The effect of combined oral contraceptives containing drospirenone and ethinylestradiol on serum levels of amino acids and acylcarnitines

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Abstract

Introduction: Metabolome variations have long been associated with normal hormonal fluctuations, and similar effects, related to the use of early generation synthetic hormones as a means of contraception, have also been identified.

Objective: We investigated the serum amino acid and acylcarnitine profiles induced by the use of combined oral contraceptives (COCs) consisting of Ethinylestradiol (EE) and a 4th generation progestin, Drospirenone (DRSP).

Method: Gas chromatography mass spectrometry and liquid chromatography with tandem mass spectrometry was used to identify and quantify the serum amino acids and acyl carnitine levels in 24 controls, 25 DRSP/20EE users and 26 DRSP/30EE users.

Results: Of the 26 amino acid compounds measured, 13 showed significant variations in abundance between the control and COC user groups. Although none of the 21 acylcarnitine compounds detected were statistically significant with regards to group variations, a trend, related the EE concentration, was observed. The detected metabolome disparities corresponded to that identified for earlier generation COCs, all pointing toward increased oxidative stress levels in the user groups.

Conclusion: These findings suggest that the clinical complications associated with these COCs could, to some extent, be alleviated by the simultaneous use of antioxidants. The study also highlights the role that targeted metabolomics could play in the elucidation of the underlying mechanisms of drug-induced severe effects.

Keywords: Combined oral contraceptives; Amino acids; Acylcarnitines; Drospirenone; Ethinylestradiol; Antioxidant

Introduction

The use of combined oral contraceptives (COCs) is becoming increasingly popular, and these drugs are currently the preferred method of contraception in sexually active females from high-income countries (Daniels & Mosher, 2013; Kavanaugh & Jerman, 2018). Some of the common side effects associated with COC use include nausea and vomiting, headache, irritability, and changes in skin pigmentation. COC use is also known to impact the haemostatic variables associated with hypercoagulability (Kluft et al., 2006; Stocco et al., 2015). The underlying mechanism driving these effects are, however, not fully comprehended and the application of newer research methodologies, such as metabolomics, could lead to a better understanding of these occurrences.

Although a number of studies have indicated variations in the metabolism due to normal hormonal fluctuations such as the menstrual cycle (Cox & Calame, 1978; Hrboticky et al., 1989) and pregnancy (Cederblad et al., 1986), very few have focussed on the effect of synthetic hormones on the human metabolome.

In a previous study, using an untargeted metabolomics approach, we identified disparities in the serum metabolite profiles of females related to the use of Ethinylestradiol (EE) combined with a 4th generation progestin, Drospirenone (DRSP).

These observations were associated with potential variations in the amino acid metabolism and increased oxidative stress, which in turn, could be linked to the prothrombotic state seen in these individuals (Swanepoel et al., 2020). The aim of the current study is to substantiate these findings using targeted metabolomics methods, including the analyses of serum amino acids and acylcarnitines, in these COC users. Similar studies have indicated variations in these metabolite classes related to the use of EE combined with 1st (Craft & Peters, 1971), 2nd (Wang et al., 2016), and 3rd (Ruoppolo et al., 2014; Wang et al., 2016) generation progestins. The androgenic characteristics of these early progestins, which can result in severe lipid and skin effects, led to the development of DRSP, a 4th generation anti-androgenic, antimineralocorticoid (Regidor, 2018). Our work is the first of its kind to assess serum amino acids and acylcarnitines in relation to the use of DRSP in combination with 30 µg and 20 µg EE, respectively. We also compared the detected variations to those previously identified for earlier generation progestins, and interpret the results based on known biochemical and physiological states induced by these COCs.

Materials and methods

Participant recruitment and sample collection

For this study, healthy females aged between 18 and 30 years, with no history of chronic illnesses (including thrombotic diseases), using no tobacco products or chronic medication (other than the relevant COCs), were recruited. These participants were classified as either controls (women not using any type of hormonal contraceptive, for a minimum of 6 months prior to sample collection), women consuming DRSP/20EE, or women consuming DRSP/30EE. In order to remove the effect of endogenous estrogen and progesterone, blood was collected from controls during menses (Swanepoel et al., 2014). The COC test groups were sampled after using the active pills for a minimum of 7 days (Reif et al., 2013). After 7 days, the levels of these exogenous hormones stabilize. Moreover, it has been indicated that the concentration of the exogenous progestin from these formulations are seven to eight fold

higher than the median endogenous progesterone during a 28-day cycle in females not using COCs. The exogenous estrogen levels for females using these formulations are comparable to the maximum level of endogenous estradiol during a 28-day cycle in non COC users (Lovett et al., 2017). These high levels of progestin, additionally decreases the targeted organs' response to estrogen. Synthetic hormone formulations therefore 'override' the actions of endogenous estrogen and progesterone.

