 14 during smoking cessation. The core promoter region of leptin contains a c/EBPalpha transcription binding site (Mosher et al., 2016), a key promoter element required for initiating transcription. Therefore, we put an additional focus on C-300 (-300 base pairs from the transcription start site), which is located in the binding site of c/EBPalpha.

To the best of our knowledge, this is the first longitudinal investigation of leptin promoter methylation in the blood of tobacco-dependent patients during smoking cessation.

2. Methods

2.1. Study design

2.1.1. Tobacco dependent patients and control group

This investigation is part of a pilot project on neurobiological mechanisms in tobacco dependence. The study adheres to the declaration of Helsinki and was approved by the local Ethics Committee of the Hannover Medical School (MHH). All participants included in the study gave written informed consent. Each patient suffered from tobacco dependence (as defined by the International Classification of Diseases-10 (ICD-10) (Dilling et al., 2014) and the Diagnostic and Statistical Manual of Mental Disorders-IV TR (DSM-IV TR) (American Psychiatric Association, 2000). Exclusion criteria were concomitant psychiatric illnesses, other substance abuse, cerebral ischemia, cerebral hemorrhage, epilepsy, cardiovascular and renal diseases, age < 18 years, pregnant women and acquired immunodeficiency syndrome. Inclusion criteria were: age 18–65 and being a current smoker (minimum 7 cigarettes per week or one cigarette per day). All patients underwent detailed physical examination, routine laboratory testing, and urine drug screening. To check for nicotine consumption, urine cotinine was checked at each collection time point. Immediately before taking the blood sample, craving was assessed using the "Questionnaire of smoking urges" (QSU) (Tiffany and Drobes, 1991). The 32 QSU items form a summed total score and two subscales that measure "reward craving" and "relief craving" (Tiffany and Drobes, 1991). The Fagerström Test for nicotine dependence (FTND) was used as a measure of tobacco dependence (Bleich et al., 2002). In total, 77 subjects were included in the study (36 smokers/41 controls). There was no therapeutic intervention during the study period. Additional information about the sociodemographic and disease-related data are summarized below in Table 1.

3. Assays

3.1. Leptin measurement

Leptin measurement was performed using the "human leptin ELISA" (Mediagnost, Reutlingen, Germany, www.mediagnost.de) according to the manufacturer's recommendations. The sandwich type ELISA essays employ two monoclonal anti-leptin antibodies, one as solid phase and one as detection antibodies. The intra-assay coefficients of variation (CVs) are < 5.2%, interassay CVs are < 19.2% whereas the least-detectable concentrations are < 0.25 ng/mL. The assay is calibrated according to the WHO International Standard for human leptin, NIBSC 97/594.

3.2. Analysis of DNA methylation

Fasted blood samples were drawn from tobacco dependent patients on the first day (T0, right after smoking the last cigarette), as well as on day 7 (T7) and day 14 (T14) after smoking cessation on T0. All samples were drawn between 8:00 am and 10:00 am. Only those patients that remained abstinent over the entire study period (14 days) after smoking the last cigarette on T0 were included into the study (Fig. S1). We included 77 subjects in the analysis (36 smoking individuals / 41 healthy controls). Ethylenediaminetetraacetic acid (EDTA)-blood samples were aliquoted and stored at −80 °C promptly after collection. The following preparation steps were the same for all blood samples as part of the analysis of CpG-methylation. Extraction from blood and cleanup of genomic deoxyribonucleic acid (DNA) was performed using the NucleoMag® Blood 200 µL DNA Kit (Macherey-Nagel, Düren, Germany).

3.3. Bisulfite conversion of genomic DNA and subsequent amplification of the leptin target region

Bisulfate conversion of DNA samples was achieved using the

Table 2
Primer list: Leptin: rev: reverse primer. Nest: nested. Leptin-Nest-F-10: primer also served for sequencing. ENSEMBL accession Number: ENSG00000174697.

Primer name	Sequence	length
Leptin	TTT GGG GTG TTA GTT AGA GAT	21
Leptin_REV	GAT AAC CTT CTA TCT AAC TAA AAC	24
Leptin-Nest-F-10	TTAGAGAAGGGGTGGGATTTTAG	23
Leptin-Nest-A-R12	TACATCCCTCCTAACTCAATTTCC	24

ENSEMBL accession Number: ENSG00000174697.

