UNIVERSITY OF LEICESTER & UNIVERSITY OF LEEDS

DETERMINATION OF MATERNAL CAFFEINE INTAKES ASSOCIATED WITH INCREASED RISK TO THE FETUS

T01032 & T01033

JANUARY 2009

THE CAFFEINE AND REPRODUCTIVE HEALTH (CARE) STUDY GROUP

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EXECUTIVE SUMMARY

Determination of maternal caffeine intakes associated with increased risk to the fetus.

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Caffeine is the most widely consumed xenobiotic in pregnancy, with the potential to adversely affect the developing feto-placental unit. For decades, pregnant women have been advised to avoid caffeine-containing beverages during pregnancy, although this was largely based on animal studies and circumstantial, rather than scientific, evidence. Caffeine intake during pregnancy has been associated with congenital malformation, low birth weight or pre-term delivery. In 2001, the Committee on Toxicity of Chemicals in Food, UK, after a thorough review of the literature, concluded that whilst caffeine intake above 300 mg/day may be associated with low birth weight and spontaneous miscarriage, the evidence was inconclusive due to wide inconsistency in the reported studies. Possible reasons for these inconsistent outcomes include: (i) inaccurate estimation of caffeine consumption; (ii) retrospective assessment of caffeine intake; (iii) assessment of effects based on consumption in individual trimesters rather than throughout pregnancy; (iv) failure to include inter-individual variations in caffeine metabolism; (v) inadequate control for confounding factors such as smoking and alcohol consumption.

The aim of this project was to develop tools for the accurate assessment of (i) caffeine intake; (ii) caffeine half-life (to provide some indication , in part, of CYP1A2 activity) and, in combination with a robust assessment of fetal growth restriction, apply these to the examination of the association between maternal caffeine intake, during pregnancy, and fetal growth restriction (FGR). The study was a prospective longitudinal observational study, based at two large UK centres (Leicester and Leeds). 2635 low risk pregnant women recruited between 8-12 weeks of pregnancy. Quantification of total caffeine intake from 4 weeks before, and throughout, pregnancy was performed using a validated caffeine assessment tool (CAT). Caffeine half-life (used as a proxy for clearance) was determined by measuring caffeine in saliva, at two defined intervals (one and five hours) after a caffeine challenge. Smoking and alcohol were assessed by self-reported status and, for smoking, salivary cotinine concentration.

A reproducible HPLC-UV method was developed for the assessment of caffeine and its metabolites in saliva and used, along with a three day diary, to validate the CAT. This method also allowed assessment of caffeine half-life. The main outcome measure was FGR, as defined by customised birth weight centile, adjusted for alcohol intake and salivary cotinine concentrations. Caffeine consumption throughout pregnancy was associated with an increased risk of FGR: OR=1.2 (95% CI, 0.9 to 1.6) for 100-199 mg/day, OR=1.5 (1.1 to 2.1) for 200-299 mg/day, and OR=1.4 (1.0 to 2.0) for over 300 mg/day compared to <100 mg/day (P_{trend}<0.001). Mean caffeine consumption decreased in the 1st and increased in the 3rd trimester. This study showed some evidence that the association between caffeine and FGR was stronger in women with a faster, compared to a slower, caffeine clearance (test for interaction, P=0.06).

Using robust and reliable tools, we have demonstrated that caffeine consumption during pregnancy is associated with an increased risk of FGR and this association is continuous throughout pregnancy. We were unable to determine a threshold for this effect. Sensible advice would be to reduce caffeine pre-conceptionally and throughout pregnancy.

1.0 Introduction

There are a number of well-established socio-economic risk factors associated with low birth weight in humans such as maternal nutritional status and smoking [1]. However, the involvement of certain environmental toxins is less well defined and caffeine (1,3,7-trimethylxanthine) is currently receiving particular attention. Caffeine is the most widely consumed xenobiotic in pregnancy, with the potential to adversely affect the developing feto-placental unit. For decades, pregnant women have been advised to avoid caffeine-containing beverages during pregnancy, although this was largely based on animal studies and circumstantial evidence rather than scientific proof [2]. Caffeine intake during pregnancy has been associated with congenital malformation, low birth weight or pre-term delivery (spontaneous abortion, or SAB, defined as the expulsion of the fetus before 20 weeks gestation or weighing less than 500 g [3]) and consumption of caffeine which is common during pregnancy [4]. The doses of caffeine associated with congenital malformation have largely only been achieved in animal studies. A number of recent literature reviews have suggested that congenital defects are unlikely to be associated with coffee/caffeine consumption in humans [5]. This has, in part, highlighted shortcomings in the animal studies with respect to the contrasting route of exposure between animal and human doses and hence prevented correct interpretation of no-observable-effect-levels [6] and the significant interspecies differences in caffeine metabolism [7]. Whilst it appears that moderate caffeine consumption (150 mg/day) has no effect on SAB [8], caffeine in excess of 300 mg/day may be associated with an increased risk of SAB [9], a conclusion supported by the findings of the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment [10]. A similar result was seen in studies examining birth weight (reviewed in Christian and Brent [6]). Where

relatively accurate estimates of caffeine ingestion had been made, it was possible to conclude that intakes over 300 mg/day have a small but measurable association with fetal growth restriction (FGR) [11-14]. However, Vlajinac et al. showed a reduction in birth weight of 114 g in infants born to mothers whose daily caffeine consumption was as little as 141 mg/day [15]. Maternal caffeine intake has been reported to be associated with a reduction in birth weight [16-20] however, the precise level of intake above which the risk is increased remains unknown. Indeed in the case of low birth weight, Shiono and Klebanoff [21] suggested that the contradictory findings of the epidemiological studies would suggest the presence of a small and perhaps limited subgroup of individuals in whom there is a greater risk from the effects of caffeine. More controversially others have shown that maternal caffeine concentrations had a negative association with birth weight but only when confounders such as smoking were taken into account [17, 22, 23]. In 2001, the Committee on Toxicity of Chemicals in Food, UK, after a thorough review of the literature, concluded that whilst caffeine intake above 300 mg/day may be associated with low birth weight and spontaneous miscarriage, the evidence was inconclusive [24]. Similar guidance is provided to pregnant women in the US [25]. Possible reasons for these inconsistent outcomes include:

(i) inaccurate estimation of caffeine consumption, including an assumption that tea and coffee are the only sources of caffeine [15, 18, 22]. In fact there is a significant mis-classification of exposure when coffee consumption alone is used as a surrogate measure of caffeine intake [26].

(ii) retrospective assessment of caffeine intake [17] [22, 27-29].

(iii) assessment of effects based on consumption in a single trimester rather than throughout pregnancy [15, 19, 22, 27].

(iv) failure to include inter-individual variations in caffeine metabolism [19, 30].

(v) inadequate control for confounding factors such as smoking and alcohol consumption [31, 32].

Smoking is another important confounding factor. On average, smokers consume more caffeine than non-smokers and smoking doubles the rate at which caffeine is metabolised [33]. Upon cessation of smoking, the induced enzymes quickly return to the levels of non-smokers, however, it may take up to six months for caffeine intake to reduce accordingly [33]. Furthermore, several studies have shown a positive association between smoking and SAB and low birth weight (reviewed in Cramer and Wise [34]; Gardella and Hill [9]). Associated with smoking, alcohol has well established teratogenic properties and may result in the fetal alcohol syndrome with subsequent low birth weight [9]. In combination these factors can complicate meaningful interpretation of studies examining the effects of caffeine in pregnancy, particularly as the consumption of large amounts of caffeine is associated with a greater risk of becoming a smoker and consuming alcohol in excess [6].

(vi) non-uniformity in defining the primary outcome measures [11, 15-17, 19, 22, 29, 30]. A major weakness of many epidemiological studies is the reliance on birth weight as the end-point for assessing fetal growth. It is well recognised that being low birth weight is not synonymous to poor growth and therefore these studies would have included a large number of appropriate-for-gestational-age babies that were low birth weight and included growth restricted babies that were of a ‘normal’ weight at birth.

A number of combined factors result in significant exposure of the fetus to caffeine. Caffeine, in part due to being fat soluble, is rapidly absorbed and crosses the placenta freely resulting in fetal plasma levels which are equivalent to maternal levels

[35]. It has been shown that after ingestion of 200 mg caffeine, intervillous blood flow in the placenta is reduced by 25 percent [36]. The rate of elimination of caffeine during pregnancy is lowered and hence serum levels are higher in pregnant women than in their non-pregnant controls. Cytochrome P4501A2 (CYP1A2), the principal enzyme involved in caffeine metabolism, is absent in the placenta and the fetus [37].

Of the four primary routes of caffeine metabolism in humans, 3-demethylation is quantitatively the most important reaction [38], converting caffeine to paraxanthine (1,7-dimethylxanthine), with CYP1A2 being identified as the enzyme responsible [39] and further acetylation by NAT2 to 5-acetylamino-6-formylamino-3-methyluracil (AFMU [40]). Although expressed constitutively in liver, human CYP1A2 is inducible, for example through cigarette smoking, in the gastro-intestinal tract, liver and brain [41].

The amount of caffeine available to the feto-placental unit and hence its effects therefore, depend upon the metabolic activity of CYP1A2 which has marked inter-individual variation due to genetic and environmental factors [42] such as nicotine [43, 44]. Alterations in CYP1A2 activity rather than blood caffeine concentrations have been more closely associated with FGR [45]. It follows from these observations that any comprehensive study into the effects of caffeine on fetal growth must include an assessment of caffeine metabolism.

In order to examine the effect of maternal caffeine intake on fetal well-being, we used a validated, robust caffeine assessment tool (CAT) to prospectively quantify total caffeine intake, from all possible sources, throughout pregnancy [46]. Using these data, and taking into account the inter-individual variation in caffeine metabolism, we aimed to establish the safe upper limit of caffeine consumption with respect to adverse pregnancy outcome (specifically FGR).

1.1 Aims of the project

In collaboration between Universities of Leicester and Leeds:

1.1.1 To develop a detailed assessment tool for caffeine intake and perform a pilot study to validate measures of salivary caffeine in a small sample of pregnant women from Leeds. Once developed, to administer the Caffeine Assessment Tool (CAT) to all subjects recruited in the main study at baseline, 28 weeks and post-delivery.

1.1.2 To undertake a prospective study to explore the link between caffeine consumption, metabolism and FGR. This unique prospective approach will ensure bias-free measures of caffeine intake and metabolism during the pregnancy rather than afterwards.

1.1.3 To obtain saliva samples from subjects to determine their caffeine and paraxanthine levels to confirm whether subjects are high or low caffeine consumers. Cotinine levels will be assessed as a measure for smoking status. These measures will be used to validate caffeine intake from the CAT, described in aim 1.1.1., and reported smoking status.

1.1.4 To perform a caffeine challenge on all participants during pregnancy to determine their phenotype for caffeine metabolism.

