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Original article

Activation of nuclear factor-kappa B subunits c-Rel, p65 and p50 by plasma lipids and fatty acids across the menstrual cycle

Gernot Faustmann[a,b], Beate Tiran[c], Slave Trajanoski[d], Barbara Obermayer-Pietsch[e], Hans-Jürgen Gruber[c], Josep Ribalta[f,g], Johannes M. Roob[b,1], Brigitte M. Winklhofer-Roob[a,*]

[a] Human Nutrition & Metabolism Research and Training Center, Institute of Molecular Biosciences, University of Graz, Graz, Austria
[b] Clinical Division of Nephrology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[c] Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria
[d] Core Facility Computational Bioanalytics, Center for Medical Research, Medical University of Graz, Graz, Austria
[e] Division of Endocrinology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[f] Unitat de Recerca en Lípids i Arteriosclerosi, Departament de Medicina i Cirurgia, Universitat Rovira i Virgili and Institut d'Investigació Sanitària Pere Virgili, Reus, Spain
[g] Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, Spain

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ABSTRACT

This study focused on a comprehensive analysis of the canonical activation pathway of the redox-sensitive transcription factor nuclear factor-kappa B (NF-κB) in peripheral blood mononuclear cells, addressing c-Rel, p65 and p50 activation in 28 women at early (T1) and late follicular (T2) and mid (T3) and late luteal (T4) phase of the menstrual cycle, and possible relations with fasting plasma lipids and fatty acids. For the first time, strong inverse relations of c-Rel with apolipoprotein B were observed across the cycle, while those with LDL cholesterol, triglycerides as well as saturated (SFA), particularly C14–C22 SFA, monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) clustered at T2. In contrast, p65 was positively related to LDL cholesterol and total n-6 PUFA, while p50 did not show any relations. C-Rel was not directly associated with estradiol and progesterone, but data suggested an indirect C22:5n-3-mediated effect of progesterone. Strong positive relations between estradiol and individual SFA, MUFA and n-3 PUFA at T1 were confined to C18 fatty acids; C18:3n-3 was differentially associated with estradiol (positively) and progesterone (inversely). Given specific roles of c-Rel activation in immune tolerance, inhibition of c-Rel activation by higher plasma apolipoprotein B and individual fatty acid concentrations could have clinical implications for female fertility.

1. Introduction

Lipids and fatty acids exert regulatory effects on different determinants of female fertility, including ovarian steroidogenesis [1], ovulation [2,3] and implantation [4]. There is further strong evidence from different animal species, including mammals, that fatty acids are an essential energy substrate during oocyte maturation [5,6]. In addition to such energy supply, fatty acids are also required for non-metabolic processes, particularly for cellular signal transduction by regulating transcription factors, either by direct binding to receptors or by indirect control of the signalling pathway such as phosphorylation, ubiquitination, or proteolytic cleavage [7]. Numerous transcription factors have been identified as potential targets of fatty acids [7]. However, the mechanisms for fatty acid control of transcription factors such as nuclear factor-kappa B (NF-κB) are still poorly understood [7]. Studies in different cell types showed that saturated fatty acids (SFA) increased

* Corresponding author. Human Nutrition & Metabolism Research and Training Center, Institute of Molecular Biosciences, University of Graz, Universitätsplatz 4, 8010, Graz, Austria.
E-mail address: brigitte.winklhoferroob@uni-graz.at (B.M. Winklhofer-Roob).
[1] Johannes M. Roob deceased during preparation of this manuscript.

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Abbreviations

ANOVA	analysis of variance
ApoB	apolipoprotein B
CV	coefficient of variation
FAME	fatty acid methyl esters
FID	flame ionization detection
hCG	human chorionic gonadotropin
LDL	low-density lipoprotein
MDRD	modification of diet in renal disease
MUFA	monounsaturated fatty acids
NF-κB	nuclear factor-kappa B
OD	optical density
P	percentile
PBMC	peripheral blood mononuclear cells
PUFA	polyunsaturated fatty acids
Q	quartile
SD	standard deviation
SFA	saturated fatty acids
TAD	transcription activation domain
Treg cells	regulatory T cells
TRI	triglycerides
WHO	World Health Organization

NF-κB p65 activation, while unsaturated fatty acids did not [8–11] or that n-6 fatty acids increased NF-κB activation, while n-3 fatty acids did not [12]. In subjects with familial hypercholesterolemia NF-κB p50 activation in peripheral blood mononuclear cells (PBMC) was positively associated with LDL cholesterol and apolipoprotein B concentrations [13]. In contrast, data on the influence of fatty acids or lipids on the activation of c-Rel have not been reported.

The redox state of a specific highly conserved cysteine residue of the DNA-binding region in all members of the NF-kB family plays an important regulatory role in NF-κB activation [14,15]. Redox-sensitivity of NF-κB has been demonstrated in different cell types, also in immune cells [16], and binding sites for NF-κB have been identified in regulatory regions of many genes that are related to oxidative stress [16].

Successful female reproduction requires a delicate regulation of the immune system and of local inflammatory processes, with NF-κB signalling being an essential modulator, as shown by its involvement in folliculogenesis [17], angiogenesis during corpus luteum development [18], corpus luteum survival [19], and in the control of regulators of the implantation process in the endometrium [20,21], with the final goal to ensure dynamic immunological responses at the receptive maternal-foetal interface for successful implantation [22–24]. Reduced NF-κB p65 activation has been shown to be related to a shift from a Th1- towards a Th2-type immune response [25], which has been implicated in the preparation of the endometrium for implantation [22,26] and was observed at luteal phase of the menstrual cycle [27]. Faustmann et al. [28] provided evidence that NF-κB p65 (but not p50) activation in PBMC is reduced under physiological conditions at luteal phase, suggesting that NF-κB p65 activation could indeed play an important role in implantation. C-Rel activation across the menstrual cycle has not been investigated so far.

While p65-and p50-containing dimers are present in most cell types, c-Rel-containing dimers are predominately found in white blood cells [29], with c-Rel playing an important role in mammalian T cell differentiation and function [29,30]. C-Rel exhibits a dual role in the regulation of inflammatory and tolerogenic responses through promoting Th1- and Th17-mediated immune responses on the one hand and promoting development of regulatory T (Treg) cells on the other [29,31]. The increase in Treg cells during pregnancy [32,33] and, as particularly relevant to the present study, also already during the luteal phase of the menstrual cycle [34], may support the immune modulatory effects of progesterone, which are relevant as gestation requires a maternal immune response shift from a Th1- and Th17-mediated towards a Th2- and Treg-mediated immune response [35].

Taken together, evidence of possible effects of lipids and fatty acids, as evidenced by directed associations, on the regulation of NF-κB activation at different time points of the menstrual cycle of healthy women has not been provided. In particular, all three subunits belonging to the canonical NF-κB activation pathway, namely p50, p65 and c-Rel [36], have not been measured in concert across the menstrual cycle to allow for elucidating whether they would exert similar or differential effects. While associations of lipids and fatty acids with p50 (in human subjects) and p65 (in cell culture) have been described, such information is entirely missing for c-Rel.

In view of metabolic and regulatory effects of lipids and fatty acids as well as NF-κB activation on determinants of fertility and potential regulatory effects of lipids and fatty acids on NF-κB signalling, we hypothesized that lipid status would have an impact on NF-κB activation and studied all 3 subunits of the canonical pathway of NF-κB activation in relation to plasma lipids and fatty acids as well as female sex hormones at 4 time points of a given menstrual cycle.

2. Subjects and methods

2.1. Study participants

For the present study, 28 women were recruited from the BIOCLAIMS cohort, comprising 1310 Austrian study subjects, 606 men and 704 women, who were investigated between 2011 and 2014 as part of the European Commission's Framework 7 collaborative project entitled “Biomarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health Claims Made on Food”, as described previously [37]. The inclusion criteria used for the entire BIOCLAIMS cohort included age of 18–85 years, body mass index of 18–36 kg/m^2 and estimated glomerular filtration rate, calculated using the Modification of Diet in Renal Disease (MDRD) formula [38], ≥60 ml/min/1.73 m^2, and the exclusion criteria were any acute inflammatory diseases, immunological diseases, cardiovascular disease events in the past 6 months and malignancies in the past 6 months as well as pregnancy. The study protocol of the cross-sectional study was approved by the Ethics Committees of both the Medical University of Graz (Approval number 23-306ex10/11, dated May 20, 2011) and the University of Graz, Austria (Approval number GZ.39/24/63ex2010/11, dated May 30, 2011). The study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all study subjects prior to study entry. All 704 women of the BIOCLAIMS cohort completed a questionnaire, including questions regarding menopause, use of hormonal contraceptives and of having a regular menstrual cycle with stable lengths of the cycles.