A trained phlebotomist collected venous blood in VACUCARE plain red tubes (with no additives), which were left to stand at room temperature for 30–60 min (for clot formation), before centrifugation (3000 rpm) at 4 °C for 5 min. The serum layer (supernatant) was then transferred to a new serum collection tube using a glass Pasteur pipette. The collected serum was again centrifuged (5000 rpm) for 5 min at 4 °C, where after the supernatant was transferred to a DNA LoBind Eppendorf tube which was immediately frozen at − 80 °C (and transported as such) until metabolomics analyses commenced at the Centre for Human Metabolomics, South Africa.

Amino acid analyses

A Phenomenex EZ: faast™ amino acid kit was applied for the preparation of the serum samples (50 µL), according to the manufacturer's instructions. For quantification purposes, additional internal standards, including; 4-methyl-DL-tryptophan (Merck, South Africa), DL-cystine-d_6 isotope (CDN isotopes, Canada), universally labelled carbon and nitrogen isotopes L-glutamine and L-asparagine, and an ALGAL amino acid mixture (Cambridge Isotope Laboratories, Inc., USA), was added. Amino acid extracts (1 µL) were analysed in splitless mode on an Agilent HP 7890A Gas chromatograph (GC) (Hewlett-Packard Company, North Carolina, USA) coupled to an Agilent 5975C Mass selective detector (MSD) with Triple-Axis Detector (Agilent Technologies, California, USA). A GC column (Zebron ZB-AAA, 10 m × 0.25 mm), which is included in the analysis kit, was used for compound separation, with ultra-pure helium as the carrier gas at a constant flow of 1.3 mL/min and pressure of 0.37 psi. The inlet temperature was kept constant at 250 °C throughout the run. The oven temperature was initially held at 60 °C for 1 min, where after it was increased by 50 °C/min to 110 °C, then with 20 °C/min to 185 °C, followed by an increase of 25 °C/min to 235 °C, and finally with 30 °C/min to an end temperature of 320 °C, where it was kept for 1 min. Peak detection, integration and quantification were done using Enhanced MSD Chemstation software (Agilent Technologies, Inc., USA, version F.01.00.1903). The data was quantified by comparing the intensities of the detected compounds to that of the corresponding internal standards. European Research Network for evaluation and improvement of screening, diagnosis and treatment of inherited disorders of metabolism, internal quality control (ERNDIM IQCS) amino acids levels 1 and 2 (MCA Laboratory, The Netherlands) were used for quality control purposes.

Acylcarnitine analyses

Acylcarnitine stable isotopes ([methyl-d_3]-L-carnitine.HCl, [d_3]acetyl-L-carnitine.HCl, [3,3,3-d_3]propionyl-L-carnitine.HCl, [d_9]isovaleryl-L-carnitine.HCl, [8,8,8-d_3]octanoyl-L-carnitine.HCl, [10,10,10-d_3]decanoyl-L-carnitine.HCl, [12,12,12-d_3]dodecanoyl-L-carnitine.HCl, [14,14,14-d_3]tetradecanoyl-L-carnitine.HCl, [16,16,16-d3]hexadecanoyl-L-carnitine.HCl, [18,18,18-d_3]octadecanoyl-L-carnitine.HCl) (VU Medical Center, Amsterdam, The Netherlands) were prepared in methanol and was used as internal standard mixture.

Samples were prepared by adding 205 µL of the internal standard mixture to 10 µL of each sample, where after it was vortexed for 30 s and centrifuged for 20 min at 12 000 g (10 °C), in order to precipitate the proteins. Hereafter, 205 µL of the resulting supernatant was transferred to a 96 well microtiter plate, and evaporated to complete dryness under a steady stream of nitrogen at 37 °C. Consequently, 100 µL of 3 N butanolic HCl was added to the dried residue followed by an incubation step of 45 min at 65 °C. The butylated samples were then evaporated to dryness under a steady stream of nitrogen at 37 °C and suspended in a water: acetonitrile solution (50:50) (v/v) containing 0.1% formic acid.

For MS analyses, an Agilent 1260 series Liquid chromatography (LC) system coupled to a 6420 triple quadrupole (QQQ) mass analyzer (Agilent Technologies, USA) consisting of a 1260 HiP degasser (G4225A) and binary pump (G1312C) was used in positive ion electrospray mode. Samples (5 µL) were injected directly at a constant flow rate of 0.05 mL/min. Acylcarnitines were analysed with a precursor ion scan of m/z 85, and scanning was done from m/z 200 to 600. Data analysis was performed with Masshunter Qualitative Analysis Software B.07 (Agilent Technologies, USA) and the acylcarnitines (C0, C2, C3, C3-DC, C4, C4-OH, C4-DC, C5, C5:1, C5-OH, C5-DC, C6, C8, C10, C14, C14:1, C16, C16-OH, C18, C18:1) were identified and quantified using the stable isotopes as a reference. These acylcarnitines are also present in the ERNDIM IQC samples (2019.1261 and 2019.1262, MCA Laboratories, The Netherlands) which were used for quality control purposes.