1.1.5 To produce a report on the findings of the studies, provide recommendations for public health and give directions for further research.

2.0 MATERIALS AND METHODS

2.1 Development of the caffeine assessment tool.

The CAT (see Appendix I) was developed in Leeds to assess caffeine intake in all women taking part in the study. The tool assessed caffeine intakes from all possible sources of caffeine in a food frequency questionnaire style, taking into account specific brand, preparation and portion size information. Brand information was collected on coffee, tea, hot chocolate, cola and energy drinks, and was categorised into types of drink e.g. instant, filter, iced, and place of consumption to remind women of beverage consumption outside the home or workplace. Further questions requested include changes in intake of tea and coffee during pregnancy, and intakes of foods which may affect caffeine metabolism e.g. cruciferous vegetables, grapefruit and barbecued foods [47-49].

The caffeine content of foods and beverages were obtained from a UK government report [50] and also from manufacturers and coffee-houses, providing caffeine values for 29 instant coffees (see table 2.1), nine filter coffees, three coffee-house filter coffees, a standard espresso shot and decaffeinated shot, eight instant beverage mixtures, seven espresso-based drinks, 18 hot chocolates, 25 teas prepared from tea bags, 17 teas prepared from tea leaves, one iced-tea, three instant teas, 36 colas, 33 energy drinks, 11 soft drinks and two alcoholic drinks. Other brands of tea and coffee where detail on caffeine content was not available had a standard caffeinated and decaffeinated value assigned based on the average caffeinated and decaffeinated content of each drink. For each of the chocolate items a value was assigned based on

the average caffeine content from various brands. The caffeine content of 59 over-the-counter drugs was accessed from manufacturer's web sites.

Table 2.1. Example of caffeine values used in CAT for instant coffee products

Product ID	Product	Source	Sample weight (g)a	Caffeine (mg/l)	Caffeine g/100g	Caffeine (mg/100g)
	Standard instant					
1	Asda granules (Farmstores) instant granules	MAFF	1.6	325	4.06	4060
2	Cafe Direct	MAFF	1.6	270	3.375	3375
3	Cafe Hag	MAFF	1.6	11	0.1375	137.5
4	Co-op powder	MAFF	1.6	295	3.687	3687.5
5	Kenco Superior Blend	MAFF	1.6	215	2.6875	2687.5
6	Kwik-Save no frills instant granules	MAFF	1.6	330	4.125	4125
7	Maxwell House	MAFF	1.6	290	3.625	3625
8	Mellow Birds	MAFF	1.6	335	4.18	4180
9	Nescafe Alta Rica	Nescafe			2.8	2800
10	Nescafe Black Gold	Nescafe			3.2	3200
11	Nescafe Blend 37	Nescafe	1.6	315	3.9375	3937.5
12	Nescafe Cap Colombie	Nescafe			2.8	2800
13	Nescafe Fine Blend	Nescafe			4.6	4600
14	Nescafe Fine Tasses	Nescafe			3.7	3700
15	Nescafe Gold Blend decaf	Nescafe			0.15	150
16	Nescafe Gran Arome	Nescafe			3.1	3100
17	Nescafe High Roast	Nescafe			3.9	3900
18	Nescafe Kenjara	Nescafe			2.4	2400
19	Nescafe Decaffeinated	Nescafe	1.6	10	0.125	125
20	Nescafe Organic	Nescafe			2.9	2900
21	Nescafe Original	Nescafe			3.4	3400
22	Nescafe New Gold Blend	Nescafe			2.3	2300
23	Nescafe Old Gold Blend	Nescafe			2.8	2800
24	Safeways Full Roast	MAFF	1.6	310	3.875	3875
25	Sainsbury's medium roast	MAFF	1.6	300	3.75	3750
26	Somerfield Medium Roast Instant Coffee Granules	MAFF	1.6	340	4.25	4250
27	Tesco Classic	MAFF	1.6	275	3.43	3437.5
28	Tesco Classic Gold	MAFF	1.6	230	2.875	2875
29	Waitrose instant coffee granules	MAFF	1.6	210	2.625	2625
30	Standard instant (mean of values above minus decaf)	MAFF				3399.6
31	Standard instant decaf (mean of values above)	MAFF				137.5
32	Half-caff coffee					1700

The CAT also assesses possible confounders e.g. smoking habits, alcohol intake, use of medication, symptoms of pregnancy. The CAT is thus the most detailed and comprehensive tool to assess caffeine intake during pregnancy which is currently available. A detailed computer algorithm was developed which assigned a value for caffeine content (mg) to all caffeinated products assessed in the CAT while taking into account portion sizes, brand information and frequency of intakes.

2.1.2 Validation of the CAT

In order to validate the newly developed CAT as an assessment of caffeine consumption, comparisons with an established method (a 3-day food and drink diary), and salivary concentrations of caffeine and paraxanthine, in pregnant women during the first trimester only, were performed. The 3-day food and drink diaries were analysed manually for each woman's daily caffeine intake. All caffeine containing foods and drinks recorded in the diaries were allocated a caffeine value depending upon the amount and type of food or drink consumed including brand level information if available. A mean caffeine value was calculated for each food and beverage source and assigned to any unbranded sources recorded in the diaries. Daily caffeine intake during weeks 5-12 of pregnancy could be calculated for each participant involved in the pilot study. Saliva samples were obtained from the same women on two consecutive days, and analysed for caffeine and paraxanthine, according to the method described below.

2.1.2.1 Validation of study methods

(Reported in Boylan et al. {Boylan, 2008 #517})

The validation study involved two aspects:

(i) Assessment of intra-individual variation in caffeine levels at different times during the day

(ii) Assessment of day-to-day (between and within day) variation in caffeine levels

All pregnant women 18 years old or over, attending the antenatal clinic at Leeds General Infirmary were eligible for inclusion in this development phase of the study. Pregnant women younger than 18 or who were receiving their maternity care elsewhere were not eligible to take part. Maternity records of women attending the clinic were checked for suitable women. Women were approached by the research assistant, informed about the study, and given an information sheet. Women who agreed to take part were given a background questionnaire, CAT, three-day food and drink diary, and nine Salivettes® (Sarstedt, Aktiengesellschaft & Co.) to take home, complete and return. The study protocol was approved by the local Research Ethics Committee and written informed consent was obtained from all subjects.

Using the CAT, the current study assessed caffeine intakes from weeks 5-12 of pregnancy only since this was the most appropriate time frame relative to the stage of pregnancy when most women attend the antenatal clinic for the first time, women were called to the clinic at about 16 weeks of pregnancy. The food and drink diary was completed at the same time as the CAT although the time frames did not overlap. The diary asked about three days of intake at the time of completion whereas the CAT requested recall of intake during weeks 5-12 of pregnancy. While completing the food and drink diary, the women collected a total of nine saliva samples over two consecutive days. Women noted both the time of consumption of foods and drinks and of the saliva collection in the diary to ensure that the saliva measurements provided biomarkers of actual consumption for comparison with the recorded dose

(food and drink diary). The saliva samples were also used to assess variation in salivary caffeine and paraxanthine concentrations at different times during the day, and between days. Each saliva sample was collected in a Salivette®. The Salivette® consists of an outer centrifuge vessel containing a suspended insert and cotton wool swab. Women were required to keep the Salivette® swab in their mouth for ten minutes to ensure adequate saliva collection. A sample interval of ninety minutes was chosen between collections to cause minimal disruption to normal daily activity. On the first day, each woman provided saliva samples every ninety minutes over a nine-hour period, involving a total of seven saliva samples. To avoid the presence of caffeine in the saliva due to recent consumption (rather than following absorption), the women were asked to avoid caffeine-containing foods and drinks, listed on a sheet provided, for one hour before collecting the first sample, and for fifteen minutes before taking each of the following six samples. The women were also asked to rinse their mouth with tap water prior to collection.

On the second day, the same women were asked to provide a further two saliva samples at approximately mid-morning and mid-afternoon to reflect likely time of sample collection in the larger study. Again the women were asked to avoid caffeine-containing foods and drinks for one hour prior to sample collection and to rinse their mouth with tap water before the samples were taken. The women were asked to refrigerate the samples until they were returned by post to the research team along with the background questionnaire, CAT and diary.

Statistical analysis was carried out using SPSS version 10.1 (SPSS Inc., Chicago, IL, USA), Stata version 8.2 (StataCorp. LP, College station, TX, USA), and MLwiN (University of bristol, Bristol, UK) to explore intra and inter individual variation in caffeine intakes. Women with caffeine intakes above the median caffeine

intake from both the CAT and the diary were categorised into high caffeine consumers, and women with caffeine intakes below the median caffeine intake from both the CAT and the diary were categorised into low caffeine consumers. For each saliva measure, women with saliva measures above the median were classed as having a high exposure, and women with saliva measures below the median were classes as having a low exposure. Kappa statistics were carried out to test the agreement between the caffeine intakes estimated by the CAT and diary, and the mean of the seven saliva caffeine and paraxanthine measures on day one, and the mean of the two saliva caffeine and paraxanthine measures on the day two. The kappa value represents the strength of the agreement: <0.2 (poor), 0.21-0.4 (fair), 0.41-0.6 (moderate), 0.61-0.8 (good), 0.81-1.0 (very good). A variance components model was used to investigate the variance structure of the data and estimate ICC agreement taking all sources of error into account.

Once validated, the CAT was used in the main study both in Leeds and Leicester.

2.2 Development of a method for the non-invasive assessment of caffeine and its metabolites.

2.2.1 Analysis of urine by HPLC-UV (Leeds)

The original protocol had included analysis of caffeine and metabolites in urine to determine phenotype, with saliva collected as a backup. The analysis was developed to initially use urine but when the first set of caffeine challenges were analysed it became apparent that urine was not the best biological fluid for assessing caffeine metabolism. There were several reasons for this, which included:

(i) Urine is a biological fluid rich in salts which interfered with the analysis of the compounds of interest.

(ii) Subjects abstained from caffeine for 12 hours prior to the caffeine challenge but this was not enough time to allow a caffeine and metabolite clearance from the urine in order to show accurately a depletion of caffeine and a production of metabolites.

(iii) The amount of fluids consumed during the challenge was variable and this was considered to have a detriment effect on the results[1].

It became apparent that saliva would be a superior matrix in which to study caffeine and its metabolites.