EpiTect® 96 Bisulfite Kit (QIAGEN AG, Hilden, Germany) according to the manufacturer's protocol.

Amplification of leptin target sequences of the purified, bisulfite-converted DNA was achieved through nested touch-down polymerase chain reaction (PCR).

The sequences of oligonucleotides used as bisulfite-primers can be found in Table 2.

Nested reaction components
0.4 µL (20 µmol) of the regarding forward primer
0.4 µL (20 µmol) of the regarding reverse primer
1 µL DNA
3.2 µL H_2O
5 µL HotStarTaq® Master Mix Kit (QIAGEN, Hilden, Germany)
Σ 10 µL Total volume

The T_m for the amplification of the bisulfite-primers was set to 64 °C in the first and second round of the semi-nested PCRs.

1 µL of the amplified product of the first PCR was used as a template for the second PCR.

Amplification products of the second PCR were purified using the Agencourt® AMPure® XP magnetic beads (Beckman Coulter, Krefeld, Germany). Sequencing of the target fragment was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems/HITACHI 3500xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

The bisulfite primer used for sequencing of the respective genes is depicted in Table 2. Products of the sequencing PCR were purified using the Agencourt® CleanSeq® XP magnetic beads (Beckman Coulter, Krefeld, Germany) and then used for sequencing. Electropherograms and sequences respectively, detected by the Genetic Analyzer, were analyzed using the specialized Epigenetic Sequencing Methylation analysis Software to determine methylation rates for every CpG locus. All polymerase chain reactions were performed in a C1000 Thermal Cycler (BIO-RAD, Hercules, CA, USA) using the respective protocols as described above. A Biomek® NXP (Beckman Coulter, Krefeld, Germany) was used for pipetting, transferring steps as well as purification of DNA and amplifying oligonucleotides.

3.4. Statistical analysis

Mean differences between the time points investigated were assessed for normality by analyzing residuals, both visually with histograms and statistically using the Shapiro-Wilk goodness-of-fit test. Log transformation of leptin protein levels leads to a parametric distribution, which we tested for differences between sexes using an independent samples *T*-Test. Since most factors (methylation results included) deviated from a normal distribution, we used nonparametric methods for all other comparisons. Mann Whitney *U* tests were used to compare individual levels of sex comparisons and Kruskal-Wallis testing with correction for multiple comparisons (Dunn's multiple comparison test for the KW) was applied to both global and specific comparisons. For the correlation of variables, Spearman's test was used.

We plotted continuous variables where we observed a possible correlation and calculated the individual linear regressions, resulting in the fit lines visible in Fig. 1b–d.