Statistical analyses

All statistical analysis were done on the quantified datasets, using MetaboAnalyst 4.0, a web server for metabolomics data analyses with functions written in R (v 3.6.3) (Chong et al., 2019). Data emanating from the amino acid and acylcarnitine analyses were evaluated separately. Compounds with zero values were assumed to be present at concentrations below the detection limit and were therefore replaced by 1/5th of the minimum value detected for that particular compound. No further filtering was applied, and the data was consequently log transformed and auto scaled. To get an overall, summative view of the natural variation in the data, principle component analyses (PCA), a multivariate, unsupervised technique, was applied to the two datasets. In an attempt to identify compounds, which are potentially significant concerning discrimination between sample groups, we applied a univariate one-way analyses of variance (ANOVA), with Fisher's Least significant difference method (LSD) as the post-hoc analyses of choice. Compounds with a p value < 0.05 were considered significantly different between the sample groups. As an additional measure to identifying compounds that drive group separation, we also applied the multivariate, supervised modelling method; Orthogonal projections to latent structures - Discriminant analyses (OPLS-DA). A permutation test was done to determine the significance of group separation, using the optimal number of components, as defined by cross validations. Compounds were considered important when the Variable import in projection (VIP) parameter, which is a sum of squares of the OPLS loadings (average of the optimal number of components), was > 1.

Results

Population

A total of 47 parameters (26 from amino acid analyses and 21 from acylcarnitine analyses), were measured from 75 participants, fulfilling the inclusion and exclusion criteria. This cohort included 24 controls, 25 DRSP/20EE users and 26 DRSP/30EE users.

Metabolite variations induced by COC use

A graphical representation of the PCA scores plots generated for each dataset for the first two principle components (PC) is given in the supplementary material (Figure S1). Although the PCA indicated probable heterogeneity between the control and the COC groups in the amino acid data, no discrimination between the two COC groups were evident. No form of discrimination between samples was apparent on the PCA scores plot emanating from the analyses of the acylcarnitine data, indicating no probable heterogeneity.

The application of OPLS-DA to the amino acid data was able to classify samples to the respective sample groups using two components (Fig. 1; Supplementary material Figure S2). Of the 26 amino acids measured, 11 had VIP values > 1 (Table 1). Based on the permutation test, the OPLS-DA model built for the acylcarnitine data was not significant, and this analysis was therefore not used as a measure to identify important compounds for this dataset (Fig. 1; Supplementary material Figure S2).