2.2.2 Analysis of saliva by HPLC-UV

2.2.2.1 Leeds method

The method has been published [46]. Caffeine and metabolites were extracted and quantified using liquid:liquid extraction and reversed phase high performance liquid chromatography (HPLC) with UV detection using a modification of the method of [40]. Salivettes were thawed and saliva isolated by centrifugation at 756 x g for 5 min at room temperature. Saliva (180 µL) was added to a 5mL screw cap tube containing 50 mg ammonium sulphate following which 20µL of a stock solution of β-hydroxyethyltheophylline (20 µg/mL) was added as an internal standard. The tube was shaken vigorously for 10 sec using a Baxter multi tube vortex (Alpha laboratories, Eastleigh, UK), setting 4, to thoroughly mix the contents and precipitate any protein. This was followed by the addition of 2 mL chloroform and 0.5 mL isopropanol, and the tube shaken again for 4 min on setting 4. After centrifugation for 5 min (84 x g) the aqueous top layer was discarded and the organic layer transferred

[1] Dr Fred Kadlubar, personal communication.

to a thick walled glass tube and dried down, under nitrogen, at 45 °C. The residue was reconstituted in 30 % (v/v) methanol in water (200 µL), mixed vigorously for 3 sec and centrifuged for 2 min (756 x g) to give a final concentration of β-hydroxyethyltheophylline of 2µg/ml. The reconstituted solution was transferred to an autosampler vial and 30 µL injected onto a Beckman Ultrasphere column (ODS 4.6 mm x 25 cm) with a short guard column (5 cm x 0.4 cm; packed in-house with Bondapak C18/corasil; Waters, Elstree, UK). The solvent delivery system, autosampler and UV detector were manufactured by Gilson, France. UV detection was set at 280 nm. Mobile phase used for elution were: solvent (A) 0.045 % (v/v) acetic acid containing 9% (v/v) methanol; and solvent (B) 100% methanol. Starting with solvent A, elution was a linear gradient over 5 min to a 2 % solution of solvent B. This was then held for10 min. Over the next 5 min there was a linear gradient increase to 5% solvent B, followed by a linear increase over 5 min to 8% B, changed to 15% B and maintained for 15 min, raised to 75% B and held at this for 10 min, followed by reversion to 100% A (the starting solvent) which was maintained for 10 min to equilibrate the column before injection of the next sample.

The flow rate was 1.2 mL/min and the retention time, in minutes, for each compound was approximately: theobromine (37X), 10.3; 1,7 dimethyluric acid (17U), 14.8; paraxanthine (17X), 16.2; theophylline (13X), 17.7; β-hydroxyethyltheophylline (β-HET), 22.4; caffeine (137X), 29.5. Retention times varied slightly from day to day and were adjusted accordingly, by the analysis software, to produce optimum identification of each analyte when processing results. A single standard containing all the above compounds at the same concentration (5 µg/mL) was made up in 30 % (v/v) methanol; which was run after every five samples.

Two ‘in house’ quality control (QC) samples were also extracted and run with each batch of 18 samples. QC samples were made by spiking ‘blanked saliva’ (i.e. saliva for which all target compounds had been previously extracted) with 5 μg/mL of each compound. ‘Blanking’ was achieved by gentle mixing of saliva with charcoal (0.1 g/ml saliva) for 24 h, then centrifuged (728 x g) for 10 min. The supernatant was then filtered through a 0.20 micron filter and stored at -20 °C until use. When in use QC material was stored at 4°C.

2.2.2.2 Leicester method

This was performed in conjunction with the Special Biochemistry section, Dept. Chemical Pathology, University of Hospitals NHS Trust. This laboratory participates in an external QC system for caffeine and theophylline. To 200 μL of each saliva sample, QC material or standard, was added 1 mL of internal standard, β-HET (1 mg/L, final concentration, in 50:50 isopropanol/chloroform), followed by 200 μL of 0.1 M phosphate buffer (pH 8.0). As this is a biological matrix, it is therefore important to retain physiological pH. From a stock concentration of each standard (100 mg/L) were prepared working standards at 1.0, 2.0 and 5.0 mg/L. Quality control samples were also prepared by dilution of the stock standard in blanked saliva (containing no caffeine or metabolites) to concentrations of 1.0 and 4.0 mg/L. For the purposes of assay evaluation additional QCs were prepared at 0.1, 0.5 and 5.0 mg/L. An additional QC was also prepared by dilution of commercially available QC material with assigned values for caffeine and theophylline (Lyphochek, Bio-Rad, Hemel Hempstead). The samples were vortexed, prior to centrifugation (5 min at 3000 rpm), prior to aspiration of the top layer to waste. The organic layer was evaporated to dryness under nitrogen at 50 °C, prior to reconstitution in 200 μL of

mobile phase. The standards also underwent this extraction procedure, as there can be significant matrix effects with saliva, or indeed urine.

The reversed phase HPLC system comprised a Gilson 231 series autosampler with 401 series dilutor, Gilson 307 series HPLC pump, Agilent 1100 series UV detector, Agilent Chemstation Integration Software. On to a Phenomenex "Gemini" HPLC column (150 x 4.6 mm, 5 μm C18; column oven at 50 °C) were injected 30 μL of sample. Mobile phase was 4 % (v/v) acetonitrile in HPLC grade water, with a flow rate of 1.4 mL/min, run time 35 min. UV detection was at 273 nm.

2.3 Inter-laboratory comparisons for HPLC-UV methods for the assessment of caffeine and its metabolites in saliva.

The two HPLC-UV methods for salivary caffeine and metabolites were compared via the single-blind, distribution of 'blanked' saliva samples, into which various concentrations of caffeine, theobromine, paraxanthine and theophylline had been added. Samples, prepared at both sites, were exchanged, analysed, and levels compared with those determined 'in-house'.

2.4 Non-invasive assessment of smoking status

This involved analysis of cotinine, the primary metabolite of nicotine, via a semi-quantitative ELISA method. The cotinine values were used as an assessment of nicotine exposure at all saliva sample collection time points, and prior to the caffeine challenge. It also provided confirmation, or otherwise, of the self-reported smoking status. The ELISA kit used was a commercial ELISA (from Cozart Bioscience Ltd, Oxfordshire, UK). Subjects were classified, based on cotinine levels, as active smoker (> 5 ng/mL saliva), passive smoker (1-5 ng/mL) and non-smoker (<1 ng/mL).

2.5 Study design

An overview of the study is given in Figure 2.5.1.

2.5.1 Power calculation

To estimate the sample size required, we assumed that the mean caffeine intake during pregnancy was 206 mg/day [19], and that caffeine followed a log normal distribution, with a coefficient of variation of 1. Assuming that 10% of births were FGR, then 3000 births gave 80% power to detect a difference of 30 mg/day in caffeine intakes between mothers of FGR births compared to babies born of appropriate weight for gestational age (AGA) with type I error set at 0.05. This also gave 80% power to detect an odds ratio for FGR of 1.4 between high and low caffeine consumers defined as being above or below the median caffeine intake.

2.5.2 Inclusion/exclusion criteria

All subjects were healthy pregnant women, with no current or past medical history (low-risk pregnant women). Selection criteria were used, to exclude factors known to affect fetal growth and/or caffeine metabolism (hypertension, previous pre-eclampsia, fetal growth restriction, antidepressants). The inclusion criteria were all women aged 18 years or more, spontaneous conception, with singleton pregnancy of less than 20 weeks gestation, and no previous or current history of medical disorder. Both primiparous and multiparous women were invited to participate. The exclusion criteria was women with multiple pregnancy, conception following IVF/ICSI, women infected with HIV/Hepatitis B, use of recreational drugs/ antidepressants at the time of recruitment, current or past history of diabetes outside or whilst pregnant, current or past history of hypertension or pre-eclampsia, past history of preterm birth (delivery at

<37 completed weeks of pregnancy, Leicester only), past history of fetal growth restriction (as per records of the last pregnancy Leicester only).

2.5.3 Background questionnaire

The same general questionnaire was used, at both sites, to ascertain various maternal characteristics (Appendix II). The questionnaire was completed by the women at recruitment and included information regarding date of birth, ethnicity, maternal height and weight at booking to calculate the body mass index (BMI), employment and education details. In addition, the questionnaire had information regarding estimated due date (EDD) based upon the dating scan, past obstetric history, family history and active or passive smoking status. The data were later entered into an MS Access database, developed specifically for the study at each respective site, for subsequent analysis. Social class was coded according to the Registrar General's classification [51].

2.5.4 Recruitment strategies

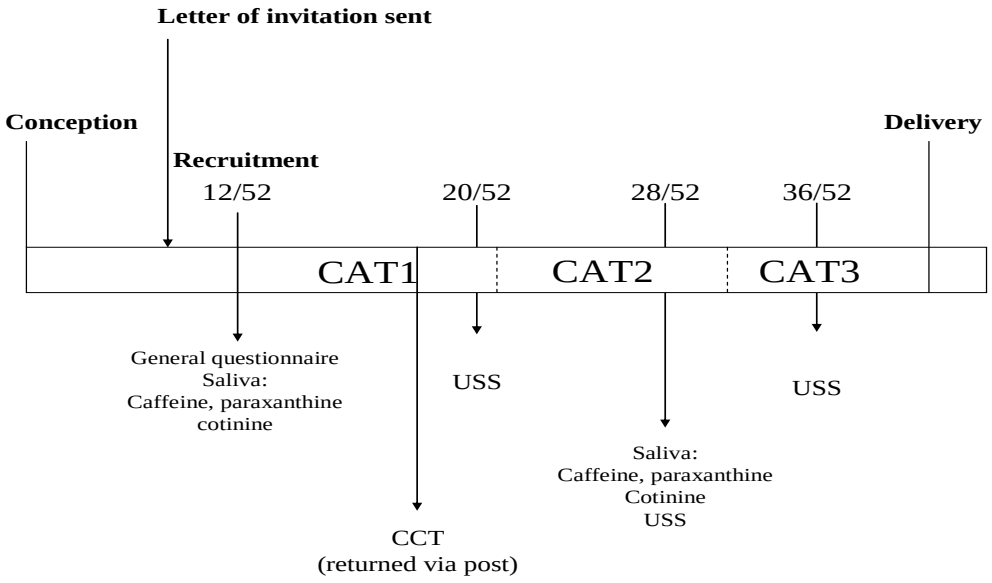


Figure 2.5.1. Overview of study.

Letters of invitation and patient information sheets, containing a simple and brief description of the study, were prepared. After identifying eligibility from the booking maternity notes, letters of invitation were posted/given to the women. Subjects were recruited in the first trimester of pregnancy between 9-14 weeks, at their pregnancy dating scan/booking visit (duration between receipt of information and participation being a minimum of one week; see Figure 2.5.1). Women, who decided to take part, phoned back to, or were phoned by, the midwives to arrange for an appointment according to their convenience. In Leicester, the majority were seen when they came for their dating scan. Those needing further information were seen by a doctor in the antenatal clinic area. Whilst in Leeds the midwives made an appointment to see the women at their homes. Reasons for declining were not recorded as it was considered ethically inappropriate and to avoid possible embarrassment/coercion of the subjects.