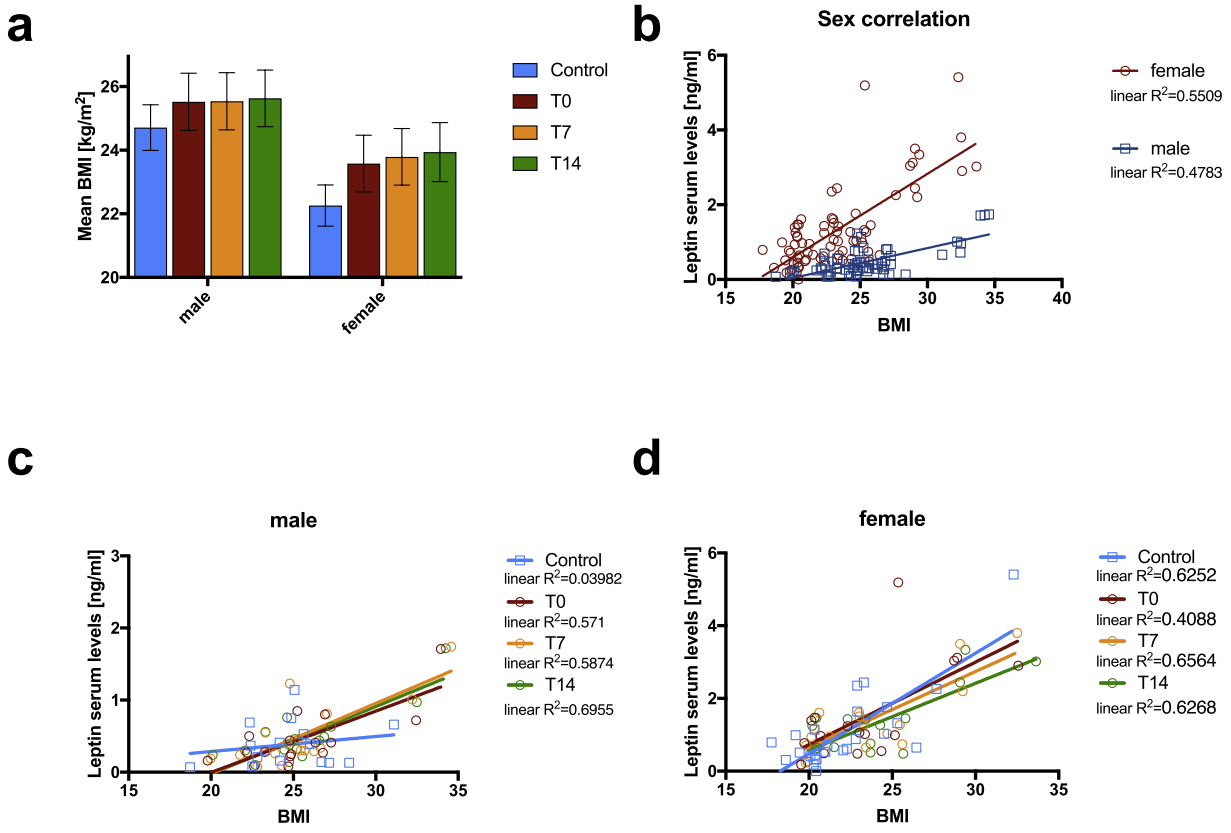

Fig. 1. (a) Body Mass Index (BMI) levels for both sexes are displayed for the different timepoints. (b) Correlation of leptin serum levels and BMI shows strong interrelationships for both men (linear R^2 = 0.478) and women (linear linear R^2 = 0.550). Looking at the progression over time, we observe a relationship between timepoint and slope of the linear correlation curve in women (d), while this is not the case for men (c). All error bars are +/− Standard error of the mean.

To assess the effect of smoking on leptin measurements over the cessation period, we calculated nicotine amounts per cigarette with cigarettes/day to receive nicotine/day levels for each participant. The full data of cigarettes/day and the preferred brand was only available for 29 of 36 participants (see Table 1). We visually binned this value into 3 equal-sized groups and compared leptin levels over the three time points assessed (Fig. 2).

All calculations were performed using either SPSS Statistics 24.0 or GraphPad Prism 7.0 (GraphPad Software. La Jolla, CA, USA).

3.5. In silico transcription factor binding site prediction

The transcription factor binding sites were predicted using the TFsearch database accessed via the Alibaba 2 online package (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). The sequence used as a template was the same EnsEMBL data entry for human leptin and was also used for primer design and analysis of Methylation data (www.ensembl.org, accession #: ENSG00000120937). Settings were set to 64% pairism percentage, 75% matrix conservation, and 95% similarity of sequence to matrix. The obtained results were cross-checked using the PROMO transcription factor prediction site (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB = TF_8.3).

4. Results

4.1. Association of smoking with leptin levels

In order to equally address both sex and time point influences regarding leptin levels, we calculated a Mixed Linear Model using leptin or C-300 methylation levels as the dependent variable with time point and sex as covariates (Table S1). While we noted a strong difference in values between the two genders, time point alone (without splitting for male and female) no longer offered significant results between time points. We, therefore, concluded that leptin expression is primarily sex-dependent, displaying the need for separation in order to compare time points effectively. Regarding C-300 methylation levels, none of the covariates showed significant effects.

Regarding the whole sample size (female/male) and the net difference between controls and smokers, serum levels of leptin did not differ significantly (data not shown). However, differentiation between the sexes led to highly significant differences: Women generally had higher leptin levels than men at all time points ($p < 0.02$, $F > 2.9$). Male smokers did not show significant differences compared to male non-smokers at any time point (Fig. 2a).