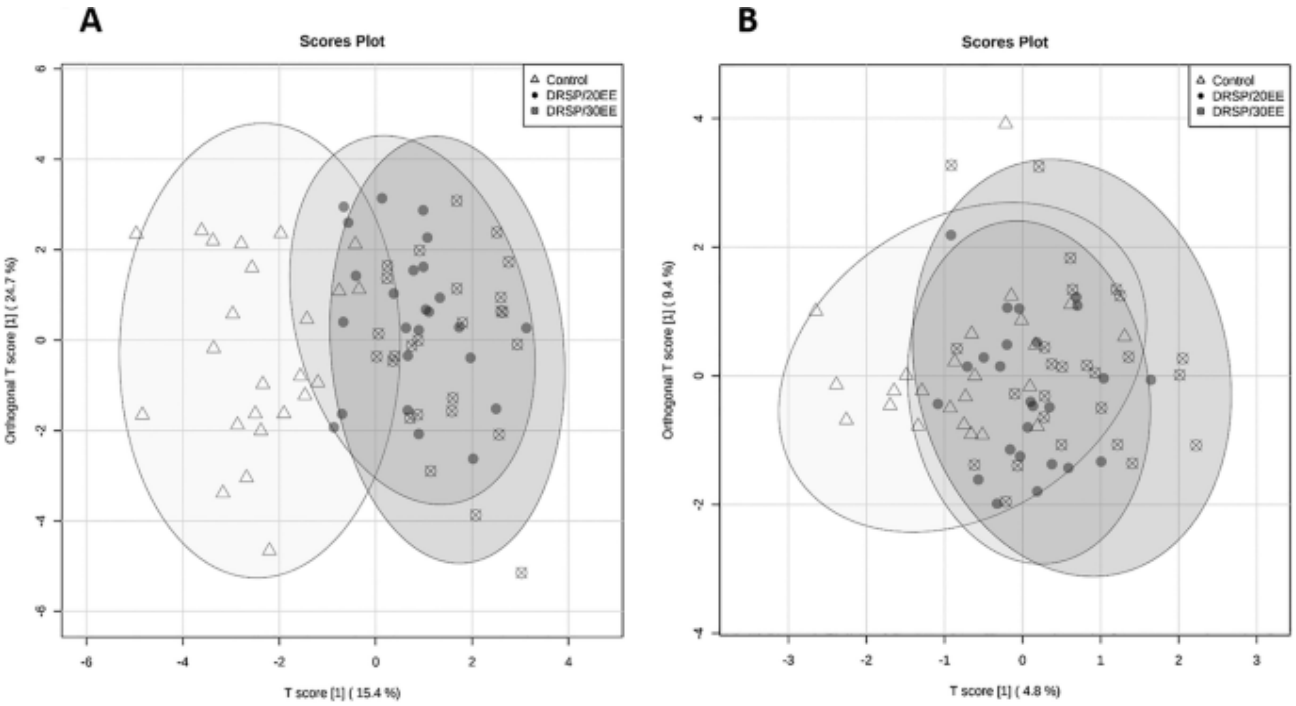

Fig 1. OPLS-DA scores plots for the two datasets investigated, with the variation explained indicated in parentheses. **A** amino acid data, **B** acylcarnitine data

Table 1 Compounds detected via the amino acid analyses

Detected compound	Control	DRSP/20EE		DRSP/30EE		One-way ANOVA		OPLS-DA (VIP) (Avg comp 1 and 2)
	Average concentration (µmol/L) (SEM)	Average concentration (µmol/L) (SEM)	Increased (↑) or decreased (↓) with COC	Average concentration (µmol/L) (SEM)	Increased (↑) or decreased (↓) with COC	P value	Fisher's LSD	
4-Hydroxyproline	17.42 (2.124)	10.35 (0.863)	↓	10.65 (0.952)	↓	5.58×10^{-4}	Control - DRSP/20EE; Control - DRSP/30EE	Not significant
Alanine	497.5 (24.13)	404.0 (9.944)	↓	398.0 (12.39)	↓	2.03×10^{-4}	Control - DRSP/20EE; Control - DRSP/30EE	1.14
Alpha-aminoadipic acid	0.330 (0.020)	0.336 (0.023)	↑	0.326 (0.029)	↓	Not significant		Not significant
Alpha-aminobutyric acid	23.11 (1.423)	17.97 (1.003)	↓	19.37 (1.272)	↓	Not significant		Not significant
Argininosuccinic acid	0.019 (0.003)	0.021 (0.003)	↑	0.018 (0.002)	↓	Not significant		Not significant
Asparagine	65.83 (2.543)	57.68 (1.452)	↓	60.33 (1.473)	↓	1.61×10^{-2}	Control - DRSP/20EE	Not significant
Aspartic acid	14.01 (0.753)	18.53 (0.578)	↑	17.89 (0.728)	↑	1.16×10^{-5}	DRSP/20EE - Control; DRSP/30EE - Control	1.03
Cystathionine	0.827 (0.099)	0.919 (0.078)	↑	0.825 (0.073)	↓	Not significant		Not significant
Cystine	29.66 (1.228)	27.55 (1.478)	↓	30.46 (1.027)	↑	Not significant		Not significant
Glutamic acid	33.61 (1.279)	37.02 (1.445)	↑	34.77 (1.765)	↑	Not significant		Not significant
Glutamine	666.1 (13.64)	527.2 (11.97)	↓	531.1 (12.01)	↓	4.05×10^{-11}	Control - DRSP/20EE; Control - DRSP/30EE	1.27
Glycine	308.3 (11.91)	209.5 (6.634)	↓	225.4 (14.19)	↓	8.51×10^{-9}	Control - DRSP/20EE; Control - DRSP/30EE	1.31
Histidine	87.78 (2.218)	87.97 (1.903)	↑	88.83 (1.875)	↑	Not significant		Not significant
Isoleucine	66.48 (3.479)	57.69 (2.872)	↓	57.17 (3.182)	↓	Not significant		1.15
Leucine	120.5 (5.208)	109.8 (4.466)	↓	107.0 (4.640)	↓	Not significant		1.09
Lysine	189.5 (8.174)	161.5 (6.456)	↓	170.3 (6.559)	↓	2.06×10^{-2}	Control - DRSP/20EE	Not significant
Methionine	30.01 (1.460)	23.72 (1.079)	↓	24.91 (1.188)	↓	1.80×10^{-3}	Control - DRSP/20EE; Control - DRSP/30EE	1.16
Ornithine	65.02 (3.363)	39.74 (2.075)	↓	40.62 (1.934)	↓	9.06×10^{-11}	Control - DRSP/20EE; Control - DRSP/30EE	1.32
Phenylalanine	66.71 (2.513)	65.79 (1.885)	↓	65.21 (1.313)	↓	Not significant		Not significant
Pipecolic acid	1.864 (0.172)	3.441 (0.347)	↑	3.004 (0.237)	↑	1.17×10^{-5}	DRSP/20EE - Control; DRSP/30EE - Control	Not significant
Proline	227.1 (11.72)	151.8 (7.126)	↓	165.0 (10.01)	↓	1.71×10^{-6}	Control - DRSP/20EE; Control - DRSP/30EE	1.17
Serine	151.3 (3.497)	131.0 (1.794)	↓	131.2 (2.679)	↓	7.92×10^{-7}	Control - DRSP/20EE; Control - DRSP/30EE	1.20