Approximately one year after the start of the cross-sectional study, women who had completed the cross-sectional study were informed about the menstrual cycle study and were invited to participate in an information session, if they were interested in participating in the study. Inclusion criteria were being premenopausal, not using hormonal contraceptives and having had reported stable lengths of their individual menstrual cycles in the questionnaires of the cross-sectional study; exclusion criteria were pregnancy and any acute inflammatory disease. The first consecutive 28 women who were interested to participate and fulfilled the inclusion criteria were enrolled between May 29, 2012 and June 19, 2013. The study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all women prior to study entry. The study protocol was approved by the Ethics Committees of both the Medical University of Graz (Approval

number 23-306ex10/11, Amendment 1, dated April 24, 2012) and University of Graz, Austria (Approval number GZ.39/23/63ex2011/12, Amendment 1, dated May 24, 2012).

2.2. Study design

All study subjects were investigated 4 times at individual time points, which were established based on the individual length of the menstrual cycles and rise in basal body temperature records, indicating that ovulation had taken place, using the average of results of at least two menstrual cycles prior to study entry. The first day of menstrual bleeding was considered as the first day of the menstrual cycle (T0). Investigations were performed at T1 (early follicular phase); T2 (late follicular phase); T3 (mid luteal phase); and T4 (late luteal phase). Basal temperature was recorded at rest in the morning under the tongue, using Cyclotest lady® ovulation thermometer (UEBE Medical, Wertheim, Germany) according to precise instructions of the volunteers. Pregnancy was excluded prior to each investigation using the human chorionic gonadotropin (hCG) Pregnancy Rapid Test (Mexacare, Heidelberg, Germany).

2.3. Pre-analytical sample collection

Blood was drawn after an overnight fast at all 4 time points at similar morning hours to exclude circadian variations. Blood was collected in BD Vacutainer® CPT™ tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for isolation of PBMC and centrifuged immediately. For all other analyses, blood was collected on different Vacuette® blood collection tubes (Greiner Bio-One, Frickenhausen, Germany) and centrifuged immediately to obtain heparin plasma for determinations of lipids, EDTA plasma for determination of fatty acids, and serum for determination of estradiol and progesterone. Plasma lipids were analysed on the same day, while the other aliquots were stored at −80 °C until analysis.

2.4. Analytical methods

2.4.1. Determination of the activation of NF-κB subunits p50, p65 and c-Rel in PBMC

As described previously [28,37], PBMC were washed with phosphatase and protease inhibitors (Active Motif, Carlsbad, CA, USA) immediately after centrifugation, frozen in liquid nitrogen and subsequently stored at −80 °C until further processing. Whole cell extracts were prepared using the Active Motif™ nuclear extract kit (Active Motif, Carlsbad, CA, USA) and volumes adjusted to achieve identical protein concentrations in each extract using Pierce™ Coomassie assay (Thermo Fisher Scientific Inc. Waltham, MA, USA). Selective binding of activated p50, p65 and c-Rel-containing NF-κB dimers to κB consensus binding sites in immobilized oligonucleotides was determined using specific antibodies against each subunit (TransAM® NF-κB family kit, Active Motif, Carlsbad, CA, USA), and a secondary horseradish peroxidase conjugated antibody was used for spectrophotometric quantification. To control for specificity of the assay, competitive binding experiments were performed, and Raji nuclear extract (Active Motif, Carlsbad, CA, USA) was applied as the positive control. Samples of the 4 individual time points of each woman were processed in the same run. Intra-assay CV were 0.92% for p50, 2.93% for p65, and 18.0% for c-Rel, and inter-assay coefficients of variation (CV) were 3.36% for p50, 11.7% for p65, and 26.9% for c-Rel.

2.4.2. Determination of plasma fatty acids

Concentrations of 21 fatty acids with chain lengths of C14–C24, including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), were determined in plasma according to Sattler et al. [39]. Prior to freeze-drying, samples were spiked with an internal standard (C17:0) for quantification. Transesterification of fatty acids to fatty acid methyl esters (FAME) was achieved by addition of boron trifluoride-methanol complex and toluene and exposure to a temperature of 110 °C for 90 min. After extraction of FAME with n-hexane, the latter was evaporated under nitrogen, and dried samples were resolved in dichloromethane. FAME were separated on an Agilent (Santa Clara, CA, USA) DB-23 GC 30 × 0.250 (122–2332) column under application of gradually increasing temperature (125–250 °C) and detected using flame ionization detection (FID). Plasma obtained from a given woman at the 4 individual time points were processed in the same run; one run was done on a given day. Intra- (within-day) and interassay (between-day) CV were for C14:0 (4.38%; 5,64%); C15:0 (6.85%; 3.74%); C16:0 (4.85%; 2.80%); C18:0 (4.61%; 1.54%); C20:0 (3.91%; 7.93%); C22:0 (5.09%; 9.39%); C24:0 (3.86%; 4.34%); C16:1n-7 (5.06%; 2.99%); C18:1n-7 (5.55%; 5.18%); C18:1n-9 (4.96%; 1.78%); C22:1n-9 (8.57%; 13.20%); C18:3n-3 (5.38%; 2.16%); C18:4n-3 (4.39%; 19.11%); C20:5n-3 (6.60%; 5.12%); C22:5n-3 (6.49%; 5.37%); C22:6n-3 (7.94%; 5.77%); C18:2n-6 (5.75%; 1.08%); C18:3n-6 (6.54%; 2.27%); C20:3n-6 (6.06%; 3.48%); C20:4n-6 (6.29%; 2.68%); C22:4n-6 (7.16%; 6.39%).

2.4.3. Determination of plasma lipids

Total cholesterol, HDL cholesterol, apolipoprotein A-I and B and triglycerides were determined in plasma using routine clinical chemistry methods. LDL-cholesterol was calculated by the Friedewald equation.

2.4.4. Determination of serum estradiol and progesterone

ImmuliteVR, an automated, random access chemiluminescent immunoassay system (DPC/Siemens, Los Angeles, CA, USA) was used for the determination of serum estradiol concentrations and an ELISA from Dia-Metra (Segrate, Italy) for determination of serum progesterone concentrations.

2.5. Statistical analysis

To study relations between different variables, Spearman rank correlation analyses were performed; to adjust for multiple comparisons $P < 0.01$ was considered significant. Study subjects were further grouped according to quartiles (Q1-Q4) of c-Rel activation in PBMC as well as plasma lipids and fatty acids, and groups were compared using one way ANOVA or Kruskal-Wallis one way ANOVA on ranks, depending on the data distributions, and Holm-Sidak or Tukey all pairwise multiple comparison procedures were used as post-hoc tests; $P < 0.05$ was considered significant. Linear regression analysis was performed on square root-transformed data (to pass normality and constant variance tests) for assessing the dependency of c-Rel activation on apolipoprotein B concentrations; $P < 0.05$ was considered significant. SigmaPlot version 13.0 (Systat Software, San Jose, CA, USA) was used for statistical analysis as well as for creating all graphs. R Statistical Software version 3.5.3 (r-project.org) was used for hierarchical clustering and visualization of data as heatmaps.

3. Results

3.1. Adherence to the study protocol and characteristics of the study participants

Investigations were performed at T1 (early follicular phase) on (mean ± SD) day 5.8 ± 1.0; at T2 (late follicular phase) on day 11.9 ± 1.8; at T3 (mid luteal phase) on day 19.6 ± 2.4; and at T4 (late luteal phase) on day 25.3 ± 2.1. None of the study participants dropped out and all completed all 4 investigations at the scheduled time points. None of the women had positive pregnancy hCG test results at any of the 4 time points, which would have led to exclusion from the study.

The study participants were (mean ± SD) 34.2 ± 6.58 years old

(median 33.4; 5th percentile 25.7; 95th percentile 46.3 years), their BMI was 22.4 ± 3.44 kg/m^2 (median 22.2; 5th percentile 17.6; 95th percentile 30.8 kg/m^2). The average length of the menstrual cycles, calculated from the last 2 cycles prior to study entry and the cycle of investigation in the present study, was 28.1 ± 3.64 days (median 27.5; 5th percentile 24.0; 95th percentile 33.8 days). Plasma lipids showed total cholesterol of (mean ± SD) 4.88 ± 0.499 mmol/L; HDL cholesterol of 1.97 ± 0.351 mmol/L; LDL cholesterol of 2.60 ± 0.456 mmol/L; triglycerides of 0.684 ± 0.206 mmol/L; apolipoprotein A-I of 1.77 ± 0.201 g/L and apolipoprotein B of 0.770 ± 0.137 g/L. Fatty acid concentrations of the study participants at T1, presented in Table 1, very well matched WHO reference ranges obtained in a US population using comparable methods [40].

3.2. Relations of c-Rel with plasma lipids and fatty acids

As shown in Table 2, panel on the left hand side, activation of c-Rel in PBMC at T2 was significantly inversely related with LDL cholesterol ($r = -0.511$, $P < 0.01$), triglyceride ($r = -0.555$, $P < 0.01$) and apolipoprotein B concentrations ($r = -0.536$, $P < 0.01$), while such relations were not observed at T1 and remained significant for apolipoprotein B also at T3 ($r = -0.505$, $P < 0.01$) and further at T4 ($r = -0.496$, $P < 0.01$).