Visual binning of nicotine consumption per day resulted in three equal-sized groups: 0–7 mg, 8–14 mg and more than 14 mg nicotine per day. In our cohort, we only observed changes in leptin levels in the group with the highest nicotine levels which consisted entirely of women (Fig. 2c, d). The change in the high consumption group did not reach statistical significance.

4.2. Leptin global methylation

Fig. S2 displays all CpGs measured, dissecting the proximal part of the promoter. Global methylation of the core promoter region showed no significant difference between smokers and non-smokers (data not shown). A detailed statistical analysis comparing the different time points during abstinence revealed no significant results.

4.3. Leptin methylation of c/EBPalpha- related C-300

The transcription factor binding site of c/EBPalpha was predicted using the TFsearch database, accessed via the Alibaba 2 online package (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) (Fig. S3). Since C-300 is part of this transcription factor binding site, which plays a major role in controlling leptin synthesis (Mosher et al., 2016), we restricted our methylation analysis to C-300. Here, the statistical analysis revealed significantly higher methylation for women on day 14 of smokers in withdrawal compared to non-smokers (Fig. 2b, overall $p = 0.021$, $F = 9.727$; adjusted significance $p = 0.049$).

4.4. Association with the QSU and FTND

At Baseline (T0), FTND correlates with QSU main score in both sexes (men: $p = 0.013$, corr.coeff. = 0.559; women: $p = 0.033$, corr.-coeff. = 0.519). In addition, mean methylation correlates in women with FTND ($p = 0.007$, corr.coeff. = 0.685), and QSU total score ($p = 0.008$, corr.coeff. = 0.679).

Mean, and C-300 methylation (located on position 28 (Fig. S2)) correlates in female smokers at all three time points and in male smokers at T7 and T14 (Table S2).

4.5. Correlation between leptin and BMI in women

BMI alone did not significantly differ within sexes at the observed time points (data not shown), but we observed a positive correlation between BMI and leptin for men and women (Fig. 1b, male R^2:0.4783, female R^2:0.5509). Consequently, we investigated the time point split in this relationship. While subgroups still display reliable coefficients for both sexes (exception: male controls R^2:0.03982, compared to male T0 R^2:0.571, T7 R^2:0.5874, T14 R^2:0.6955; female Control R^2:0.6252, T0 R^2:0.4088, T7 R^2:0.6564, T14 R^2:0.6268), only women displayed a pattern of decreasing leptin/BMI slope over cessation time (Fig. 1c and d).

5. Discussion

Recent studies have demonstrated that circulating leptin is involved in the pathophysiology not only of alcohol but also of tobacco dependence (Aguiar-Nemer et al., 2013; al'Absi, 2006; Donahue et al., 1999; Hillemacher et al., 2015). Previously, our group investigated changes in leptin promoter methylation in patients undergoing alcohol withdrawal for the first time (Hillemacher et al., 2015). Concerning tobacco dependence, no data concerning epigenetic regulation of the leptin gene was available until now.

With leptin expression being c/EBPalpha dependent (Mosher et al., 2016), we observed significant hypermethylation of C-300 in smokers on day 14 compared to healthy controls. By dividing the population according to sex, female smokers show hypermethylation of C-300 whereas male smokers showed no difference in methylation status compared to non-smokers.

Leptin`s core promoter region has previously been identified as differentially methylated (Stoger, 2006) and contains c/EBPalpha transcription factor binding sites. c/EBPalpha is a key promoter element required for initiating transcription of its protein leptin (Kadonaga, 2012) and is associated with a final step in adipogenesis for energy storage (Mason et al., 1998). Previous studies reported that increased methylation in this region is correlated with decreased gene expression (Mosher et al., 2016). Female smokers had significantly higher serum levels of leptin compared to male smokers at baseline level. At that point, the methylation status of C-300 did not significantly differ between smokers and non-smokers. Nevertheless, female smokers showed significantly higher methylation of C-300 on day 14 during smoking cessation compared to female non-smokers. Furthermore, a trend of decreasing serum leptin levels could be detected in female smokers during the 14 days of abstinence (Fig. 2a). These findings could support the hypothesis that increasing methylation is correlated with decreasing leptin expression and vice versa. This relationship was also confirmed regarding the regulation of leptin expression through transcription factor c/EBPalpha. The governing principles of promoter methylation regulating leptin expression in humans had not been fully confirmed until now. In vitro demethylation of the promoter leads to an