CATs were completed at 3 time points: at booking (12-18 weeks gestation), at 28 weeks and either 36 weeks gestation (Leicester only) or 4-6 weeks post-partum (Leeds only). Saliva samples, to monitor exposure to caffeine and nicotine, were collected at approximately 14-18 weeks, 28 weeks and, in Leeds, 4-6 weeks post-partum. A caffeine challenge was administered between 12-18 weeks and, in Leeds six weeks post-partum. Serial ultrasound scans (USS) were performed at 20 weeks gestation (both Leicester and Leeds) and also at 28 weeks and 36 weeks (Leicester only). A 24 hr dietary recall questionnaire was completed at both 16 and 28 weeks gestation (Leeds only)

2.5.5 Administration of the CAT

Previous work has demonstrated that dietary questionnaires provide a much better assessment of caffeine consumption than any biological marker such as plasma caffeine. In the absence of a robust pharmacokinetic model it is difficult to calculate caffeine intake, based solely on the basis of plasma concentrations. Thus, for a detailed and accurate assessment of habitual caffeine intake from various beverages/food and drinks, an assessment tool for caffeine intake (Caffeine Assessment Tool) was developed by the Nutritionists at Leeds University, UK as described in the earlier section of this report. The CAT was validated by measures of salivary caffeine in a small sample of pregnant women [46]. It is essentially a prospective diary of caffeine intake, smoking patterns, food intake and pregnancy symptoms (Appendix I). Furthermore, it takes into account the varying caffeine content of beverages and other sources of caffeine such as chocolate, energy drinks and medications that may contain caffeine. The information recorded included:

1. Caffeine intake in coffee: cups/mugs, frequency per day/per week/per month, brand of coffee, caffeinated or decaffeinated, type of coffee (instant, filter, espresso), size of cups/mugs, method of preparation including number of teaspoon of instant coffee per mug size.
2. Caffeine intake in tea: cup/mug/teapot, frequency per day/per week/per month, brand of tea, caffeinated or decaffeinated, type of tea (tea bags, tea leaves, iced tea), number of tea bags in a mug/teapot, method of preparation.
3. Cola drinks: brand names, caffeinated or decaffeinated, frequency per day/per week/per month.
4. Energy and other soft drinks: brand names, caffeinated or decaffeinated, frequency per day/per week/per month.
5. Chocolates, including energy bars, mini or full-size bars.
6. Other sources of chocolate, such as cakes, biscuits, brownies, ice-cream, milk shakes.

Other information collected included:

1. Alcohol intake: type/ frequency/ number of pints/glasses.
2. Intake of grapefruit juice, oily fish, barbequed foods and cruciferous vegetables (which have been reported may affect caffeine metabolism).
3. Smoking details: type (cigarettes manufactured or hand-rolled, filtered/non-filtered, paan tobacco, cigars, pipe), amount, frequency, duration, quitting (at what gestation).
4. Over the counter medications for fever, cold, asthma, cough etc.(source of caffeine): preparation, amount per day and duration.
5. Information regarding symptoms of pregnancy and level of physical activity (mild, moderate and severe).

All the information per subject was entered in Microsoft Word Access database developed, at each site, specifically for this purpose. The information was then extracted in Excel format and analysed by SPSS. There were 81 brands of coffee, 54 brands of tea, 19 brands of hot chocolate, 39 brands of cola drinks and 34 brands of energy drinks with caffeine content identified. For the purpose of calculating the amount of caffeine consumed per subject, every 7-8 weeks of pregnancy, a unique algorithm developed by the University of Leeds' team to calculate the amount of caffeine in mg/day from the beverages and food mentioned in the CAT [46].

2.5.6 Biological sample collection and distribution

At both sites, saliva samples for determining caffeine and nicotine exposure were collected at 12-16 weeks gestation (i.e. recruitment) and 28 weeks gestation using a Salivette®, kept in the mouth for 5-10 minutes to ensure adequate saliva collection. Post-partum samples (4-6 weeks post partum) were also collected in Leeds. Urines continued to be collected at 12 and 28 weeks gestation, at Leicester. Ethical approval had been granted for the collection of maternal blood samples at both sites, for future genotyping analyses, and this was performed at 28 weeks gestation (Leicester).

2.5.7 Caffeine challenge test (CCT),

Assessment of CYP1A2 enzyme activity required a caffeine challenge test at 12-16 weeks gestation (both sites) and, in Leeds only, 6 weeks postpartum - this was the subset of women with FGR babies and a matched number of AGA. The caffeine challenge was performed, by the subject, at home. Subjects were provided with

instructions, caffeine source, labelled salivettes and a questionnaire. The CCT involved overnight fasting, prior to ingesting a known amount of caffeine (500 mL diet cola, containing 63.5 mg caffeine, to be ingested over a period of 20 minutes), following which saliva was collected at one and five hours post-challenge. Precise sample collection times and any caffeine-containing drink or food consumed during the test period were recorded by questionnaire. The samples were posted through first class mail and once received; these were stored at -20 ºC until isolation of the saliva. Periodically, batches of salivettes were processed to isolate the saliva. This was achieved by centrifugation, at 3000 rpm for 10 min, at room temperature, during which the saliva transferred into the 5mL salivette plastic tube. These tubes were then labelled and stored at -80 ºC until further analysis. Periodically, batches of samples from Leicester were thawed and, 750 μL (where this volume was achievable, failing that 500 μL) aliquots transferred to 1.0 mL Eppendorfs, prior to transport to the Molecular Epidemiology Unit (University of Leeds), on dry ice. The minimum volume required for analysis was 500 μL. For analytical reasons, the protocol was modified from analysis of caffeine and its metabolites in urine, to analysis of saliva [see 2.2.1 Analysis of urine by HPLC-UV (Leeds)].

2.5.8 Outcome assessment

Information regarding delivery events and birth was obtained from the maternity notes. Birth weight centiles were calculated using the computer software (version 5.2) provided by the Perinatal Institute Birmingham (www.gestation.net). This calculator takes into account maternal height (centimetres), maternal weight (kilograms), ethnicity, parity, gestation at delivery, gender and weight of the baby. Gestational age was calculated from the early dating scan in all cases. Babies born at

< 37 weeks completed gestation were defined as preterm. All babies measuring less than 10th centile on customised centile charts were considered growth restricted for the primary analysis.

2.6 Statistical analysis

(See Appendix III for statistical analysis plan.)

The primary outcome measure was fetal growth restriction (FGR) defined as birth weight <10th centile on customised centile charts which takes into account maternal height, weight, ethnicity, parity and neonatal birth weight and gender (www.gestation.net). Secondary outcome measures were birth weight centile, birth weight, late miscarriage (spontaneous pregnancy loss between 12 and 20 weeks), pre-term labour (delivery <37 completed weeks), gestational hypertension (BP of 140/90 mmHg on more than one occasion 4 hours apart anytime >20 weeks), proteinuric hypertension (gestational hypertension and significant proteinuria of 300 mg protein/24 hr) and stillbirth (delivery at 24 weeks with no signs of life at birth).

Unconditional logistic regression modelling was used for FGR and general linear modelling for birthweight, with stratification for the two centres, using Stata version 10 survey facilities [52]. Maternal height, weight, ethnicity, parity at booking, gestation at delivery and gender of the neonates were taken into account in the definition for FGR, and were adjusted for in the model for birthweight. Statistical adjustment was also made for salivary cotinine levels and self-reported alcohol consumption in all models. Sensitivity analyses was performed to assess the robustness of the results to adjustment for nausea, exclusion of high risk pregnancies, multiparity, extremely high or low caffeine intakes, and the centre. Furthermore, the relation between the risk of FGR and maternal caffeine intake during pregnancy was

assessed by taking caffeine intake as a continuous variable (mg/day), adjusting for the factors mentioned above, and modelling was performed using the best-fitting second – order fractional polynomial with 95% confidence intervals.

Maternal caffeine half-life as assessed by the CCT was not normally distributed, therefore, based upon the median, we categorised women as having a shorter half-life (faster clearance) or longer half-life (slower clearance) of caffeine (i.e. in those with a slower clearance caffeine remained longer in their circulation and vice-versa for those with a faster clearance). Stratification of the OR for FGR by caffeine half-life (proxy for clearance) and intake was performed after taking account of maternal age, weight, height, ethnicity, parity, gestation, sex of the baby, and adjusted for smoking status, amount smoked (cotinine concentration), and alcohol intake.

3.0 RESULTS

3.1 Development and validation of the caffeine assessment tool and accompanying algorithm.

Sixty three pregnant women were recruited from Leeds for this pilot study but only 24 completed all aspects of the study. Subjects needed to be highly motivated to comply with the study protocols. The original recruitment target had been 30 women but this proved impossible within the time constraint.

Caffeine intakes

The highest mean daily caffeine intakes calculated from the CAT and diary were among women who were of later gestation (Table 3.1.1). On average, the daily caffeine intake from the CAT at 128 mg/day (SD=129mg/day) was 15 mg more than the diary at 113 mg/day (SD=97 mg/day). This difference is small being approximately 1/5 of a cup of instant coffee. The caffeine intakes from the CAT and the diary among the total sample (n=24) showed adequate agreement (ICC=0.5). Ten of the women did not provide complete brand information on sources of caffeine intake in the diary despite doing so in the CAT. For these women, the mean daily caffeine intake from the CAT was 156 mg/day (SD=77 mg/day) which was 27 mg/day more than the diary at 129 mg/day (SD=80 mg/day). However, for the women who did provide complete brand information in the diary, the mean daily caffeine intake from the CAT was 108 mg/day (SD=156 mg/day), this was only 6mg/day more than caffeine intake assessed from the diary at 102 mg/day (SD=109 mg/day). It is also evident that these women had lower caffeine intakes from both the CAT and diary than those who did not provide complete brand information in the diaries.

Table 3.1.1. Caffeine intake and time of CAT completion.

Tool	Time of CAT and diary completion								
	Total group (n=24)			≤ 15 weeks gestation (n=13)			> 15 weeks gestation (n=11)		
	mg/day	SD	κ	mg/day	SD	κ	mg/day	SD	κ
(mg/day)	128.2	129.2	0.50	123.4	90.7	0.69	133.8	168.7	0.29
/ (mean intake over 3 - mg/day)	113.2	97.0		98.4	91.2		130.1	105.0	

K = kappa statistic comparing CAT and diary

Figure 3.1.1 illustrates the agreement between mean caffeine intake (mg/day) from the CAT and diary. The level of agreement between the two methods was greater for women who were ≤ 15 weeks gestation (ICC=0.69) compared to those between 16-37 weeks gestation (ICC=0.29) (Table 3.1.2). A greater level of agreement was also apparent when women who left education earliest were considered (ICC=0.69) compared to those who left education later (ICC=0.20). Level of agreement between the two methods were similar for both young and old women (ICC=0.50 and 0.46 respectively).