Regarding the plasma fatty acids, c-Rel activation at T2 was also inversely related with total fatty acids ($r = -0.718$, $P < 0.001$), total SFA ($r = -0.681$, $P < 0.001$), total MUFA ($r = -0.528$, $P < 0.01$), total PUFA ($r = -0.617$, $P < 0.001$) as well as total n-6-PUFA ($r = -0.586$, $P < 0.01$), but not with total n-3-PUFA. Total fatty acids ($r = -0.498$, $P < 0.01$), total PUFA ($r = -0.499$, $P < 0.01$) and total n-6-PUFA ($r = -0.617$, $P < 0.01$) were inversely related with c-Rel activation also at T3. Regarding the individual plasma fatty acids, a clustering of significant inverse relations of c-Rel with SFA with chain length between C14 and C22 (all $P < 0.01$), with the exception of C15:0 was observed, along with further significant relations for C18:1n-9 ($r = -0.582$, $P < 0.01$), C18:3n-3 ($r = -0.506$, $P < 0.01$) and C22:5n-3 ($r = -0.558$, $P < 0.01$) as well as C18:2n-6 ($r = -0.539$, $P < 0.01$), all starting at T2. Further significant inverse relations were present for C20:0 ($r = -0.501$, $P < 0.01$) and C22:5-n3 ($r = -0.550$, $P < 0.01$) at T3.

When looking at possible clustering for specific groups of fatty acids regarding chain length, SFA, MUFA, n-3 versus n-6 PUFA, it is noteworthy that at T2 of those with significant relations ($P < 0.01$), including, besides LDL cholesterol, apolipoprotein B and triglycerides, 9 individual fatty acids, a total of 5/9 were SFA (C14:0, C16:0, C18:0, C20:0, C22:0), 1/9 were MUFA, 2/9 were n-3 PUFA, and 1/9 were n-6 PUFA. At T3 apolipoprotein B, C20:0 and C22:5n-3 remained significant and at T4 only apolipoprotein B showed significant negative relations.

Fig. 1 shows the heatmap of individual plasma lipids and fatty acids tested for implication in c-Rel activation (not including total lipids and total fatty acids and different groups of fatty acids). The colour bars indicate correlation coefficients (Spearman rank correlations) of relations of lipids and fatty acids with c-Rel activation from negative (marked in red, strongest r-value, −0.647) to positive relations (marked in blue, strongest r-value, 0.313), at the 4 time points (T1-T4) of investigation across the menstrual cycle. Cluster analysis showed that (i) the vast majority of relations were negative across all time points; (ii) T2 showed the highest number of strong (and statistically significant) inverse relations, followed by T3 and T4 (for P values see Table 2); and (iii) overall, more similarities between T2 and T3 compared to T4 and T1.

3.3. Relations of p65 with plasma lipids and fatty acids

As shown in Table 2, panel on the right hand side, activation of the p65 subunit of NF-κB was positively related to LDL cholesterol ($r = 0.536$, $P < 0.01$), n-6-PUFA ($r = 0.496$, $P < 0.01$) and C18:2-n6 ($r = 0.482$, $P < 0.01$). Also, total cholesterol was positively related to activation of p65 ($r = 0.549$, $P < 0.01$). All associations were restricted to T2. Thus, in contrast to c-Rel, the relations of plasma lipids and fatty acids with p65 were positive and significant only at T2.

Fig. 2 shows the heatmap of individual plasma lipids and fatty acids tested for implication in p65 activation (not including total lipids and total fatty acids and different groups of fatty acids). The colour bars indicate correlation coefficients (Spearman rank correlations) of relations of lipids and fatty acids with p65 activation from negative

Table 1
Individual plasma fatty acid concentrations (μmol/L) at T1 compared with WHO reference ranges [40] presented as mean, median, 5th (P5) and 95th (P95) percentiles.

	Early follicular phase, T1 $n = 28$				WHO reference ranges $n = 66$			
	Mean	Median	P5	P95	Mean	Median	P5	P95
Individual plasma fatty acids								
C14:0	134	136	71.8	200	96.3	72.8	36.6	253
C15:0	35.3	35.0	22.8	48.1	ND	ND	ND	ND
C16:0	2328	2293	1762	2937	2272	2106	1335	3663
C18:0	682	683	533	872	678	648	415	1027
C20:0	22.0	21.8	15.3	27.7	21.5	20.7	13.3	31.7
C22:0	45.8	45.3	33.9	60.7	53.7	51.9	26.6	90.6
C24:0	39.8	38.4	29.6	55.1	52.7	47.3	29.6	92.7
C16:1n-7	211	213	105	371	205	162	76.4	477
C18:1n-7	128	124	81.1	186	143	134	79.1	249
C18:1n-9	1738	1731	1098	2241	1785	1604	958	3268
C22:1n-9	2.87	2.78	2.34	3.62	ND	ND	ND	ND
C18:3n-3	56.9	46.3	32.0	215	54.6	45.7	23.5	117
C18:4n-3	6.60	6.31	4.86	11.4	ND	ND	ND	ND
C20:5n-3	54.4	48.3	27.9	116	42.9	37.4	22.6	68.6
C22:5n-3	27.5	26.6	18.2	37.8	40.6	36.7	22.6	64.9
C22:6n-3	119	118	68.9	181	110	103	62.1	168
C18:2n-6	2982	2939	2398	3818	3230	3142	2053	4483
C18:3n-6	48.0	42.4	27.8	96.5	52.5	46.6	24.9	102
C20:3n-6	119	115	74.9	168	127	118	66.4	201
C20:4n-6	528	514	379	773	799	795	444	1196
C22:4n-6	13.0	13.3	8.05	18.0	27.9	27.0	14.9	46.5

ND: Not determined.

Table 2
Spearman rank correlations of activation of c-Rel and p65 NF-κB subunits in peripheral blood mononuclear cells (PBMC) with blood lipids and plasma fatty acids in 28 women across the menstrual cycle.

	NF-κB c-Rel				NF-κB p65			
	T1	T2	T3	T4	T1	T2	T3	T4
Plasma lipids								
Total cholesterol	−0.146	−0.456	−0.446	−0.238	−0.097	0.549[a]	0.158	0.149
HDL cholesterol	0.083	0.244	−0.009	−0.029	0.167	0.081	0.045	0.186
LDL cholesterol	−0.223	−0.511[a]	−0.449	−0.199	−0.140	0.536[a]	0.121	0.165
Triglycerides	−0.313	−0.555[a]	−0.289	−0.145	−0.334	0.096	−0.128	0.001
Apolipoprotein A-I	0.158	0.019	−0.043	0.044	−0.031	0.276	−0.118	0.151
Apolipoprotein B	−0.331	−0.536[a]	−0.505[a]	−0.496[a]	−0.164	0.448	0.090	0.222
Plasma fatty acids								
Total fatty acids	−0.132	−0.718[b]	−0.498[a]	−0.389	−0.128	0.453	0.032	0.159
Total SFA	−0.119	−0.681[b]	−0.402	−0.323	−0.295	0.357	−0.088	0.161
Total MUFA	−0.111	−0.528[a]	−0.392	−0.164	−0.232	0.334	−0.067	0.162
Total PUFA	−0.228	−0.617[b]	−0.499[a]	−0.391	0.141	0.472	0.149	0.201
Total n-3-PUFA	−0.274	−0.398	−0.069	−0.108	−0.140	0.143	−0.094	0.066
Total n-6-PUFA	−0.176	−0.586[a]	−0.511[a]	−0.373	0.135	0.496[a]	0.171	0.153
Individual plasma fatty acids								
C14:0	−0.117	−0.580[a]	0.026	−0.296	−0.378	0.066	−0.192	0.092
C15:0	−0.135	−0.448	−0.420	−0.448	0.005	0.383	0.403	0.362
C16:0	−0.052	−0.647[b]	−0.457	−0.276	−0.285	0.335	−0.045	0.117
C18:0	−0.280	−0.581[a]	−0.331	−0.367	−0.271	0.314	−0.050	0.136
C20:0	−0.373	−0.571[a]	−0.501[a]	−0.467	0.015	0.435	0.155	0.299
C22:0	−0.374	−0.480[a]	−0.371	−0.433	−0.026	0.333	0.061	0.184
C24:0	−0.291	−0.384	−0.252	−0.315	−0.061	0.250	0.042	0.102
C16:1n-7	−0.007	−0.116	−0.076	0.152	−0.129	0.004	−0.177	−0.033
C18:1n-7	−0.134	−0.288	−0.429	−0.068	−0.004	0.373	0.090	0.092
C18:1n-9	−0.181	−0.582[a]	−0.383	−0.218	−0.237	0.332	−0.049	0.194
C22:1n-9	−0.001	0.081	0.004	0.313	−0.275	0.132	−0.066	−0.143
C18:3n-3	−0.107	−0.506[a]	−0.332	−0.418	0.192	0.250	0.254	0.339
C18:4n-3	−0.028	−0.398	−0.209	−0.259	−0.320	0.024	−0.054	0.058
C20:5n-3	−0.308	−0.342	0.162	−0.110	−0,226	0.036	−0.083	−0.007
C22:5n-3	−0.314	−0.558[a]	−0.550[a]	−0.252	−0.064	0.373	0.178	0.221
C22:6n-3	−0.213	−0.245	0.039	0.031	−0.106	0.119	−0.234	−0.030
C18:2n-6	−0.207	−0.539[a]	−0.451	−0.412	0.193	0.482[a]	0.263	0.198
C18:3n-6	−0.041	−0.025	0.066	0.064	−0.234	−0.332	−0.241	−0.013
C20:3n-6	−0.076	−0.197	−0.113	0.096	−0.273	0.012	−0.317	−0.170
C20:4n-6	−0.074	−0.157	−0.254	−0.128	−0.007	0.259	−0.040	−0.244
C22:4n-6	0.060	−0.246	−0.316	0.082	−0.128	0.245	−0.049	−0.174

[a] $P < 0.01$, [b] $P < 0.001$.