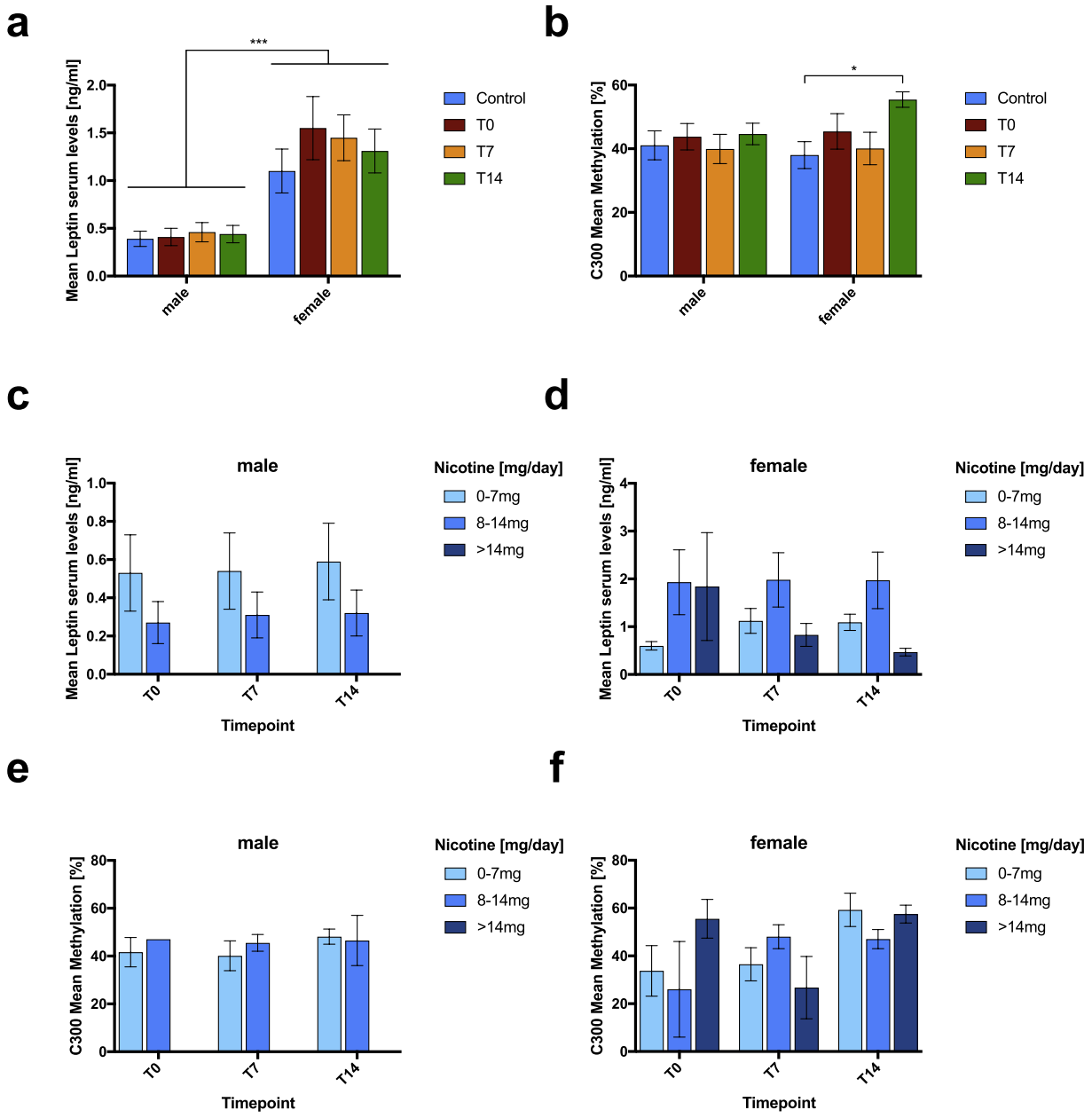

Fig. 2. Levels for the different time points (Control baseline, smoker baseline T0, smoker T7 (day 7) and T14 (day 14) of overall leptin (a) split for sex (after independent samples *T*-test $p < 0.01$, $df = 79.152$). (b) C-300 mean methylation for time points and sex (female T0-T14: adjusted significance of KW test after Wilcoxon test with Bonferroni correction $p = 0.049$, $Z = -17.682$). Below, values for different timepoints are displayed for men (c,e) and women (d,f) according to binned daily nicotine levels (blue = 0–7 mg, green = orange = > 14 mg) for leptin serum levels (c,d) and C300 methylation percentage (e,f). All error bars are +/− Standard error of the mean, significant results are marked: * < 0.05, *** < 0.001. Fig. 2e: T0 consists of three participants with equal methylation levels. Therefore, no error estimation is possible (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

increase of leptin expression (Melzner et al., 2002). Our results are in line with these findings (even though there are other mechanisms that regulate leptin expression in humans, such as the transcription factor function of the androgen receptor (Lenz et al., 2010)). Keeping in mind the pilot character of our study with relatively small sample size, larger studies are necessary to confirm these exciting results, especially with the focus on the transcription factor binding site c/EBPalpha. Our study concentrated on those who remained abstinent over the whole study period. Since recent studies pointed out changes in serum leptin levels in female abstainers but neither in female relapsers nor male smokers (Lemieux et al., 2015), future studies should focus on the c/EBPalpha related C-300 methylation status in smokers who relapse compared to the ones who remain abstinent.

As the main result, we found a significant group difference between female and male smokers at every time point during the study, thereby indicating that female smokers not only showed significantly higher serum levels of leptin (baseline: T0, Fig. 2a) compared to female non-smokers but also compared to male smokers. On the other hand, male smokers showed no significant difference compared to male non-smokers, as well as during the two weeks of abstinence (Fig. 2a). Lemieux and colleagues (Lemieux et al., 2015) reported in a sample of 15 female abstainers that leptin concentrations were increasing only in female abstainers whereas relapsers and male smokers showed stable leptin levels. Our study provides further evidence of substantial sex differences. When binned for nicotine amount, the highest-consuming group consisted only of women. Furthermore, this group showed the strongest