Threonine	162.2 (7.271)	146.1 (5.658)	↓	163.0 (5.026)	↑	Not significant		Not significant
Tryptophan	61.38 (2.561)	60.60 (2.018)	↓	63.38 (2.851)	↑	Not significant		Not significant
Tyrosine	68.90 (3.284)	53.48 (1.976)	↓	55.18 (2.972)	↓	5.01×10^{-4}	Control - DRSP/20EE; Control - DRSP/30EE	1.16
Valine	234.1 (8.488)	218.3 (7.124)	↓	220.6 (7.391)	↓	Not significant		Not significant

ANOVA analyses of variance, *COC* combined oral contraceptive, *DRSP* drospirenone, *EE* ethinylestradiol, *LSD* least significant difference, *PLS-DA* partial least squares-discriminant analyses, *SEM* standard error of the mean, *VIP* variable import in projection

A total of 13 of the amino acids measured had p values < 0.05, which highly corresponds to the OPLS-DA list of important features (Table 1). Fisher's LSD indicated that the levels of variation for these compounds were between the control and respective COC groups, and not amid the two COC groups. No significant p values (< 0.05), discriminating between sample groups, were detected for the acylcarnitine data via ANOVA. Boxplots for all compounds detected via the amino acid and acylcarnitine methods are given in the Supplementary material as Figures S3 and S4, respectively.

Discussion

Amino acid variations

DRSP/EE use resulted in a significant reduction in several amino acids, including 4-hydroxyproline, alanine, asparagine, glutamine, glycine, lysine, methionine, ornithine, proline, serine, and tyrosine when compared to the controls. Aspartic acid and pipecolic acid were significantly increased in the DRSP/EE groups compared to the control group. It is interesting to note that the variations, statistically significant or not, did not follow a dose dependent pattern. DRSP/20EE with the lowest estrogen concentration, had the greatest effect on the majority of the amino acids analysed, which is similar to the results of our untargeted study (Swanepoel et al., 2020).

Several studies have associated fluctuations in the plasma and serum amino acid levels with the different menstrual cycle phases (Cox & Calame, 1978; Hrboticky et al., 1989; Sawai et al., 2018). Moller et al. indicated that, in control females (not using hormonal contraceptives), the sum of amino acids significantly decreased in the luteal phase (high progesterone) compared to the follicular phase (low progesterone), showing the effect of hormonal changes on these metabolites (Moller et al., 1996). Consequently, various groups have studied the effect of synthetic sex hormone (COCs) use on blood amino acid levels (Table 2). Our results, which describes variations related to the use of EE in combination with a 4th generation progestin, is highly comparable to findings from previous studies focussing on COCs containing earlier generation progestins. These results also correlates to some extent, to that identified for Cyproterone acetate (CPA), a steroidal anti-androgenic progestin like DRSP, which was originally classified as a treatment for hyperandrogenism, and has never been categorized according to generation (Regidor, 2018).

Table 2 Compounds detected via the acylcarnitine analyses (no statistically significant variations detected)