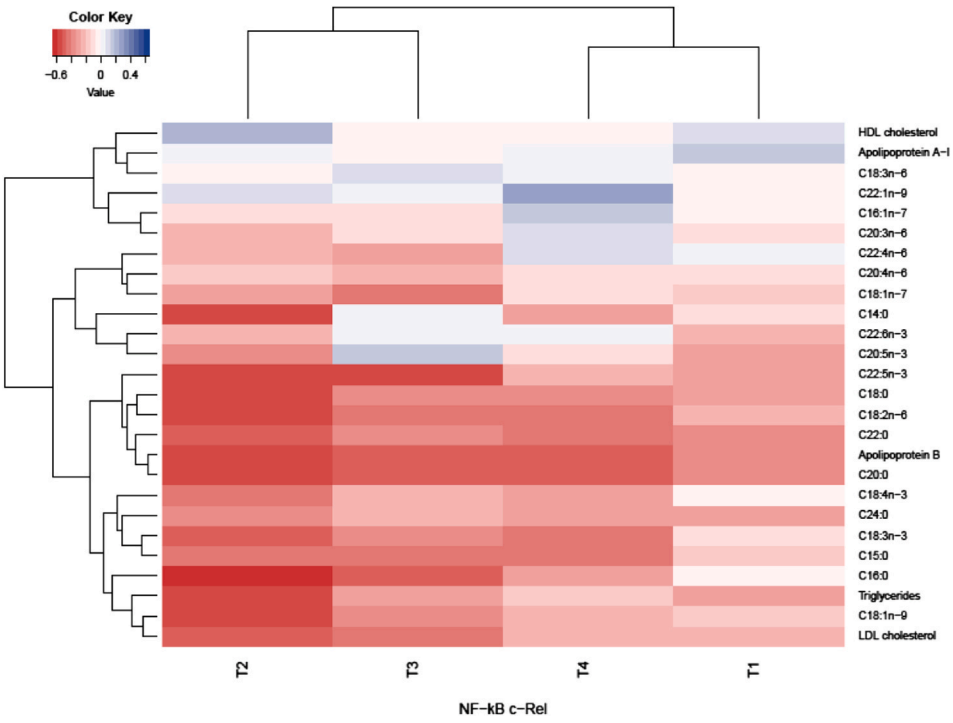

Fig. 1. Heatmap of Spearman rank correlations between individual plasma lipids as well as individual plasma fatty acids and c-Rel activation in peripheral blood mononuclear cells. The colour bars indicate positive (blue) and negative (red) correlation coefficients at the 4 time points (T1-T4) of investigation across the menstrual cycle in 28 women. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

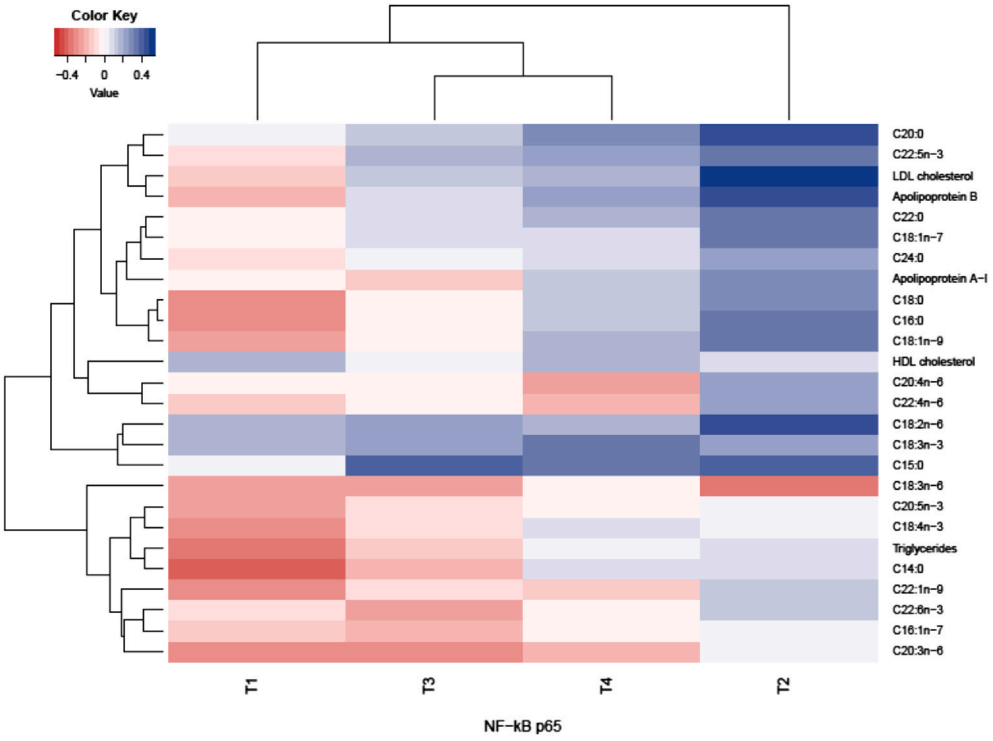

Fig. 2. Heatmap of Spearman rank correlations between individual plasma lipids as well as individual plasma fatty acids and p65 activation in peripheral blood mononuclear cells. The colour bars indicate positive (blue) and negative (red) correlation coefficients at the 4 time points (T1-T4) of investigation across the menstrual cycle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(marked in red, strongest *r*-value, −0.378) to positive relations (marked in blue, strongest *r*-value, 0.536), at the 4 time points (T1-T4) of investigation across the menstrual cycle. Cluster analysis showed opposite directions of relations between T1 (tendency towards inverse relations) and T2 (tendency towards positive relations). Overall, activation of p65 at T2 showed opposite relations with plasma lipids and fatty acids compared to c-Rel.

3.4. Relations of p50 with plasma lipids and fatty acids

No significant relations were observed between the activation of the p50 subunit and plasma lipids or fatty acids (data not shown).

3.5. Relations of c-Rel, p65 and p50 with estradiol and progesterone

There were no statistically significant relations of c-Rel, p65 or p50 activation with estradiol or progesterone concentrations at any of the 4 time points (Table 3).

3.6. Relations of estradiol with plasma lipids and fatty acids

As shown in Table 4, panel on the left hand side, there were no significant relations of estradiol with plasma lipids. Regarding plasma fatty acids, at T1 strong positive relations of fatty acids, including total fatty acids ($r = 0.567$, $P < 0.01$) and total MUFA ($r = 0.668$, $P < 0.001$), with estradiol were observed. Of individual fatty acids, C18:0 ($r = 0.518$, $P < 0.01$), C18:1n7 ($r = 0.559$, $P < 0.01$) and C18:1n9 ($r = 0.619$, $P < 0.001$) as well as C18:3n3 ($r = 0.572$, $P < 0.01$) were positively related to estradiol. In contrast, at T3 a significant inverse relation was confined to C18:3n-6 ($r = -0.504$, $P < 0.01$).

Fig. 3 shows the heatmap of individual plasma lipids and fatty acids tested for associations with estradiol (not including total lipids and total fatty acids and different groups of fatty acids). The colour bars indicate correlation coefficients (Spearman rank correlations) of relations of lipids and fatty acids with estradiol from negative (marked in red, strongest *r*-value, −0.504) to positive relations (marked in blue, strongest *r*-value, 0.619), at the 4 time points (T1-T4) of investigation across the menstrual cycle. Cluster analysis showed opposite directions of relations between T1 (tendency towards positive relations) and T3 (tendency towards inverse relations). As shown in Table 4, all significant relations were confined to fatty acids with C18 chain lengths.