alteration in serum leptin levels as well as leptin promoter methylation (Fig. 2c–f). While not being statistically significant due to the group size in this cohort, this group is the modulating factor leading to related results we observed in the female abstainers.

While leptin changes with menstruation have been documented (Ajala et al., 2013; Asimakopoulos et al., 2009; Gianisslis et al., 2011), a study of al'Absi et al. (al'Absi et al., 2015) discovered that there was no significant difference between women in the luteal phase compared to women in the follicular phase during testing and that there was no association between menstrual phase and relapse status. However, the higher serum leptin levels in women may also be associated with unknown hormonal changes in leptin levels after smoking cessation.

Craving is reported to be an important factor potentially contributing to early relapse. To the best of our knowledge, this is the first study demonstrating a positive correlation between global methylation and the QSU sum score at baseline level ($p = 0.008$, Corr.coeff: 0.679) in women. The correlation of C-300 and the QSU sum score in female smokers only nearly missed significance level ($p = 0.056$, Corr.coeff:0.522). Furthermore, in female smokers, we saw a highly significant positive correlation ($p = 0.007$, Corr.coeff:0.685) between the global methylation and the degree of tobacco dependence measured by the Fagerström sum score. Although recent research has pointed out the role of leptin in food intake and energy regulation, there is evidence that it is also related to the urge to consume alcohol (Hillemacher et al., 2015) and nicotine (Aguiar-Nemer et al., 2013; al'Absi et al., 2011). Leptin as an adipocyte-derived signaling molecule is responsible for the modulation of the hypothalamic pituitary adrenal (HPA) axis by inhibiting cortisol response to stress, presumably acting at the hypothalamic level (Gomes Ada et al., 2015). Leptin also inhibits signaling of dopamine in the nucleus accumbens by binding to specific receptors located on dopamine neurons in the ventral tegmental area (VTA). The simultaneous occurrence of modulation of the HPA axis and the inhibition of dopamine transmission simultaneously contributes to increased craving (Friedman and Halaas, 1998; Inui, 1999; Palmiter, 2007). It is proposed that perturbed HPA activity during early smoking abstinence exacerbates withdrawal symptoms and may contribute to the rapid relapse observed in the majority of smokers (al'Absi, 2006; al'Absi et al., 2011).