Detected compound	Control	DRSP/20EE		DRSP/30EE		Previous study
	Average concentration (umol/L) (SEM)	Average concentration (umol/L) (SEM)	Increased (↑) or decreased (↓) with COC	Average concentration (umol/L) (SEM)	Increased (↑) or decreased (↓) with COC	EE combined with 3rd generation progestin (Ruoppolo et al., 2014 - *HPLC*). Increased (↑), decreased (↓) or no change (—) with COC
Free carnitine (C0)	33.94 (1.137)	30.58 (1.000)	↓	29.57 (0.698)	↓	↓
Acetyl-carnitine (C2)	5.497 (0.373)	6.000 (0.357)	↑	5.052 (0.399)	↓	↓*
Propionyl-carnitine (C3)	0.198 (0.027)	0.153 (0.012)	↓	0.133 (0.010)	↓	↓
Butyryl- / Isobutyryl-carnitine (C4)	0.112 (0.008)	0.169 (0.020)	↑	0.141 (0.017)	↑	↑
Tiglyl- / 3-Methylcrotonylcarnitine (C5:1)	0.029 (0.003)	0.030 (0.002)	↑	0.025 (0.002)	↓	↓
Isovaleryl- / 2-methylbutyryl- / pivaloyl-carnitine (C5)	0.045 (0.005)	0.046 (0.003)	↑	0.040 (0.003)	↓	↓
3-Hydroxybutyrylcarnitine (C4-OH)	0.057 (0.007)	0.058 (0.004)	↑	0.063 (0.009)	↑	NA
Hexanoylcarnitine (C6)	0.026 (0.003)	0.032 (0.003)	↑	0.026 (0.001)	↓	↓
3-Hydroxyisovaleryl- / 2-methyl-3-hydroxybutyryl-carnitine (C5-OH)	0.039 (0.004)	0.033 (0.002)	↓	0.037 (0.002)	↓	NA
Octanoylcarnitine (C8)	0.097 (0.012)	0.196 (0.060)	↑	0.093 (0.012)	↓	—
Malonyl- (C3:DC) / 3-OH-octanoyl-carnitine (C8-OH)	0.034 (0.004)	0.032 (0.003)	↓	0.030 (0.002)	↓	↑
Decanoyl-carnitine (C10)	0.138 (0.022)	0.309 (0.098)	↑	0.115 (0.017)	↓	—
Methylmalonyl- / Succinyl-carnitine (C4-DC)	0.019 (0.002)	0.026 (0.002)	↑	0.024 (0.002)	↑	NA
Glutaryl- (C5-DC) / 3-OH-decanoyl-carnitine (C10-OH)	0.111 (0.012)	0.116 (0.008)	↑	0.115 (0.010)	↑	NA
Cis-5-Tetradecenoyl-carnitine (C14:1)	0.067 (0.008)	0.076 (0.008)	↑	0.062 (0.006)	↓	NA
Myristoylcarnitine (C14)	0.060 (0.006)	0.052 (0.005)	↓	0.061 (0.006)	↑	↓
Palmitoylcarnitine (C16)	0.098 (0.011)	0.097 (0.006)	↓	0.089 (0.006)	↓	↓
3-Hydroxypalmitoylcarnitine (C16-OH)	0.146 (0.014)	0.141 (0.014)	↓	0.119 (0.010)	↓	NA
Oleylcarnitine (C18:1)	0.127 (0.012)	0.128 (0.011)	↑	0.115 (0.009)	↓	↓
Stearoyl-carnitine (C18)	0.090 (0.010)	0.091 (0.008)	↑	0.094 (0.006)	↑	↓
3-Hydroxystearoyl-carnitine (C18-OH)	0.138 (0.010)	0.159 (0.011)	↑	0.139 (0.012)	↑	NA

The analytical method used for metabolite detection, is indicated after the respective reference

COC combined oral contraceptive, ***DRSP*** drospirenone, ***EE*** ethinylestradiol, ***HPLC*** high performance liquid chromatography, ***NA*** not analysed, ***SEM*** standard error of the mean

*Statistically significant variation; Grey blocks indicate similar results compared to the current study

The majority of the essential amino acids were detected in decreased concentrations in the serum of the COC users. This occurrence, which is consistent with our previous findings (Swanepoel et al., 2020), could be related to the anabolic (intensified protein synthesis) properties of EE and DRSP (Lecocq et al., 1967), the progestin-induced enhancement of the amino acid metabolism (Smith et al., 2014), as well as estrogen related insulin resistance (Godsland et al., 1992).

In addition to the global changes, variations to the levels of individual amino acids suggest increased oxidative stress levels in the COC users (Table 3). Proline, a well-known antioxidant that can readily scavenge free radicals (Kaul et al., 2008) through the synthesis of hydroxyproline, glutamate and arginine (Wu et al., 2011), was detected in significantly decreased concentrations in the COC users compared to controls. Similar to proline, other amino acids with antioxidant properties (including, 4-hydroxyproline, alanine, tyrosine, glutamine, glycine, lysine, methionine, ornithine, and serine) were all reduced in the serum of COC users comparatively (Table 3). In contrast, aspartic acid, which has been shown to induce oxidative stress, as well as pipecolic acid, which is known to produce H_2O_2, were significantly elevated in the participants using the COCs. These findings confirm results obtained from our previous, untargeted study (Swanepoel et al., 2020), where we speculated that the detected metabolome changes indicated increased oxidative stress as an underlying cause for the biophysical changes seen in the coagulatory profile of DRSP/EE users.