3.7. Relations of progesterone with plasma lipids and fatty acids

As shown in Table 4, panel on the right hand side, there were no significant relations of progesterone with plasma lipids. At T1 strong inverse relations of fatty acids with progesterone, including total MUFA ($r = -0.488$, $P < 0.01$), C20:0 ($r = -0.493$, $P < 0.01$) and one individual n-3-PUFA, namely C18:3n-3 ($r = -0.515$, $P < 0.01$), were observed. In contrast, relations of total n-3-PUFA ($r = 0.482$, $P < 0.01$) as well as the individual n-3-PUFA C22:5n-3 ($r = 0.487$, $P < 0.01$) with progesterone were positive at T3.

Fig. 4 shows the heatmap of individual plasma lipids and fatty acids

Table 3
Spearman rank correlations of activation of c-Rel, p65 and p50 NF-κB subunits in peripheral blood mononuclear cells (PBMC) with serum estradiol and serum progesterone concentrations in 28 women across the menstrual cycle (Time points T1-T4). There were no significant ($P < 0.01$) relations.

	Estradiol				Progesterone			
	T1	T2	T3	T4	T1	T2	T3	T4
NF-κB c-Rel	0.291	−0.025	−0.042	0.070	0.027	0.020	−0.018	0.160
NF-κB p65	−0.130	0.170	0.182	−0.093	0.071	−0.104	−0.107	−0.117
NF-κB p50	0.084	0.282	0.080	−0.060	−0.283	−0.234	0.189	−0.140

Table 4
Spearman rank correlations of serum estradiol and serum progesterone with blood lipids and plasma fatty acids in 28 women across the menstrual cycle (Time points T1-T4).

	Estradiol				Progesterone			
	T1	T2	T3	T4	T1	T2	T3	T4
Plasma lipids								
Total cholesterol	0.296	0.209	−0.344	−0.183	−0.257	−0.203	−0.010	−0.238
HDL cholesterol	0.046	0.149	−0.225	0.029	0.108	−0.079	−0.017	0.149
LDL cholesterol	0.394	0.213	−0.191	−0.151	−0.326	−0.225	−0.054	−0.302
Triglycerides	0.280	−0.159	−0.113	0.020	−0.454	0.182	0.098	−0.125
Apolipoprotein A-I	0.123	0.119	−0.220	−0.045	−0.054	−0.006	−0.062	0.147
Apolipoprotein B	0.400	0.239	−0.092	−0.191	−0.356	−0.157	0.139	−0.294
Sum of plasma fatty acids								
Total fatty acids	0.567[a]	0.055	−0.269	−0.128	−0.438	0.018	−0.030	−0.180
Total SFA	0.476	0.019	−0.359	−0.161	−0.447	0.014	−0.039	−0.268
Total MUFA	0.668[b]	−0.046	−0.261	−0.139	−0.488[a]	0.022	−0.071	−0.277
Total PUFA	0.417	0.202	−0.203	−0.068	−0.272	−0.040	−0.026	−0.009
Total n-3-PUFA	0.141	0.155	−0.147	0.070	−0.280	−0.023	0.482[a]	0.038
Total n-6-PUFA	0.397	0.220	−0.169	−0.067	−0.243	−0.046	−0.089	−0.004
Individual plasma fatty acids								
C14:0	0.441	−0.084	−0.209	−0.071	−0.464	0.056	−0.143	−0.119
C15:0	0.175	−0.120	0.016	−0.225	−0.404	−0.072	−0.200	−0.313
C16:0	0.453	−0.023	−0.327	−0.223	−0.384	0.025	0.011	−0.322
C18:0	0.518[a]	0.197	−0.353	−0.084	−0.420	−0.030	0.020	−0.056
C20:0	0.307	0.322	−0.205	−0.199	−0.493[a]	−0.170	−0.113	−0.368
C22:0	0.107	0.244	−0.225	−0.117	−0.145	−0.138	−0.206	−0.135
C24:0	−0.013	0.153	−0.216	−0.121	0.024	0.083	−0.144	−0.104
C16:1n-7	0.373	−0.157	−0.450	−0.251	−0.236	0.078	−0.188	−0.394
C18:1n-7	0.559[a]	−0.025	−0.205	0.005	−0.351	0.039	0.043	−0.273
C18:1n-9	0.619[b]	−0.044	−0.262	−0.091	−0.460	0.018	−0.037	−0.210
C22:1n-9	0.015	−0.242	−0.066	0.041	−0.149	0.316	0.019	−0.008
C18:3n-3	0.572[a]	−0.137	0.175	0.129	−0.515[a]	0.048	0.084	−0.223
C18:4n-3	0.431	0.017	−0.478	−0.148	−0.238	0.007	0.478	−0.054
C20:5n-3	0.020	0.064	−0.241	−0.066	−0.166	0.102	0.253	−0.323
C22:5n-3	0.374	0.153	0.139	−0.290	−0.281	−0.151	0.487[a]	0.028
C22:6n-3	−0.016	0.246	−0.106	0.070	0.224	−0.065	0.116	0.119
C18:2n-6	0.351	0.194	−0.035	0.003	−0.160	0.019	−0.131	0.075
C18:3n-6	0.157	0.177	−0.504[a]	−0.394	−0.186	−0.001	−0.029	−0.280
C20:3n-6	0.194	0.140	−0.466	−0.302	−0.148	−0.088	−0.076	−0.347
C20:4n-6	0.265	0.407	−0.316	−0.441	−0.253	−0.265	0.128	−0.356
C22:4n-6	0.357	0.055	−0.355	−0.245	−0.021	0.072	−0.002	−0.271

[a]$P < 0.01$, [b]$P < 0.001$.

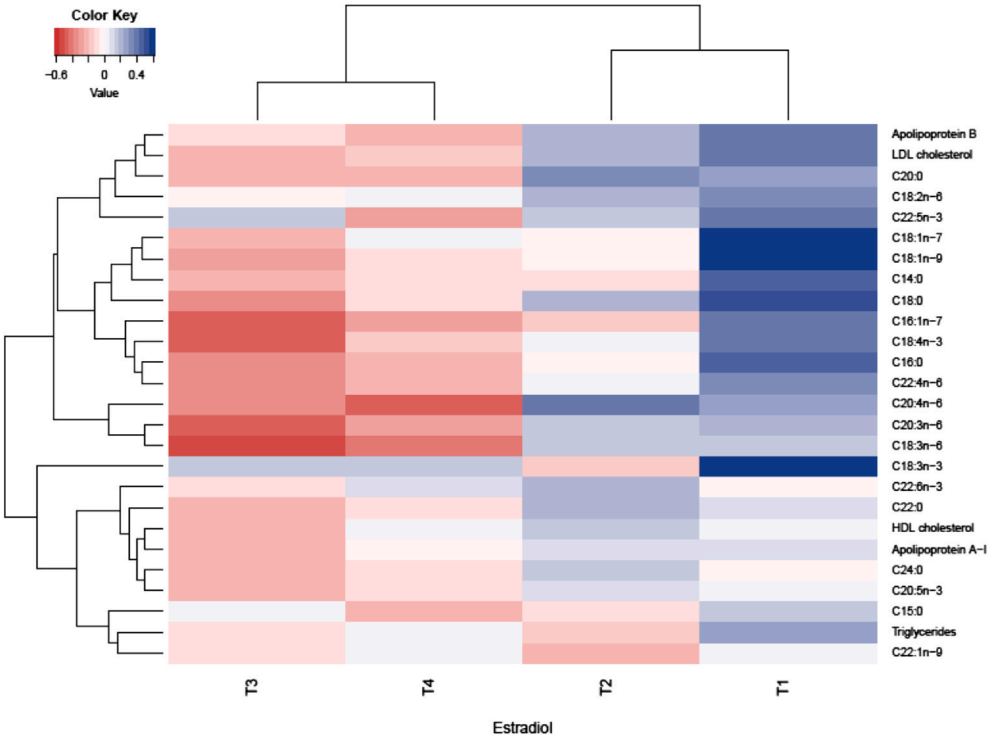

Fig. 3. Heatmap of Spearman correlations between individual plasma lipids as well as individual plasma fatty acids and serum estradiol. The colour bars indicate positive (blue) and negative (red) correlation coefficients at the 4 time points (T1-T4) of investigation across the menstrual cycle in 28 women. Abbreviations: T1, time point 1, early follicular phase; T4, time point 4, late luteal phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