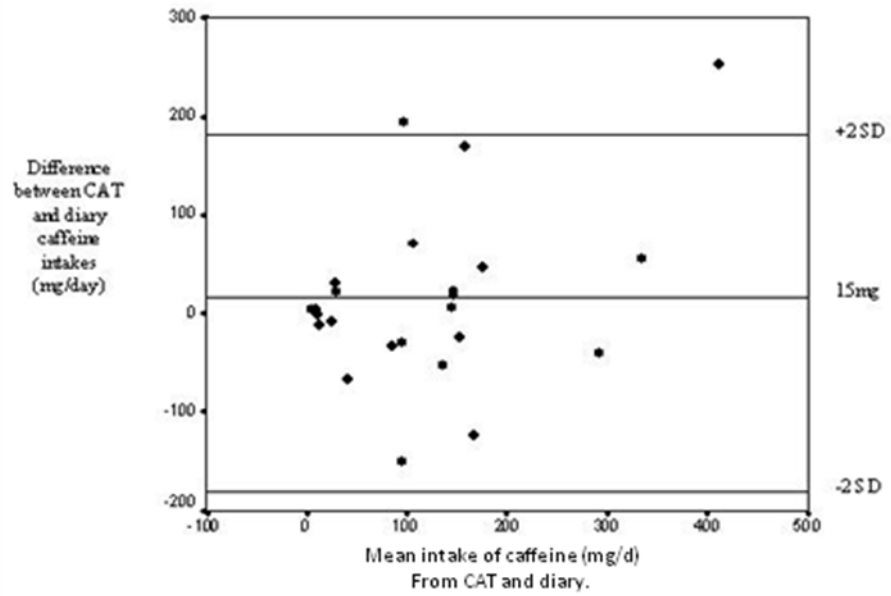


Figure 3.1.1. Bland Altman scatter plot of difference in caffeine intakes (mg/day) between CAT and food and drink diary.

Table 3.1.2. Agreement between caffeine intakes and saliva caffeine and paraxanthine measures

Tool	Day of sample collection	Kappa coefficient (κ)	
		Caffeine	Paraxanthine
CAT	1	0.50	0.33
	2	0.47	0.65
Diary (intake on day of saliva collection)	1	0.74	0.57
	2	0.45	0.64
Total of 3 days of diary	1	0.67	0.50
	2	0.30	0.48

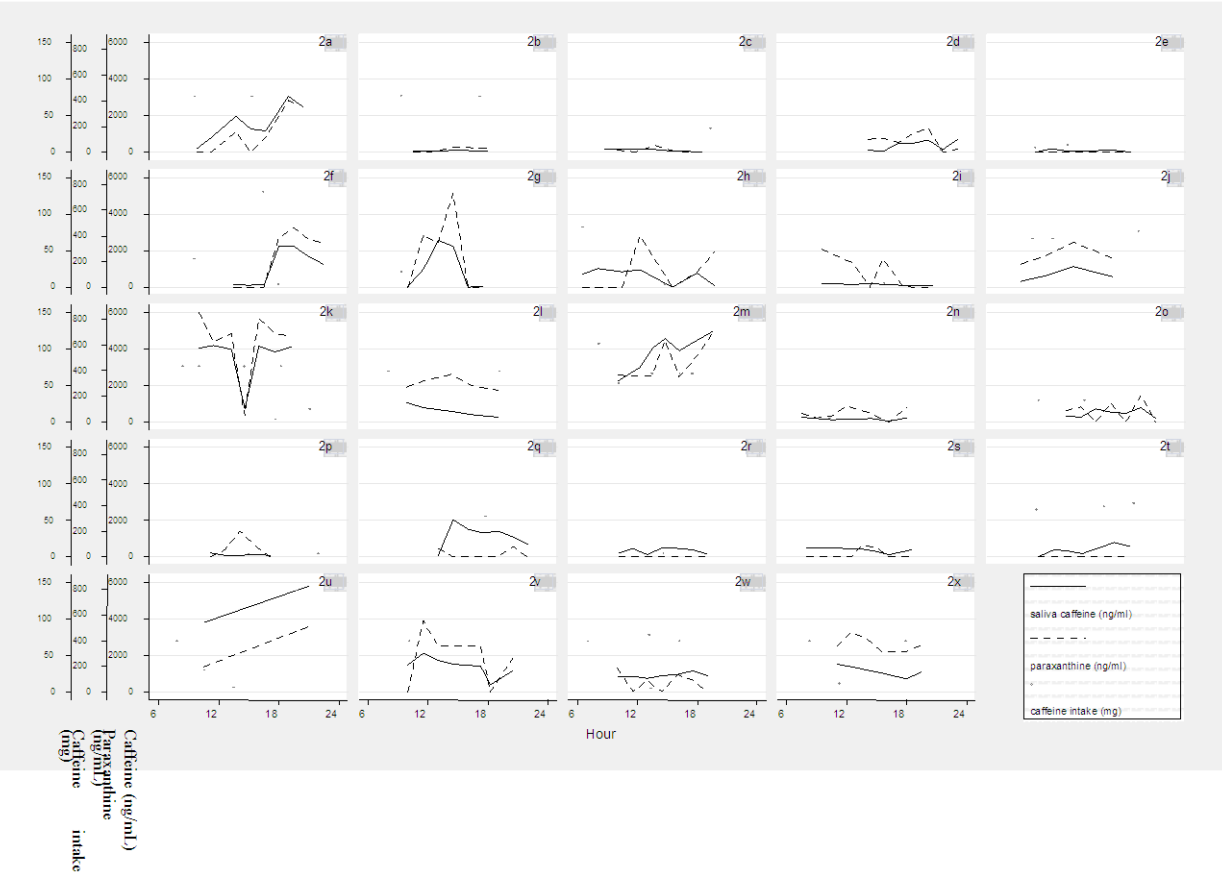
Variability of saliva measures

All nine saliva samples were available from 16 women. The mean saliva caffeine and paraxanthine concentrations, determined by HPLC-UV (see section 3.2) were 454 ng/mL (95% CI:367-561) and 198 ng/mL (95% CI:165-237) respectively. There was a good agreement between days one and two of saliva collection for both saliva caffeine and paraxanthine (ICC = 0.66 and 0.65, respectively). Figure 3.1.2 illustrates the saliva sample concentrations and caffeine intake over the first day of saliva collection. It is evident from Figure 3.1.2, that saliva caffeine and paraxanthine concentrations reflected each other closely for most women e.g. Figure 3.1.2 (a,b,f,j,k,u), however this was not the case for a few of the women e.g. Figure 3.1.2 (i,p). It is also evident from Figure 3.1.2, that salivary caffeine and paraxanthine concentrations reflected caffeine intake for some of the women e.g. Figure 3.1.2 (a,g,h,m). For some women, irrespective of level of caffeine intake, there was a sudden increase in saliva concentrations after caffeine intake e.g. Figure 3.1.2 (a,g,v). For others however, saliva concentrations did not parallel caffeine intake e.g. Figure 3.1.2 (b,o), or had a delay in the development of peaks e.g. Figure 3.1.2 (h). Caffeine intakes were low for some women, therefore, peaks in saliva caffeine and paraxanthine concentrations were not as marked e.g. Figure 3.1.2 (i,r).

For both saliva caffeine and paraxanthine, the between-sample (i.e. within the same woman) variation was 50% and 61% of total variation, respectively e.g. Figure 3.1.2 (k) which shows considerable variation in saliva caffeine and paraxanthine between samples. Between-women variation for salivary caffeine and paraxanthine was 39% and 38% of total variation, respectively. Figure 3.1.2 (b,k) shows how variable these concentrations are between women. Between-day variation for saliva caffeine and paraxanthine was relatively low at 11% and 0.1% of total variation,

respectively. Despite this variation however, the CAT agreed with the saliva measures just as well as with the food and drink diary. Using the kappa statistic, there was a moderate agreement between the CAT and saliva caffeine collected on both day one and two (0.50 and 0.47, respectively), with an even better agreement between the CAT and saliva paraxanthine collected on day two (0.65). Using the mean caffeine intake calculated over 3 days from the diary, a moderate agreement existed between the diary and saliva paraxanthine collected on both day one and two (0.50 and 0.47, respectively), with a greater agreement between the diary and saliva caffeine collected on day one (0.66). As expected, this agreement was even better when saliva concentrations were compared to caffeine intake from the diary on the same day of sample collection (Table 3.1.1). (These results have been published in Boylan et al. [52].)

Figure 3.1.2. Salivary measures collected on day one and caffeine intake (mg) on the same day per person.



3.2 Development of a method for the non-invasive assessment of caffeine and its metabolites.

3.2.1 Analysis of urine by HPLC-UV (Leeds)

A typical HPLC-UV chromatogram of caffeine and selected metabolites, is shown in Figure 3.2.1. In 60% of the pregnant women the levels of caffeine and metabolites did not increase above baseline sufficiently, post challenge, to give an accurate evaluation of caffeine clearance. Therefore caffeine clearance could not be calculated from urine using our conditions of 12 hours abstinence from caffeine prior to challenge and dose of caffeine (equivalent to 1 cup of coffee).