Table 3 Comparison of blood amino acid variations in response to different generations of progestins

Detected compound	Current study EE combined with 4th generation progestin		Previous study (untargeted metabolomics): EE combined with 4th generation progestin (Swanepoel et al., 2020 – *GCxGC-TOFMS*)	EE combined with 1st generation progestin (Craft and Peters, 1971 - *IEC*)	EE combined with 2nd generation progestin (Wang et al., 2016 - *NMR*)	EE combined with 3rd generation progestin (Ruoppolo et al., 2014 - *HPLC*; Wang et al., 2016 - *NMR*)	EE combined with 1st, 2nd and 3rd generation progestins (mixed model) (Moller et al., 1995 - *IEC*; Moller et al., 1996 - *IEC*)	EE combined with CPA (Wang et al., 2016 - *NMR*)
	DRSP/20EE	DRSP/30EE						
4-Hydroxyproline	↓*	↓*	NA	NA	NA	↓*	NA	NA
Alanine	↓*	↓*	↓*	↓	↑	↓↑*	NA	↑*
Alpha-aminoadipic acid	↑	↓	NA	NA	NA	NA	NA	NA
Alpha-aminobutyric acid	↓	↓	NA	↓	NA	NA	NA	NA
Argininosuccinic acid	↑	↓	NA	NA	NA	NA	NA	NA
Asparagine	↓*	↓*	↓*	↓	NA	↑	↓	NA
Aspartic acid	↑*	↑*	NA	↓	NA	↑	NA	NA
Cystathionine	↑	↓	NA	NA	NA	NA	NA	NA
Cystine	↓	↑	↓*	NA	NA	NA	NA	NA
Glutamic acid	↑	↑	↓*	↓*(sum)	NA	↑	NA	NA
Glutamine	↓*	↓*	↓*	↓*(sum)	↓*	↓*	NA	↓*
Glycine	↓*	↓*	↓*	↓*	↓*	↓*	↓	↓*
Histidine	↑	↑	NA	↓	↑*	↑*	NA	↑*
Isoleucine	↓	↓	↓*	↓*	↑	↑*	↓	↑
Leucine	↓	↓	↓*	↓	↑	↓↑*	↓	↑*
Lysine	↓*	↓*	NA	NA	NA	NA	NA	NA
Methionine	↓*	↓*	↓*	↓	NA	NA	NA	NA
Ornithine	↓*	↓*	↓*	↓	NA	NA	NA	NA
Phenylalanine	↓	↓	NA	↓	↑*	↓↑*	↓	↑*
Pipecolic acid	↑*	↑*	↑*	NA	NA	NA	NA	NA
Proline	↓*	↓*	↓*	↑	NA	↓*	NA	NA
Serine	↓*	↓*	↓*	↓	NA	↓	↓**	NA
Threonine	↓	↑	NA	↓	NA	NA	↓	NA
Tryptophan	↓	↑	NA	↑	NA	↓	↓	NA
Tyrosine	↓*	↓*	↓*	↓*	↓*	↓*	↓*	↓*
Valine	↓	↓	NA	↓	↓	↓↑	NA	↓

The analytical method used for metabolite detection, is indicated after the respective reference

CPA cyproterone acetate, steroidal anti-androgenic progestin, ***GCxGC-TOFMS*** two dimensional gas chromatography time-of-flight mass spectrometry, ***HPLC*** high performance liquid chromatography, ***IEC*** ion exchange chromatography, ***NA*** not analysed, ***NMR*** nuclear magnetic resonance

*Statistically significant variation. Grey blocks indicate similar results compared to the current study

Oxidative stress occurs when pro-oxidant molecules such as Reactive oxygen species (ROS) and reactive nitrogen species, outweigh the body's antioxidant defences. Oxidative stress contributes to the pathogenesis of various conditions, including cardiovascular disease (Cervantes Gracia et al., 2017), venous thrombosis (Gutmann et al., 2020), as well as female specific reproductive diseases including polycystic ovarian syndrome, endometriosis and unexplained infertility (Agarwal et al., 2012). Other factors that are known to produce free radicals, such as obesity (Marseglia et al., 2014), cigarette smoking (Ambrose & Barua, 2004), the use of alcohol and recreational drugs (Kovacic, 2005), could thus, when used by DRSP/EE users, cause an oxidative overload, which could trigger or augment the conditions as mentioned above. The importance of antioxidant therapy to scavenge free radicals and ROS (Uttara et al., 2009) should therefore be emphasized to the DRSP/EE users, as well as maintaining a healthy lifestyle.

Acylcarnitine variations

Although acylcarnitines were not statistically different in the DRSP/EE groups when compared to the control group, a dose dependent effect was evident with DRSP/30EE (with the highest estrogen concentration) showing an overall greater effect than DRSP/20EE, except for 3-hydroxybutyrylcarnitine (C4-OH), 3-hydroxyisovaleryl carnitine (C5-OH), myristoylcarnitine (C14), stearoylcarnitine (C18), where DRSP/20EE showed the greatest effect. The changes in acylcarnitine levels in the current study are comparable to that of 3rd generation progestins in combination with EE (Ruoppolo et al., 2014), with reduced free carnitine levels and augmented levels of saturated acylcarnitines, as summarised in Table 4. The dose-dependent nature of these variations could potentially be linked to the previously mentioned estrogen-induced insulin resistance, since a dysregulated fatty acid metabolism (decreased free carnitine levels) is highly associated with the development of type 2 diabetes and insulin resistance (Bene et al., 2018).