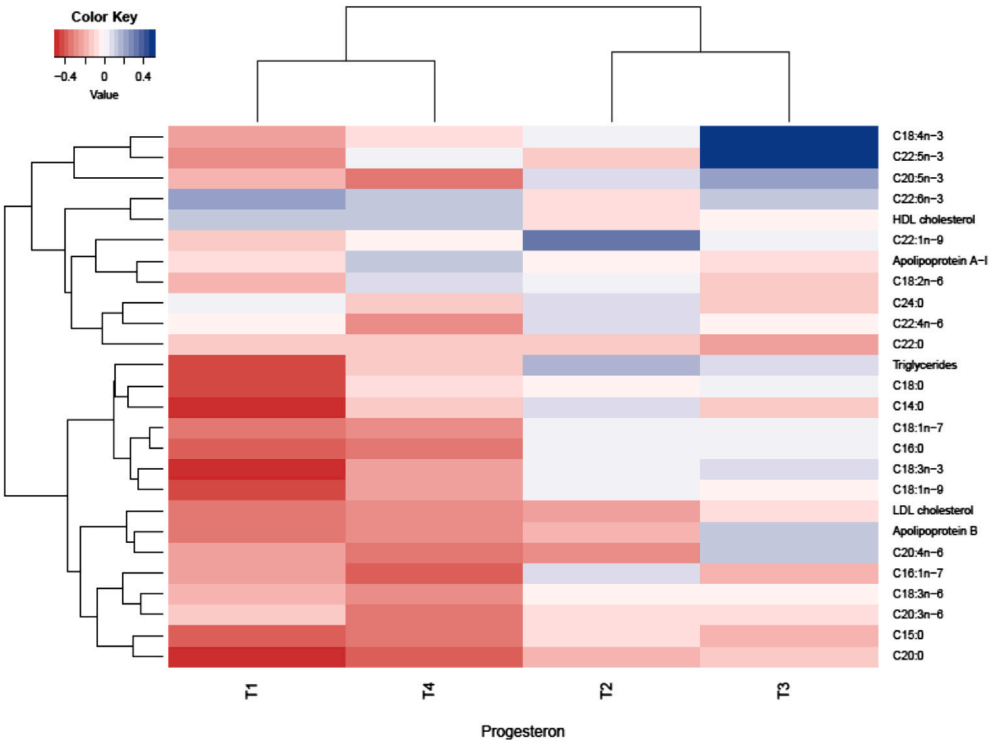

Fig. 4. Heatmap of Spearman correlations between individual plasma lipids as well as individual plasma fatty acids and serum progesterone. The colour bars indicate positive (blue) and negative (red) correlation coefficients at the 4 time points (T1-T4) of investigation across the menstrual cycle in 28 women. Abbreviations: T1, time point 1, early follicular phase; T4, time point 4, late luteal phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

tested for associations with progesterone (not including total lipids and total fatty acids and different groups of fatty acids). The colour bars indicate correlation coefficients (Spearman rank correlations) of relations of lipids and fatty acids with progesterone from negative (marked in red, strongest *r*-value, −0.515) to positive relations (marked in blue, strongest *r*-value, 0.487), at the 4 time points (T1-T4) of investigation across the menstrual cycle. Cluster analysis showed predominantly inverse relations of individual lipids and fatty acids with progesterone at

c-Rel (OD)		ApoB (g/L)	LDL (mmol/L)	TRI (mmol/L)	C14:0 (µmol/L)	C16:0 (µmol/L)	C18:0 (µmol/L)	C20:0 (µmol/L)	C22:0 (µmol/L)	C18:1-n9 (µmol/L)	C18:3-n3 (µmol/L)	C18:2-n6 (µmol/L)	C22:5-n3 (µmol/L)	
Q 1 Mean, 0.005 SD, 0.009	0.001	0.700	1.91	1.42	148	2841	711	22.4	44.4	2599	59.5	2514	34.1	Q 1
	0.001	1.100	3.44	1.23	167	2754	749	26.1	47.8	2204	71.9	3672	36.5	Q 2
	0.001	0.820	2.56	0.960	183	2361	831	25.6	54.7	2148	314	3860	36.7	Q 3
	0.001	0.870	2.95	0.475	89.2	2345	699	26.3	57.2	1475	32.1	3862	31.3	Q 4
	0.001	0.910	2.84	1.01	162	2507	669	19.0	43.3	1932	55.6	2995	32.3	
	0.004	0.820	3.18	0.735	139	2686	747	25.7	50.2	2007	53.2	3273	34.7	
	0.025	0.710	2.17	0.757	184	2856	798	24.0	67.6	1692	67.4	3524	31.5	
Q 2 Mean, 0.050 SD, 0.014	0.028	0.730	2.12	0.825	127	2185	592	24.6	40.0	1894	64.2	2464	34.3	
	0.037	0.770	2.92	1.36	227	2998	747	21.5	48.2	2457	59.6	3568	28.7	
	0.051	0.870	2.84	0.915	139	2691	722	26.2	53.5	2240	58.5	3269	29.5	
	0.052	0.780	2.77	0.396	98.5	2254	672	24.1	52.5	1373	54.3	3784	22.4	
	0.053	0.750	2.17	0.836	165	2425	754	21.6	51.2	1691	42.4	3682	31.5	
	0.064	0.910	2.61	0.723	137	2365	672	25.0	52.7	1681	48.6	3048	22.7	
	0.067	0.970	2.74	0.588	117	2537	733	23.5	51.2	2091	44.8	3240	34.5	
Q 3 Mean, 0.115 SD, 0.018	0.095	0.620	2.43	0.328	116	1875	521	16.6	37.4	1002	37.8	2580	26.6	
	0.103	0.530	1.73	0.463	65.3	1523	504	14.1	30.5	1167	20.1	2666	12.9	
	0.103	0.830	2.38	0.463	99.4	2300	627	20.9	43.8	1605	56.8	3156	38.5	
	0.113	0.580	1.78	0.554	95.0	1892	560	16.5	32.0	1469	54.8	2643	21.8	
	0.119	0.780	2.25	0.655	122	2297	606	22.7	47.9	1824	34.7	2814	22.3	
	0.129	0.740	2.53	0.407	87.3	2107	607	22.0	46.6	1664	28.8	2629	27.0	
	0.146	0.890	2.84	0.893	132	2426	621	22.3	44.6	1779	58.4	2846	39.6	
Q 4 Mean, 0.262 SD, 0.096	0.157	0.820	2.82	0.576	128	2141	588	20.1	45.3	1424	34.2	2500	21.1	
	0.194	0.700	2.51	0.689	109	2052	587	22.1	39.7	1523	33.1	2536	22.9	
	0.223	0.680	2.56	0.576	142	2240	725	23.5	45.2	1614	42.1	2916	26.9	
	0.230	0.510	1.66	0.780	108	2399	714	19.2	45.8	1703	33.5	3196	29.7	
	0.255	0.570	1.68	0.452	120	2008	607	20.0	44.2	1517	77.4	2865	29.5	
	0.339	0.540	1.68	0.486	86.7	1784	574	17.0	41.4	1374	46.5	2354	19.0	
	0.439	0.620	1.86	0.463	72.8	1998	563	19.5	38.1	1385	25.4	2556	20.0	
Mean	0.108	0.754	2.43	0.715	127	2316	660	21.9	46.3	1733	57.5	3036	28.5	
SD	0.109	0.143	0.492	0.287	36.9	348	85.5	3.26	7.65	375	52.3	475	6.64	
P 5	0.001	0.519	1.67	0.358	68.7	1641	512	15.2	31.2	1076	22.4	2403	15.6	
P 25	0.026	0.635	1.97	0.466	98.7	2066	589	19.6	41.9	1471	34.3	2592	22.5	
Median	0.081	0.760	2.52	0.672	125	2323	670	22.2	45.5	1686	50.8	2955	29.5	

Fig. 5. Study subjects were grouped according to quartiles (Q1-Q4, from top to bottom) of NF-κB c-Rel activation. Plasma lipids and individual plasma fatty acids, which were significantly related ($P < 0.01$) to c-Rel activation, were colour-coded according to quartiles (Q1-Q4) of plasma concentrations, with values above median (Q3 + Q4) marked in 2 grades of red, while values below median (Q1 + Q2) marked in 2 grades of blue. From this figure, each individual participant's lipid and fatty acid concentrations can be depicted and related to the quartiles of the individual c-Rel activations. At the bottom of the figure, results of descriptive statistics are provided for c-Rel and the plasma lipids and individual fatty acids of the 28 women. Abbreviations: ApoB, apolipoprotein B; LDL, LDL cholesterol; OD, optical density; P, percentile; Q, quartile; SD, standard deviation; TRI, triglycerides. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

T1 and T4, while no clear tendency was observed at T2 and T3.

Of note, opposite relations with plasma lipids and fatty acids were found for estradiol (positive) and progesterone (inverse) at T1 (Figs. 3 and 4); they were significant for the positive relations of estradiol with total MUFA and C18:3n-3, and for the negative relations of progesterone with total MUFA and C18:3n-3 (Table 4).