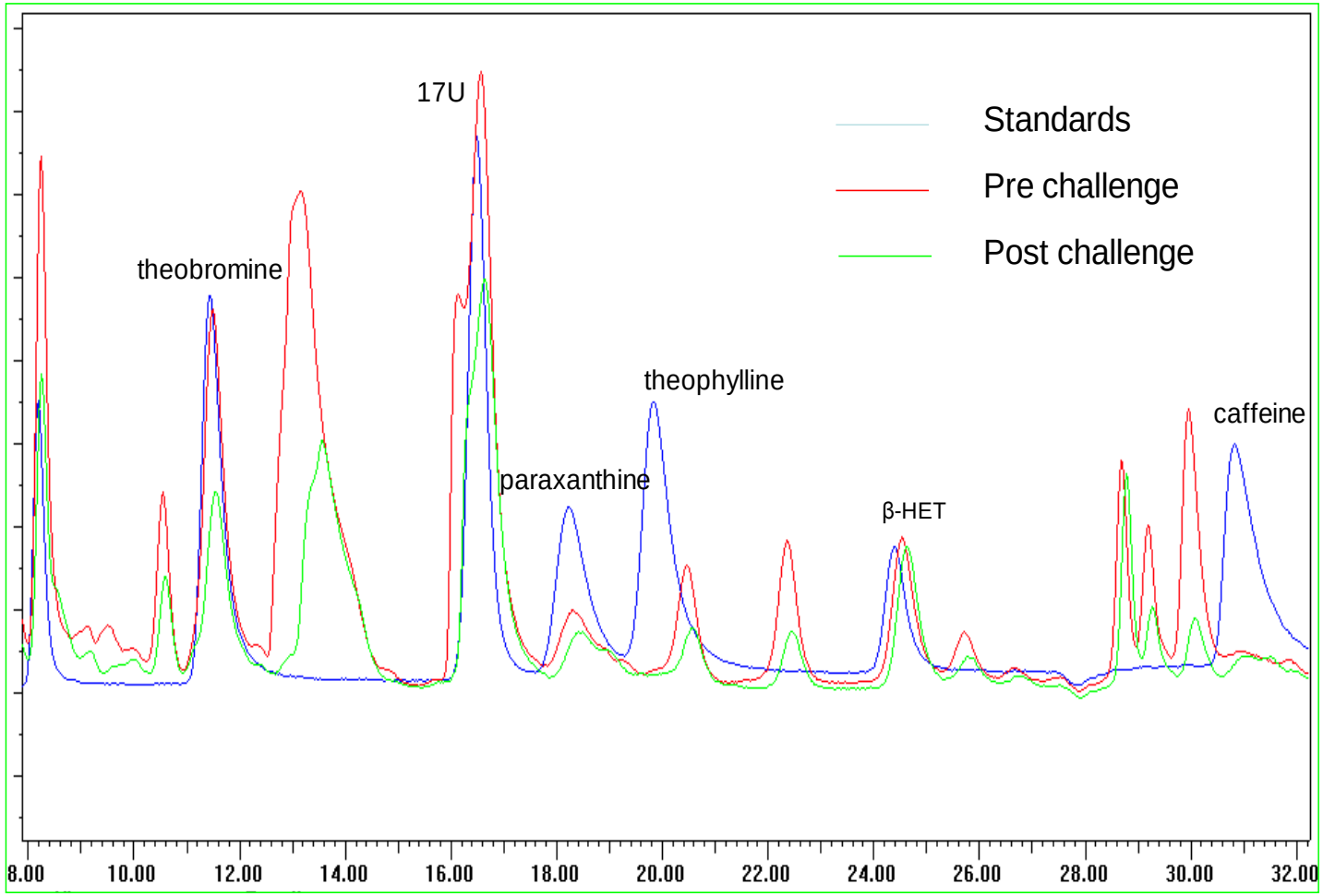


Figure 3.2.1. Representative HPLC-UV chromatogram of caffeine and selected metabolites, in urine, pre- and post-caffeine challenge.

3.2.2 Analysis of saliva by HPLC-UV

3.2.2.1 Leeds method

Figure 3.2.2.1 is a representative HPLC-UV chromatogram of salivary caffeine and selected metabolites pre- and post-caffeine challenge (1 and 5 hours post challenge). From these values the caffeine half-life could be calculated and used as a surrogate for rate of caffeine clearance (see section 3.3.1) thus overcoming the problems encountered when using urine samples (see section 3.2.1). Note that the chromatogram for saliva has fewer additional peaks, compared to that for urine.

Within batch CV (%) were 37X (2.3); 17U (2.4); 17X (2.2); 13X (2.5); β-HET (3.1) and 137X (2.7). Between batch CV (%) were 37X (2.8); 17U (2.5); 17X (2.2); 13X (3.0); β-HET(2.4) and 137X (3.8). The limit of quantification was 50 ng/mL for all compounds, calculated from standards made up in 30 % methanol with no extraction. The assay was linear over the range 50-10,000 ng/mL for all the above compounds.

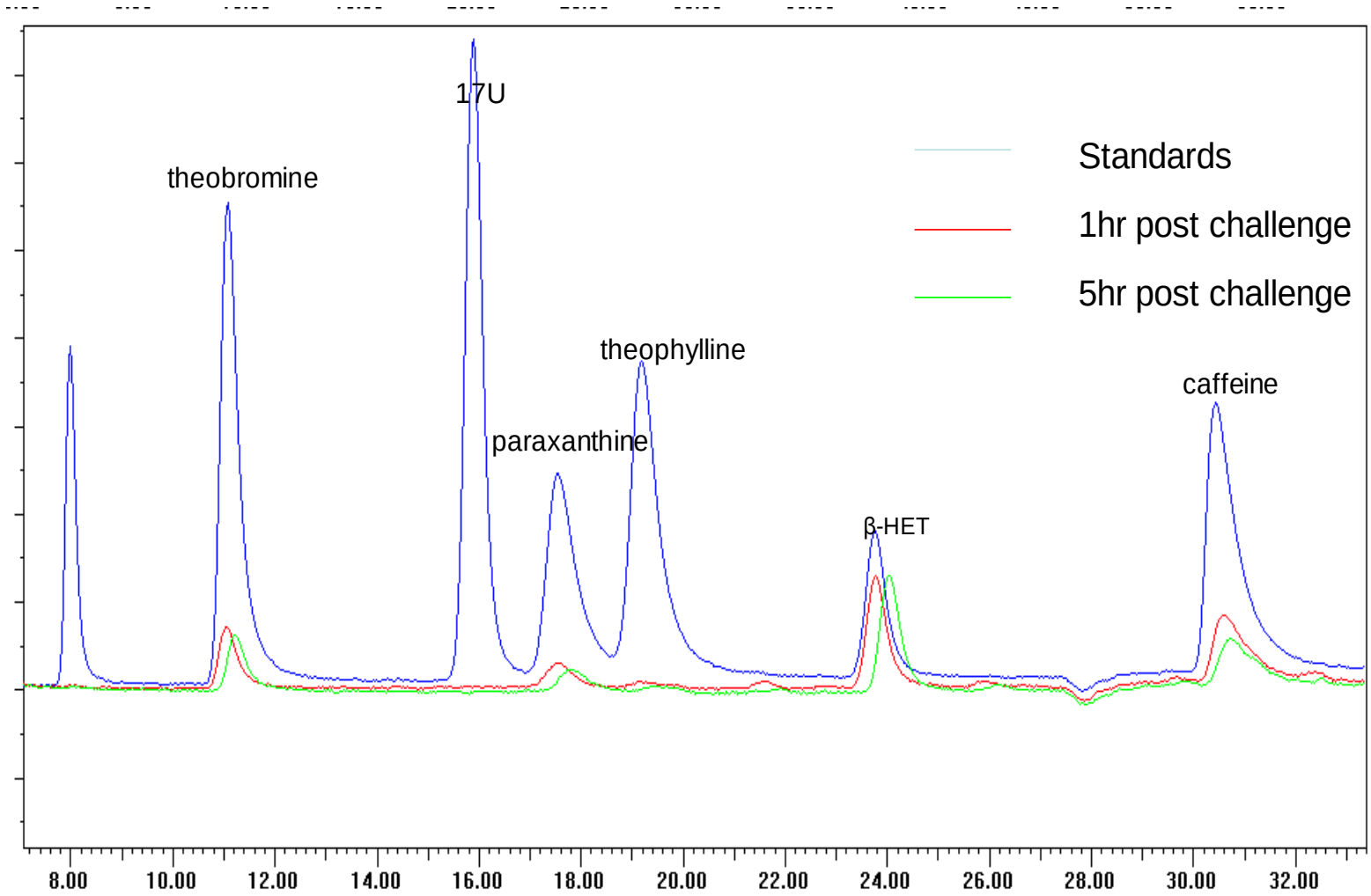


Figure 3.2.2.1. Representative HPLC-UV chromatogram of salivary caffeine and selected metabolites pre- and post-caffeine challenge.

3.2.2.2 Leicester method

The performance of the HPLC-UV assay at Leicester, used in the inter-laboratory study, is demonstrated in Table 3.2.2.2.

Table 3.2.2.2. Performance of HPLC-UV assay of caffeine and selected metabolites (Leicester).

Compound	Limit of detection (ng/mL; signal to noise ratio 3:1)	Limit of quantification (ng/mL; signal to noise ratio 10:1)	Inter-batch CV (%)	Intra-batch CV (%)
Theobromine	5	17	2.75	0.3
Paraxanthine	9	30	3.37	1.75
Theophylline	11	37	3.38	0.76
Caffeine	25	83	4.06	4.90

3.3 Inter-laboratory comparisons of HPLC-UV methods for the assessment of caffeine and its metabolites in saliva

The results for the inter-laboratory assay comparisons were promising (representative data are shown in Figure 3.3.1). Agreement was particularly close for theobromine, paraxanthine and theophylline, however sufficient agreement could not be reached for caffeine. In order to circumvent any discrepancies which might arise from two laboratories performing the analyses, it was agreed that a single centre should perform all salivary determinations.

Subsequent to the decision to undertake all salivary caffeine analyses at Leeds, a rolling study of the effects of (i) long-term storage and (ii) freezer/thaw, upon salivary caffeine and metabolites, was performed to investigate stability. Table 3.3.1 relates to long term stability at – 20 ºC.

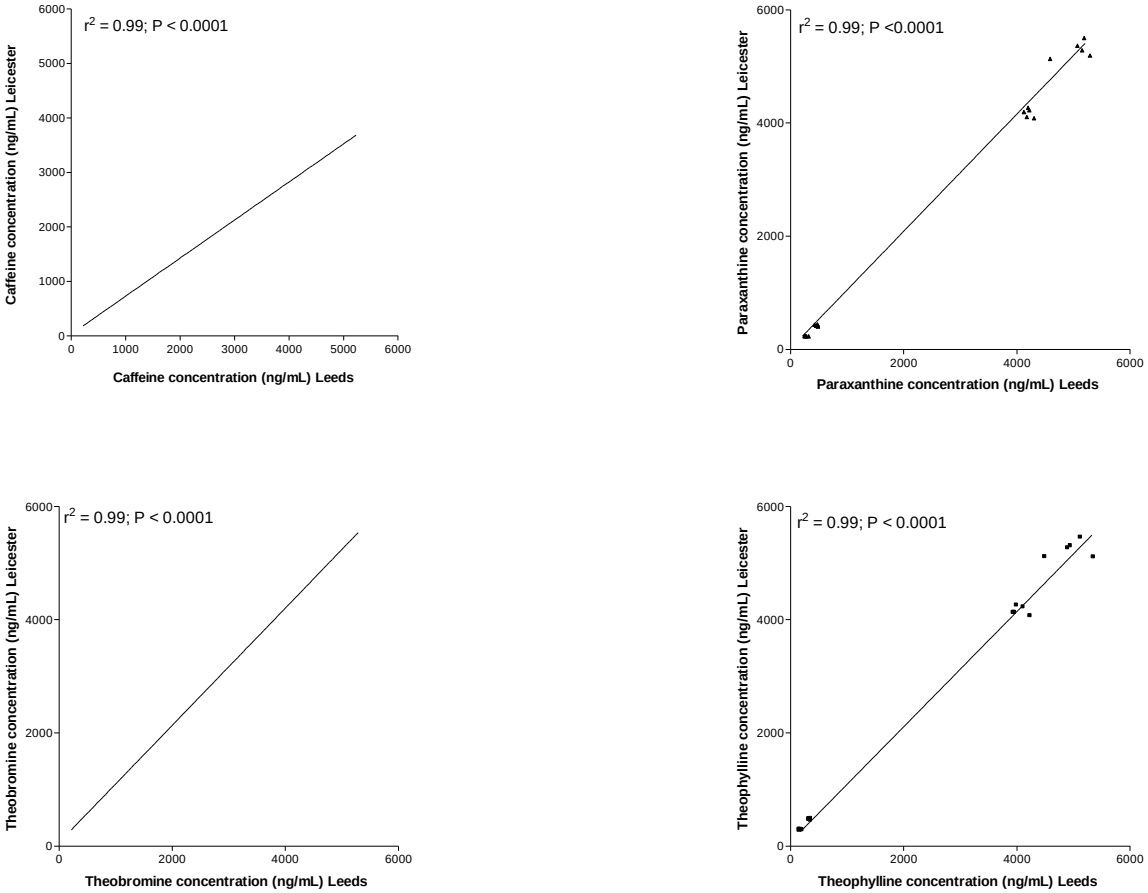


Figure 3.3.1 Comparison of caffeine and metabolite concentrations in spiked saliva samples sent from Leeds to Leicester, determined by HPLC-UV at both sites.