Table 4 Pro-oxidant and antioxidant properties of compounds identified as being significantly different between control and COC user groups

Detected compound	Increased (↑) or decreased (↓) with COC	Pro-oxidant/antioxidant properties	References
4-Hydroxyproline	↓*	Oxidant scavenger	(Phang et al., 2008, 2010)
Alanine	↓*	Enhance GSH reductase activity	(Katayama & Mine, 2007)
Tyrosine	↓*	Cytoprotective antioxidant function	(Moosmann & Behl, 2000)
Glutamine	↓*	Precursor of GSH	(Amores-Sánchez & Medina, 1999; Matés et al., 2002)
Glycine	↓*	Reduce oxidative stress, increase GSH biosynthesis	(Díaz-Flores et al., 2013; El-Hafidi et al., 2018)
Lysine	↓*	Precursor of Carnitine, Carnitine reduce oxidative stress	(Lee et al., 2014; Terruzzi et al., 2019)
Methionine	↓*	Reactive oxygen species scavenger	(Luo & Levine, 2009)
Ornithine	↓*	Precursor of the free radical scavenger spermine	(Ha et al., 1998; Ushmorov et al., 1999)
Proline	↓*	ROS scavenger	(Kaul et al., 2008; Liang et al., 2013)
Serine	↓*	Synthesis of GSH, antioxidant	(Maralani et al., 2012; Parker & Metallo, 2016)
Pipecolic acid	↑*	Produce H_2O_2	(Dalazen et al., 2014)
Aspartic acid	↑*	Induce oxidative stress (rat model)	(Chandrashekar & Muralidhara, 2010)

*Statistically significant variation between controls and COC users detected in this study

In addition, lowered free carnitine levels have been associated with pregnancy (Cederblad et al., 1986) and polycystic ovary syndrome (Fenkci et al., 2008), confirming the significance of female sex hormones in carnitine handling. Furthermore, substantiating the oxidative state described for COC users previously, free carnitine is known to stimulate the expression of antioxidants and has been suggested in the treatment of pathological conditions characterized by oxidative stress (Calò et al., 2006).

Low carnitine levels brought on by COC use could potentially have a clinically significant effect if the individual has an underlying condition associated with carnitine deficiency (Lerner et al., 1993), or if medications are prescribed that induce carnitine deficiency (Flanagan et al., 2010; Magoulas & El-Hattab, 2012; Tamai, 2013). Although the association between COC use and other carnitine lowering factors have not yet been studied, the potential of such interactions suggests that that DRSP/EE users should avoid drugs that reduce carnitine levels as well as drugs that can cause a reduction of other serum antioxidants. Also, DRSP/EE users should be aware of lifestyle choices that can contribute to the strain on the antioxidant system.

Conclusions

Oral contraceptive pills are currently used by millions of women worldwide as a method for preventing pregnancy. Hence, comprehensive knowledge on the risks and benefits of these therapies is important for the prescribing clinicians as well as the users. Our study confirms that DRSP/EE use induces oxidative stress and brings about a reduction in serum antioxidants, which is in keeping with literature. Oxidative stress plays a key part in the development of pathological conditions such as cardiovascular disease, venous thrombosis as well as female reproductive complications. It is generally known that estrogen has antioxidant properties and therefore reduces oxidative stress (Razmara et al., 2008). Yet, when estrogen and progesterone are co-administered, the beneficial effects of estrogen is known to decrease (Irwin et al., 2008), which is noted in this study as well as in our previous

publication where there is an increase in oxidative stress following combined oral contraceptive use (Swanepoel et al., 2020).

Changes due to chronic use of COCs might not always present clinically but the changes that do occur on a molecular level, as shown here, can have long term effects on the normal physiology of the human body. Awareness of these negative effects of COC use should be brought to the female user's attention to ensure informed decision-making. The users should be educated on the contraindicated medications and conditions that might have long-term effects. This also provides the clinician prescribing COC's with more insight and awareness on the types of complications that might stem from chronic COC use.

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Contributions

AS and IDP conceived and designed the research. AS and JB managed the sample collection and ethical aspects. YK and ED conducted experiments. IDP analysed the data. AS, JB and IDP interpreted the data and wrote the paper. All authors read and approved the manuscript.

Ethics declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in this study, involving human participants, were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The ethical committee of the University of Pretoria approved this study (No: 307/2016).

Consent to participate

All collected specimens were anonymised at the time of collection, and participants gave written informed consent.

Data availability

Raw data were generated at the Centre for Human Metabolomics, North-West University, South Africa. Derived data supporting the findings of this study are available from the corresponding author IDP on request.

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