3.8. Comparisons of subjects with high versus low c-Rel activation

In addition to correlation analysis, study subjects were grouped (i) according to quartiles (Q1-Q4) of c-Rel activation and (ii) quartiles of those plasma lipids and individual fatty acids which showed significant relations ($P < 0.01$) with c-Rel and thus could be regarded as possible determinants of c-Rel activation (Fig. 5). From this figure, each individual participant's lipid and fatty acid concentrations can be depicted and related to the quartiles of the individual c-Rel activations. Higher than median levels (Q3 + Q4, marked in 2 grades of red) of plasma lipids were present in 11/14 study subjects for triglycerides and 10/14 for both LDL cholesterol and apolipoprotein B in subjects with lower than median (Q1 + Q2) c-Rel activation. Regarding SFA, 12/14 subjects with higher than median C16:0 and C18:0 and 11/14 subjects with higher than median C14:0, 20:0 and 22:0 showed lower than median (Q1 + Q2) c-Rel activation. Of the 14 subjects with higher than median C18 MUFA (C18:2n-6; C18:1n-9 and C18:3n-3) 12, 11 and 10 subjects, respectively, showed lower than median c-Rel activation, as was the case for C22:5n-3 in 11/14 subjects. ANOVA showed significant differences in c-Rel activation of quartiles Q1-Q4 for apolipoprotein B ($P = 0.005$), triglycerides ($P = 0.026$), C14:0 ($P = 0.032$), C16:0 ($P = 0.005$), C18:0 ($P = 0.014$), C20:0 ($P = 0.023$), C18:1n-9 ($P = 0.026$), C18:3n-3 ($P = 0.033$), C18:2n-6 ($P = 0.010$) and C22:5n-3 ($P = 0.019$), but not for LDL cholesterol ($P = 0.053$) and C22:0 ($P = 0.056$). Post hoc tests revealed significant differences between Q1 and Q4 for all plasma lipids and fatty acids except C22:5n-3.

Table 5
Plasma apolipoprotein B concentrations (5th – 95th Percentile, P5 – P95) and the corresponding c-Rel activation levels in PBMC of 28 women at T2 (calculated from the regression equation shown in Fig. 6).

Apolipoprotein B (g/L)		c-Rel (OD)
P5	0.519	0.206
P25	0.635	0.149
Median (P50)	0.760	0.076
P75	0.860	0.031
P95	1.04	0.005

3.9. Regressions of c-rel activation on apolipoprotein B concentrations

Apolipoprotein B, as a candidate of the plasma lipids with strong relations with c-Rel activation (significant results of Spearman rank correlations at more than one time point) was tested as a possible predictor of c-Rel activation in linear regression analysis. To pass normality and equal variance tests, the square root of c-Rel data was used. As shown in Fig. 6, there were strong inverse linear dependencies of the square root of c-Rel activation on apolipoprotein B concentrations at all time points, with $P < 0.05$ used as the threshold of significance. From the regression equation at T2, i.e. the time point with the strongest relation, the c-Rel activations were calculated for the 5th - 95th percentile of plasma apolipoprotein B concentrations of the study participants (all showing apolipoprotein B concentrations within the normal range [41]) and are presented in Table 5.

4. Discussion

This is the first study focusing on a comprehensive analysis of the canonical activation pathway of NF-κB by addressing all 3 subunits, c-Rel, p65 and p50 [36], across the menstrual cycle of healthy women, and, for the first time, showing strong inverse associations between

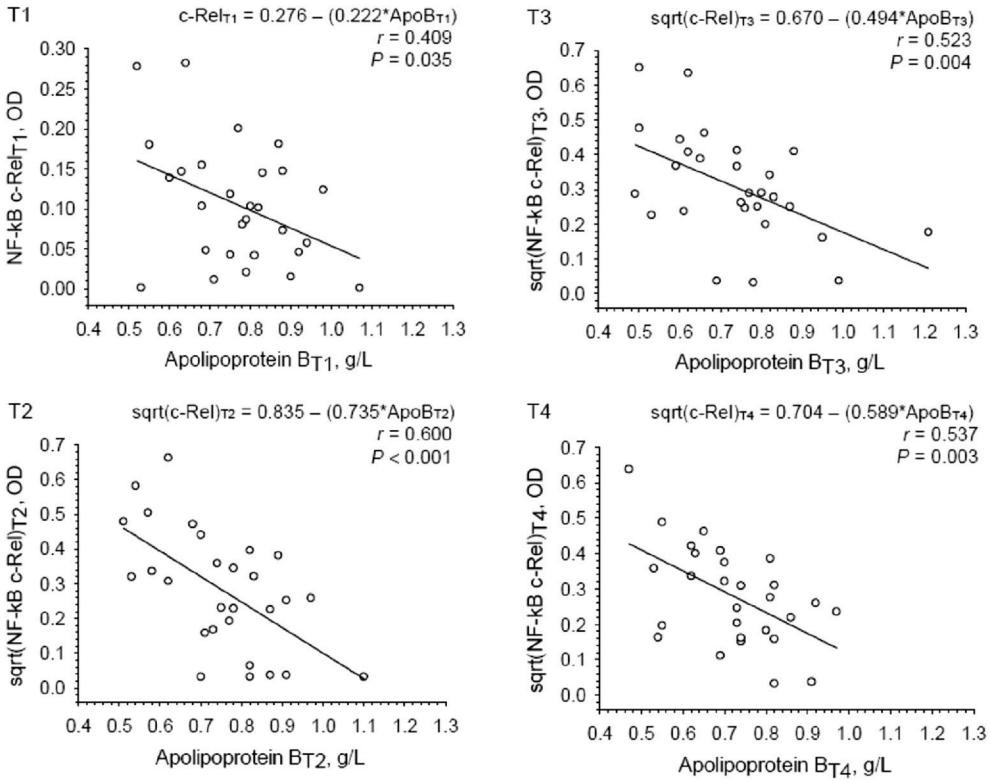

Fig. 6. Linear regressions of c-Rel activation in peripheral blood mononuclear cells on apolipoprotein B (ApoB) concentrations in plasma at the 4 time points (T1-T4) of a given menstrual cycle in 28 women. To pass normality and equal variance tests, the square route (sqrt) of c-Rel data was used.

plasma lipid and fatty acid concentrations with c-Rel activation, cumulating at late follicular phase. Interestingly, while significant associations of plasma lipid and fatty acid concentrations with c-Rel activation were exclusively negative, those with p65 activation were all positive, while associations were entirely absent for p50. The differential effects of lipid and fatty acid concentrations on c-Rel activation as compared to p65 have not been reported in any study involving human subjects so far.

The inverse relations of plasma lipid and fatty acid concentrations with c-Rel were particularly strong and consistent at late follicular phase (T2). In addition, relations of apolipoprotein B as well as total fatty acids, total PUFA, total n-6 PUFA and the 2 individual fatty acids, C20:0 and C22:5n-3, with c-Rel, remained significant also at mid luteal phase (T3), and continued to be so for apolipoprotein B also at late luteal phase (T4), suggesting that the initiation of the effects of lipid and fatty acid concentrations on c-Rel activation starts at T2 and covers the period of oocyte maturation, ovulation as well as the implantation window.

Concerning fatty acids that showed significant inverse associations with c-Rel activation it is interesting to note that strong relations were observed with fatty acids of all classes, be it SFA, MUFA, n-3 or n-6 PUFA. However, the fact that 5/9 individual fatty acids with significant relations were SFA, including C14:0, C16:0, C18:0, C20:0 and C22:0, representing fatty acids that are endogenously produced by the fatty acid synthase complex and which through elongase activity are converted to longer chain fatty acids, could suggest a possible specific inhibitory effect of these SFA on c-Rel activation.

When identifying subjects in lower (Q1 + Q2) versus higher quartiles (Q3 + Q4) of c-Rel activation in the presence of higher (Q3 + Q4) versus lower (Q1 + Q2) plasma lipid and individual fatty acid concentrations, a clustering towards higher plasma lipid, SFA and 18 carbon fatty acid concentrations in subjects with lower c-Rel activation became also evident when using this approach, which was statistically supported by significant results of group comparisons.

In view of potential physiological implications of inverse associations of lipid and fatty acid concentrations with c-Rel activation, which is involved in Th1- and Th17-mediated immune responses as well as in the development of Treg cells [29,31], it could be hypothesized that elevated lipid and fatty acid concentrations might contribute to inadequate immune modulation in the course of the menstrual cycle. This hypothesis is supported by observations that women with elevated serum cholesterol or elevated total serum lipid concentrations showed increased time to pregnancy, even when adjusted for BMI [42], and that total cholesterol concentrations ≥200 mg/dL were associated with reduced fecundability [43]. Data on apolipoprotein B and fatty acids were not reported. In this context, it is interesting to note that a potential regulatory role of apolipoprotein B in embryonic development of rodents has been demonstrated [44].