Analysis date	Internal Std		Caffeine		Paraxanthine		Theobromine		Theophylline		17U	
	ng/ml	µg/ml	ng/ml	µg/ml	ng/ml	µg/ml	ng/ml	µg/ml	ng/ml	µg/ml	ng/ml	µg/ml
2005 May-June [Mean of 8 runs]	1999.5	2.0	4952.0	5.0	4952.0	5.0	4769.1	4.8	4949.7	4.9	4818.9	4.8
2005 September	2079.2	2.1	4946.2	4.9	4925.9	4.9	4662.0	4.7	5013.9	5.0	4838.0	4.8
	1995.8	2.0	5218.2	5.2	5050.9	5.1	4643.5	4.6	5120.4	5.1	4796.3	4.8
2005 December	2008.3	2.0	4935.2	4.9	4773.1	4.8	4671.3	4.7	4939.8	4.9	4763.9	4.8
	1958.3	2.0	4759.3	4.8	4773.1	4.8	4615.7	4.6	5013.9	5.0	4763.9	4.8
2006 April	2008.3	2.0	4699.1	4.7	5310.2	5.3	4685.2	4.7	5004.6	5.0	4685.2	4.7
	1920.8	1.9	4916.7	4.9	5268.5	5.3	4578.7	4.6	5083.3	5.1	4787.0	4.8
2006 December	2070.8	2.1	5259.3	5.3	5277.8	5.3	4768.5	4.8	5439.8	5.4	4967.6	5.0
	2000.0	2.0	5138.9	5.1	4912.0	4.9	4634.3	4.6	5069.4	5.1	4782.4	4.8
2007 May	1946.7	1.9	4782.6	4.8	4642.6	4.6	4446.1	4.4	5428.6	5.4	4141.0	4.1
	1874.0	1.9	4934.0	4.9	4785.2	4.8	4355.3	4.4	5065.1	5.1	4066.3	4.1
Mean	1987.4	2.0	4958.3	5.0	4970.1	5.0	4620.9	4.6	5102.6	5.1	4673.7	4.7
SD	60.4	0.1	182.1	0.2	230.3	0.2	124.8	0.1	172.7	0.2	290.4	0.3
CV%	**3.0**	**3.0**	**3.7**	**3.7**	**4.6**	**4.6**	**2.7**	**2.7**	**3.4**	**3.4**	**6.2**	**6.2**

Table 3.3.1 Effect of storage at – 20 ºC on concentration of salivary caffeine and its metabolites in QC samples over a two year period.

3.3.1 Development of CCT

Figure 3.3.1.1 demonstrates how the time points of 1 and 5 hours fitted into the caffeine challenge and the time taken post challenge to reach maximum absorption of caffeine in three subjects. The figure illustrates that maximum absorption of caffeine was reached 15 minutes after taking the caffeine dose. This indicates that waiting one hour after finishing the caffeine drink allowed sufficient time for maximum absorption of caffeine, subsequent distribution and commencement of removal of caffeine by CYP1A2. The calculation of caffeine half life is based on two measurements of caffeine at approximately 1 and 5 hours post challenge. The calculation of half life is:

$$\frac{((\ln [\text{caffeine}]\ (1\ \text{h}) - \ln(1/2 \times [\text{caffeine}]\ (1\ \text{h}))) \times \text{exact time between measures}}{(\ln[\text{caffeine}\ ](1\ \text{h}) - \ln[\text{caffeine}](5\ \text{h}))}$$

It was necessary to use half-life as a surrogate for rate of clearance because rate of clearance requires to factor in maternal body weight (at the time of the challenge), information that we did not have.

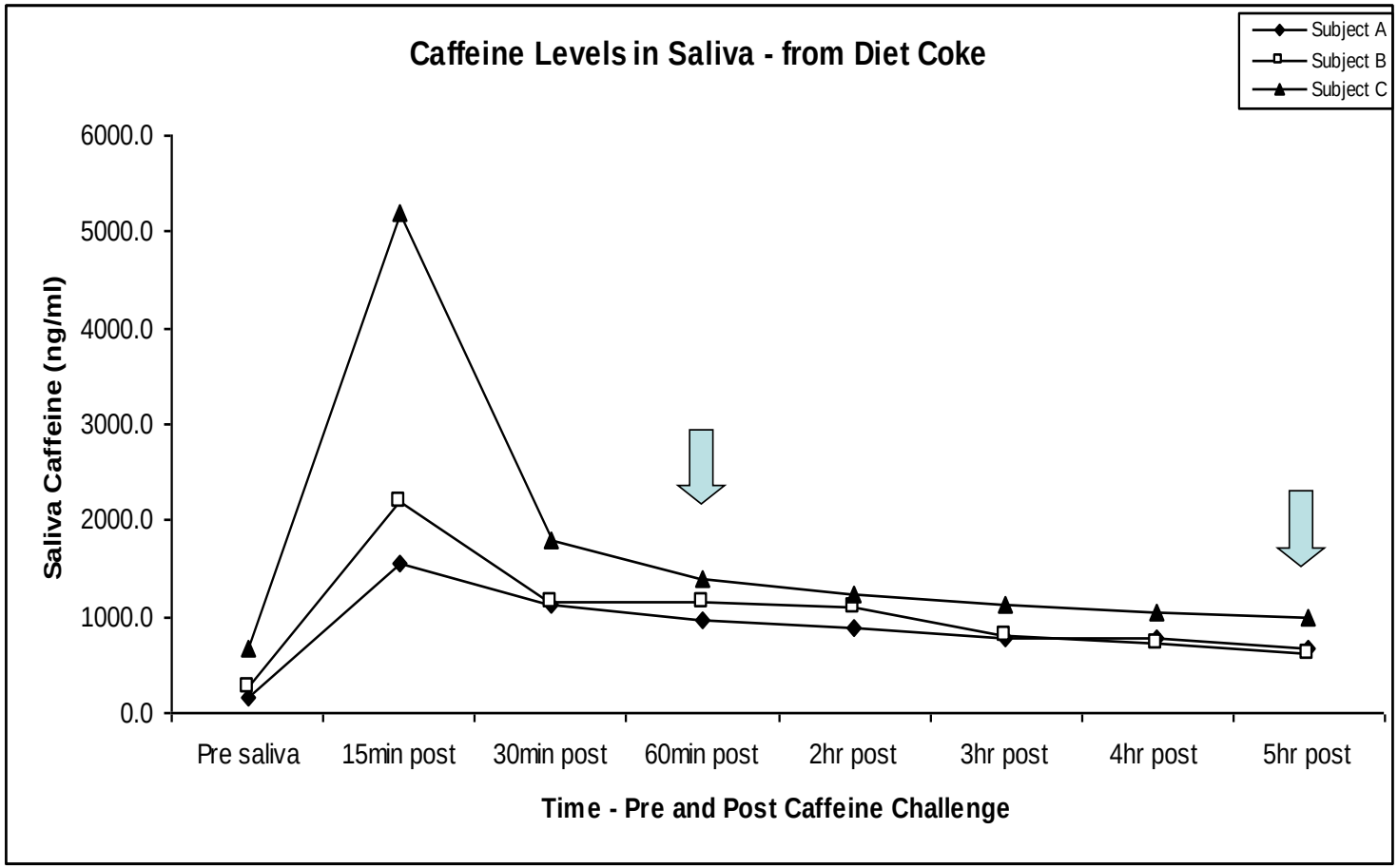


Figure 3.3.1.1. Development of the caffeine challenge. Establishment of maximum absorption time and rate of clearance of caffeine from saliva.

3.3.2 HPLC-UV QC data arising throughout study.

These are shown in Table 3.3.2.1.

Table 3.3.2.1 HPLC-UV QC data arising throughout study. All values are in ng/mL.

Leeds Exposures at Booking

	B-Het	137X	17X	37X	13X	17U
Mean	2009.4	4988.2	5065.6	5021.0	4994.9	4828.5
SD	86.9	141.2	161.8	174.5	143.4	150.9
CV%	4.3	2.8	3.2	3.5	2.9	3.1

Leeds Caffeine Challenges at Booking

	B-Het	137X	17X	37X	13X	17U
Mean	2005.8	4955.7	5037.3	5022.9	4929.6	4828.0
SD	82.0	153.9	192.3	172.4	157.0	185.0
CV%	4.1	3.1	3.8	3.4	3.2	3.8

Leeds Exposures at 28 Weeks

	B-Het	137X	17X	37X	13X	17U
Mean	2039.6	5038.4	5124.0	5129.0	5074.7	4865.1
SD	78.7	127.7	160.1	180.7	115.4	128.3
CV%	3.9	2.5	3.1	3.5	2.3	2.6

Leicester Exposures at Booking

	B-Het	137X	17X	37X	13X	17U
Mean	2029.8	5005.5	5005.2	5038.9	5056.2	4837.7
SD	73.7	132.8	183.9	140.5	139.9	166.6
CV%	3.6	2.7	3.7	2.8	2.8	3.4

Leicester Caffeine Challenges at Booking

	B-Het	137X	17X	37X	13X	17U
Mean	2029.9	4987.1	5070.6	5081.8	5058.6	4892.5
SD	75.8	221.9	261.2	168.3	150.3	189.9
CV%	3.7	4.4	5.2	3.3	3.0	3.9

Leicester Exposures at 28 Weeks

	B-Het	137X	17X	37X	13X	17U
Mean	1990.8	5038.9	4999.3	5002.7	5117.6	4692.8
SD	84.7	122.0	172.2	181.2	155.9	177.6
CV%	4.3	2.4	3.4	3.6	3.0	3.8

Leeds Exposures Post Partum

	B-Het	137X	17X	37X	13X	17U
Mean	1991.8	4972.9	5036.2	5046.1	5033.7	4740.3
SD	76.2	138.4	174.2	148.5	173.6	171.2
CV%	3.8	2.8	3.5	2.9	3.4	3.6

Leeds Caffeine Challenges Post Partum

	B-Het	137X	17X	37X
Mean	2034.1	5043.5	5083.6	5071.5
SD	75.4	184.5	206.2	194.0
CV%	3.7	3.7	4.1	3.8

Saliva QC Storage Trial May 2005 - May 2007

	B-Het	137X	17X	37X	13X	17U

3.4 Smoking status

(see outcome measures)

3.5 Outcomes

3.5.1 Data merging and cleaning

CATs from both sites were converted into same category definitions prior to merging. Duplicates and errors in subject identification numbers were corrected, and the formatting of clinical data, from both sites, made consistent. Follow-up and re-examination of hard copies located data for incomplete variables, and data entry errors for "headline" analyses corrected.