The interconnection between Body Mass Index (BMI) and leptin levels has been a matter of investigation for over two decades (Havel et al., 1996). A recent comprehensive investigation of the relationship of tobacco dependence and weight regulation illustrates the contrasting sex-dependent relationship of BMI and smoking (Koopmann et al., 2011). In our study, BMI did not change significantly over time, which is most likely due to the considerably short observation period. Observing a positive linear correlation in our cohort, we plotted BMI against leptin values and discovered that the slope gets smaller with increasing time (Fig. 1b). We are not in a position to validate or refute further explanations of this phenomenon but would like to offer two hypotheses to be tested in further experiments. The altered leptin/BMI slope is documenting the nicotine-dependent deregulation of the satiety system as described in other studies (Hodge et al., 1997). If this is the case, the slope should adapt back to control levels with prolonged abstinence. Alternatively, manifest changes in leptin regulation have been partly compensated by nicotine. Both theories could be investigated by additional follow-up measurements that would indicate the direction of the slope over the coming weeks and months. Concerning BMI, an extended period of observation would yield a more meaningful picture as well.

There are several limitations with regards to the scope of this study. Hormone levels tend to vary in cycles depending on many factors such as age, sex, and general physiology. The interindividual differences in hormone regulation can lead to variations in measurements, especially concerning serum levels. To confirm that this was indeed a leptin effect, future studies are needed to investigate other hormones, i.e., the opposite anorexic hormone ghrelin. Leptin is not directly involved in nicotine metabolism but rather controls the peripheral reactions on a more holistic level and is therefore influenced by a multitude of other regulative stimuli.

Moreover, further enhancement and differentiation of sample sizes are necessary to better focus on the interaction between sex and relapse groups. The current study puts the focus on analyzing methylation status during smoking cessation. There was neither a direct assessment of alcohol consumption and eating patterns nor was there an assessment of insulin sensitivity which should be done in further studies. This could lead to further understanding of the mechanisms behind abnormal drinking or eating behavior during smoking cessation and a possible relationship to craving (Hillemacher et al., 2015; Kiefer et al., 2005).

Furthermore, tobacco smoke consists of a large number of different agents, a fact that is often not sufficiently accounted for in studies analyzing smokers. Thus, animal studies and studies including abstinent smokers receiving nicotine replacement therapy as a control group should be done to find out if the results are nicotine-driven. Despite these shortcomings, our study points to a possible pathophysiological role of alteration in DNA promoter methylation of the leptin gene in tobacco dependence and its potential application as a biomarker for individualized treatment.

In summary, our results support previous studies with female smokers showing higher serum leptin levels compared to male smokers (Lemieux et al., 2015) and non-smokers. To the best of our knowledge, this is the first longitudinal investigation of promoter methylation of the leptin gene in the blood of nicotine dependent patients during smoking cessation. Our study demonstrates significant hypermethylation of C-300 within the transcription factor binding site c/EBPalpha, which plays an essential role in controlling leptin synthesis (Mosher et al., 2016). Furthermore, epigenetic changes seem to be linked to craving as mean methylation in female smokers was positively correlated to craving and the degree of dependence.

Contributors

AG, MM, FD and MR performed the analyses and interpretation of the data and drafted the manuscript. TH, SB, FD and HF qualify as authors based on their contribution to interpretation of the data, drafting and revising the manuscript. SB, MM, MR qualify as author based on his contribution for design and conceptualization of the study and interpretation of the data. TH, HF, MM and FD participated in the study design and coordinated and edited the manuscript. All authors read and approved the final manuscript.

Conflict of interests

All authors report no biomedical financial interests or potential personal conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2018.09.022.

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