When apolipoprotein B was tested as a possible predictor of c-Rel activation, strong significant dependencies of c-Rel activation on apolipoprotein B concentrations were elucidated across all time points, starting at T1 and remaining so up to T4, indicating that c-Rel activation was dependent on apolipoprotein B concentrations irrespective of a specific phase of the menstrual cycle. These data, for the first time, suggest that apolipoprotein B concentrations could play a role in modulating c-Rel activation levels. Whether such dependency as observed in the current study is restricted to pre-menopausal women or occurs in the general population, and downregulation of c-Rel activation could thus be regarded as a consequence of higher apolipoprotein B concentrations, remains to be further elucidated. Overall, apolipoprotein B concentrations in the present study were lower (5th percentile, 0.52 g/L; 95th percentile, 1.04 g/L) compared with reference ranges of women of a comparable age range, which were (5th −95th percentile) 0.61–1.40 g/L for 20-29 year-old and 0.65–1.53 for 30-39 year-old women described as being to the large majority subjectively healthy [41].

Among the most interesting findings of the present study are differential associations of LDL cholesterol and C18:2n-6 as well as total n-6 PUFA concentrations with the activation of p65 (positive) in contrast to c-Rel (negative) and p50 subunits (no significant association) at T2. Previous studies in different primary and cultured cells suggested that SFA with chain lengths of 16 and 18 carbon atoms activate p65 [8–11], while shorter chain SFA [11] and different classes of unsaturated fatty acids did not or even reduced p65 activation [8–10]. Regarding underlying mechanisms, activation of p65 in these studies was shown to be mediated by Toll-like receptors (TLR) [8–10], which are known to play important roles in T [45] and B cell-mediated [46] immune function.

In an attempt to explain these opposite associations in the present study it is noteworthy that, while all three subunits studied share an N-terminal Rel homology domain responsible for DNA binding and homo- and heterodimerization of subunits, a transcription activation domain (TAD), necessary for the regulation of gene expression, was shown to be present only in p65 and c-Rel, but not in p50 [47], and TADs further showed only little similarity in amino acid composition between c-Rel and p65 subunits [48], suggesting that their activation pathways are different, that they have different transactivation functions and that they target different genes [49]. Additionally, evidence from naive T cells suggests that c-Rel and p65 are differentially regulated by forming distinct complexes with IκBs, which are known as inhibitors of nuclear translocation of NF-κB, with c-Rel being primarily associated with IκBβ, while p65 is primarily associated with IκBα, and of which IκBβ is more rapidly degraded compared to IκBα [29,50]. However, the underlying mechanisms for the differential effects of lipids and fatty acids on the activation of different subunits of the canonical NF-κB activation pathway in the present study deserve further elucidation.

The absence of associations of plasma lipids and fatty acids with the activation of the p50 subunit may be surprising at the first sight, given that in patients with familial hypercholesterolemia p50 activation was significantly higher compared to normolipidemic controls and positively associated with LDL cholesterol concentrations [13], but could be explained by the fact that cholesterol concentrations of patients with familial hypercholesterolemia were up to about 200% of those of the women of the present study.

The absence of significant correlations of circulating estradiol and progesterone concentrations with any of the 3 subunits of NF-κB activation in PBMC at any of the time points across the menstrual cycle suggests that there were no direct hormonal effects. However, indirect lipid-mediated effects of estradiol on NF-κB activation cannot be ruled out. In this context, the findings of a significant positive association between progesterone and C22:5n-3 and an inverse association of C22:5n-3 with c-Rel activation at T3 could implicate a link between the hormonal effects on fatty acids and the effects of fatty acids on c-Rel activation.

Effects of oestrogens on lipid metabolism are well known [51], and lowering of apolipoprotein B and LDL cholesterol concentrations in postmenopausal women with oral administration of oestrogens has been reported [52,53]. More recently, it was found that endogenous oestrogens reduce apolipoprotein B and LDL cholesterol in pre-menopausal women via induction of hepatic LDL receptors [54]. Possible effects of oestrogens on plasma fatty acid concentrations in pre-menopausal women have not been reported.

A novel finding of the present study are the differential, i.e. strong positive associations with estradiol and strong inverse associations with progesterone of total MUFA as a class of fatty acids and C18:3n-3 as the only individual fatty acid (r = 0.572 and r = -0.515, respectively), which were confined to the early follicular phase, suggesting that C18:3n-3 may have been specifically and differentially affected by both estradiol and progesterone. In an attempt to explain these findings, data recently published by Hu et al. [55], showing significantly accelerated human oocyte maturation upon treatment with 50 μmol/L (of the test range of 0–200 μmol/L) C18:3n-3 in the *in vitro* maturation medium,

are of particular interest. Notably, the plasma concentrations of C18:3n-3 in the women of the present study, which were (mean ± SD) 56.9 ± 52.2 μmol/L, very well matched those *in vitro* concentrations. In line with improvement of porcine [56] and bovine [57] oocyte maturation by C18:3n-3 in previous animal studies, the results derived from experiments in human oocytes [55] could explain a possible specific modulatory role of C18:3n-3 in this early phase of the menstrual cycle in the present study. This role could be either exerted by (i) direct metabolic effects through serving as a substrate for complete oxidation and use for de novo fatty acid synthesis, with C18:3n-3 having been shown to be converted to SFA (C16:0, C18:0) and MUFA (C16:1n-7, C18:1n-9) in humans [58], or (ii) indirect effects through its function as the precursor of long-chain n-3 PUFA [59].

The present study further showed associations between estradiol and fatty acids with opposite directions of relations at T1 (overall tendency towards positive relations) as compared to T3 (overall tendency towards inverse relations). At T1, the most interesting findings in this regard were strong positive relations with all classes of fatty acids, including SFA (C18:0), MUFA (C18:1n-7, C18:1n-9) and n-3 PUFA (C18:3n-3), all of which were confined to C18 fatty acids. In contrast, at T3 a significant inverse relation with estradiol was restricted to C18:3n-6. Whether these results, i.e. changes across the menstrual cycle, resulted from possible changes in estradiol concentrations and/or conversions of fatty acids to long-chain PUFA remains unknown. Progesterone, but not estradiol, was associated with (i) increased expression of FADS2 (the d6 desaturase responsible for conversion of C18:3n-3 and C18:2n-6 to C18:4n-3 and C18:3n-6, respectively) in non-pregnant rats [60], and (ii) increased conversion of 18:3n-3 to 20:5n-3, 22:5n-3 and 22:6n-3 in HepG2 cells and primary hepatocytes, which was further supported by increased mRNA expression in HepG2 cells for FADS2, FADS1, ELOVl5 and ELOVl2 [61]. Estradiol was shown to upregulate *ELOVL5* (the enzyme responsible for elongation of C18:4n-3 to C20:4n-3 and C18:3n-6 to C20:3n-6, respectively) in laying hens [62]. Data on human subjects and women across the menstrual cycle are entirely missing.

Given that desaturases (as well as elongases) show a higher affinity for n-3 compared to n-6 fatty acids, modulation of FADS2 by estradiol and/or progesterone could be expected to result in differential effects on conversion of C18:3n-3 to C18:4n-3 as compared to conversion of 18:2n-6 to C18:3n-6. In this context, the finding of a significant inverse relation with estradiol at T3 that was confined to C18:3n-6, while plasma lipids and other fatty acids did not reach statistical significance, should be noted. Conversely, C22:5n-3, with desaturation (FADS1 and FADS2) as well as elongation (ELOVI5) steps involved in its endogenous generation, was the only fatty acid that showed a significant positive association with progesterone at T3. Whether the observed differences across the 4 time points of the menstrual cycle of healthy women might also reflect changing physiological needs across the menstrual cycle, remains to be elucidated.

Taking these results together, the present study, for the first time, showed strong inverse associations of plasma lipids and individual fatty acids, in particular but not exclusively of C14–C22 SFA, with c-Rel activation. Most of these associations were confined to late follicular phase, while the dependency of c-Rel activation on apolipoprotein B concentrations was visible across the 4 time points of the menstrual cycle. In contrast, associations with p65 activation at late follicular phase were positive and reached statistical significance for total and LDL cholesterol and total n-6 PUFA and C18:2n-6, while p50 activation did not show any relations. Even though estradiol and progesterone were not directly related to c-Rel activation, C22:5n-3, with a positive association with progesterone and an inverse association with c-Rel activation, could be implicated in an indirect fatty acid-mediated hormonal effect of progesterone on c-Rel activation. C18:3n-3, which might play a specific role in oocyte maturation, stood out as the fatty acid that was differentially associated with estradiol and progesterone at early follicular phase. Given the specific roles of c-Rel activation in immune tolerance, inhibition of c-Rel activation by higher plasma concentrations of apolipoprotein B and individual, mainly SFA, at and beyond the late follicular phase could have clinical implications for female fertility.

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Author contributions

B. M. Winklhofer-Roob, J. M. Roob and G. Faustmann contributed to conception and design of the study; G. Faustmann, B. M. Winklhofer-Roob, B. Tiran, B. Obermayer-Pietsch, H. J. Gruber contributed to sample analysis and acquisition of data; G. Faustmann, B. M. Winklhofer-Roob, S. Trajanoski and J. Ribalta contributed to analysis and interpretation of data; G. Faustmann and B. M. Winklhofer-Roob wrote the paper. All authors critically revised the manuscript for important intellectual content and approved the final version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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