The CAT syntax was refined to include over-the-counter medication and strengths of tea/coffee. The consistency of centile definitions was resolved, using the software version applicable at time of data entry. Half-life data were cleaned, excluding 89 women, who consumed caffeine during challenge, from the half-life analysis. Values outside pre-defined feasible limits for key variables were double-checked and cleaned, if necessary. Cross-checks were made for some variables, to ensure consistency, e.g. BMI, smoking status.

3.5.2 Demographic and clinical characteristics

Over a period of three years, 13071 eligible women were invited to participate from the two centres and 2635 consented to participate. Table 3.5.2.1 contains the demographic and clinical characteristics of the study population, according to outcome.

Table 3.5.2.1 Demographic and clinical characteristics of 2635 subjects, according to outcome (fetal growth restriction, FGR, and average for gestational age, AGA).

Characteristic	**FGR** **N=343**	**AGA** **N=2292**	**All** **N=2635**
Mother's mean age (years) (SD)	30.0 (6.6)	29.8 (6.5)	30 (6.6)
Mother's mean pre-pregnant weight (kg) (SD)	66.7 (13.2)	66.8 (12.6)	66.8 (13.1)
Mother's pre-pregnant BMI (kg/m^2) (SD)	24.5 (4.5)	24.5 (4.6)	24.5 (4.5)
Primiparous (%)	186 (55%)	1042 (46%)	1228 (47%)
Mean gestational age at delivery (weeks) (SD)	40 (3)	40 (2)	40.0 (2)
Mean birth weight (g) (SD)	2750 (520)	3560 (470)	3450 (550)
Gender (male) (%)	172 (50%)	1152 (52%)	1324 (51%)
Pre-term labour	29 (8%)	77 (3%)	106 (4%)
Gestational hypertension / pre-eclampsia (%)	25 (7%)	42 (2%)	67 (3%)
Stillbirths (%)	3 (0.9%)	6 (0.3%)	9 (0.3%)
Late miscarriages (%)	3 (0.9%)	16 (0.7%)	19 (0.7%)

Tables 3.5.2.2-5 provide a breakdown of mother's age, parity, pre-pregnant BMI and ethnicity, by centre, and combined, and according to CAT for trimester. These data reiterate the lack of difference in population, between the two centres.

Table 3.5.2.2.

			Mother's age (yr)			
CAT for trimester	Centre	n	mean	SD	min	max
1	Combined	2496	29.8	5.1	17.3	45.5
	Leeds	1179	29.9	5.1	17.7	45.5
	Leicester	1317	29.7	5.0	17.3	44.3
2	Combined	1948	30.1	4.9	17.3	44.3
	Leeds	821	30.3	4.8	17.7	42.3
	Leicester	1229	30.1	4.9	17.3	44.3
3	Combined	1360	29.9	4.9	17.3	44.3
	Leeds	384	29.5	5.1	17.7	42.8
	Leicester	900	29.9	4.9	17.3	44.3

Table 3.5.2.3.

			Parity			
CAT for trimester	Centre	n	mean	SD	min	max
1	Combined	2475	0.72	0.84	0	6
	Leeds	1168	0.70	0.82	0	6
	Leicester	1307	0.72	0.84	0	5
2	Combined	1948	0.7	0.83	0	5
	Leeds	821	0.63	0.77	0	5
	Leicester	1229	0.7	0.83	0	5
3	Combined	1362	0.7	0.84	0	5
	Leeds	384	0.63	0.81	0	5
	Leicester	900	0.7	0.84	0	5

Table 3.5.2.4

			Pre-pregnant BMI (kg/m^2)			
CAT for trimester	Centre	n	mean	SD	min	max
1	Combined	2456	24.5	4.5	13.4	58.0
	Leeds	1160	24.7	4.9	16.9	58.0
	Leicester	1296	24.3	4.2	13.4	43.2
2	Combined	1921	24.5	4.5	15.0	58.0
	Leeds	810	24.6	4.9	16.9	58.0
	Leicester	1229	24.5	4.5	15.0	58.0
3	Combined	1346	24.5	4.6	15.5	58.0
	Leeds	382	24.8	4.6	15.4	46.8
	Leicester	900	24.5	4.6	15.5	58.0

Table 3.5.2.5

		Black and ethnic minorities*	
CAT for trimester	Centre	n	%
1	Combined	2500	7.2
	Leeds	1179	6.1
	Leicester	1321	8.3
2	Combined	1952	6.4
	Leeds	781	4.5
	Leicester	1229	
3	Combined	1363	6.5
	Leeds	366	3.9
	Leicester	900	

Including other/mixed.

3.5.3 Caffeine, alcohol and cotinine levels by FGR status

The FGR rate in the cohort was 343/2635 (13%). The mean alcohol intake during pregnancy was 0.4 (95% CI 0-9) units/day, with the highest consumption occurring, as expected, pre-pregnancy and during the first four weeks of pregnancy. The mean caffeine intake during pregnancy was 159 mg/day (Table 3.5.3.1). It decreased from 238 mg/day, pre-pregnancy, to 139 mg/day between weeks 5 and 12 and remained approximately at this level until the 3rd trimester, when it gradually increased to 153 mg/day. Approximately 62% of the caffeine ingested in pregnancy in this study was from tea. Other important sources were coffee (14%), cola drinks (12%), chocolate (8%) and soft drinks (2%). Hot chocolate, energy and alcoholic drinks contributed 2%, 1% and <1%, respectively. Over the counter medications made a negligible contribution to the total caffeine intake.

Whilst 15% of the total sample were considered from their cotinine values to be current smokers, 24% of those women who delivered a baby with FGR were classified as current smokers. On average, women were drinking less than one unit of

alcohol per day throughout pregnancy.

Table 3.5.3.1. Mean caffeine, alcohol and cotinine levels by FGR status

Characteristic	**FGR**	**AGA**	**All**
	n=343	**n=2292**	**n=2635**
Mean caffeine intake (mg/day) (SD)			
Total caffeine consumed	200 (202)	153 (145)	159 (154)
First trimester	201 (206)	157 (160)	163 (167)
Second trimester	184 (207)	141 (144)	147 (156)
Third trimester	197 (222)	143 (146)	153 (164)
Caffeine intake over pregnancy (%)			
<100 mg/day	122 (36%)	1000 (46%)	1122 (44%)
100-199 mg/day	90 (27%)	601 (27%)	691 (27%)
200-299 mg/day	63 (19%)	313 (14%)	376 (15%)
300+ mg/day	63 (19%)	284 (13%)	347 (14%)
Mean alcohol intake (units/day) (SD)			
Throughout pregnancy	0.4 (0.7)	0.4 (0.5)	0.4 (0.6)
First trimester	0.6 (0.9)	0.4 (0.7)	0.5 (0.8)
Second trimester	0.2 (0.4)	0.2 (0.5)	0.2 (0.5)
Third trimester	0.3(0.4)	0.2 (0.5)	0.3 (0.5)
Cotinine status (n=2509) (%)			
Non-smoker (<1ng/ml)	213 (64%)	1622 (75%)	1835 (73%)
Passive smoker (1-5ng/ml)	39 (12%)	268 (12%)	307 (12%)
Current smoker (>5ng/ml)	79 (24%)	288 (13%)	367 (15%)

3.5.4 Caffeine intake and risk of FGR

The relationship between total caffeine intake in pregnancy and FGR showed a statistically significant trend with increasing caffeine intake (P_{trend}=0.02; Table 3.5.4.1). Compared to those consuming <100 mg/day, the odds ratio of having a growth restricted baby increased to 1.2 (95% CI, 0.9 to 1.6) for intakes between 100 and 199 mg/day, to 1.5 (95% CI, 1.1 to 2.1) for those taking between 200 and 299 mg/day and to 1.4 (95% CI, 1.0 to 2.0) for those consuming over 300 mg/day. Consuming more than 100 mg/day of caffeine therefore increased the odds of having a growth restricted fetus by 40 to 50%. The relationship was consistent across all three trimesters.

Table 3.5.4.1 Unadjusted and adjusted odds ratios for caffeine intake, in mg/day, throughout, and at various stages of, pregnancy and FGR[2].

	Caffeine (mg/day)	**Unadjusted OR**	**(95% CI)**	**P_{trend}**	**Adjusted OR***	**(95% CI)**	**P_{trend}**
Average over pregnancy	<100	1	-		1	-	
	100-199	1.2	(0.9 to 1.6)		1.2	(0.9 to 1.6)	
	200-299	1.6	(1.2 to 2.3)		1.5	(1.1 to 2.1)	
	300+	1.8	(1.3 to 2.5)	P<0.001	1.4	(1.0 to 2.0)	P=0.02
Weeks 5 - 12	<100	1	-		1	-	
	100-199	1.2	(0.9 to 1.6)		1.1	(0.8 to 1.5)	
	200-299	1.4	(1.0 to 2.0)		1.3	(0.9 to 1.9)	
	300+	1.8	(1.3 to 2.5)	P<0.001	1.4	(1.0 to 1.9)	P=0.05
Weeks 13 - 28	<100	1	-		1	-	
	100-199	1.5	(1.1 to 2.0)		1.4	(1.0 to 2.0)	
	200-299	1.8	(1.3 to 2.6)		1.7	(1.2 to 2.4)	
	300+	1.6	(1.1 to 2.4)	P=0.001	1.3	(0.9 to 2.0)	P=0.02
Weeks 29 - 40	<100	1	-		1	-	
	100-199	1.4	(1.0 to 1.9)		1.4	(1.0 to 2.0)	
	200-299	1.9	(1.3 to 2.8)		1.8	(1.2 to 2.7)	
	300+	1.9	(1.3 to 2.8)	P<0.001	1.6	(1.0 to 2.4)	P=0.004

To examine possible threshold effects, the estimated risk of delivering a growth restricted fetus was compared to caffeine intake during pregnancy (mg/day)

[2] Unadjusted odds ratios take account of maternal age, weight, height, ethnicity, parity, gestation and sex of the baby, through the definition of FGR. Adjusted odds ratio additionally adjust for amount smoked (cotinine concentration) and alcohol intake.

measured as a continuous variable (Fig. 3.5.4.1). The best fitting second-order fractional polynomial was plotted with 95% confidence limits. There was a rapid increase in associated risk from zero intake up to approximately 30 mg/day. Thereafter, estimated risk continued to rise approximately linearly over the remaining range of intake, demonstrating a dose-response relationship. At no point in the curve did the estimated risk cease to increase and there was no observed plateau effect.

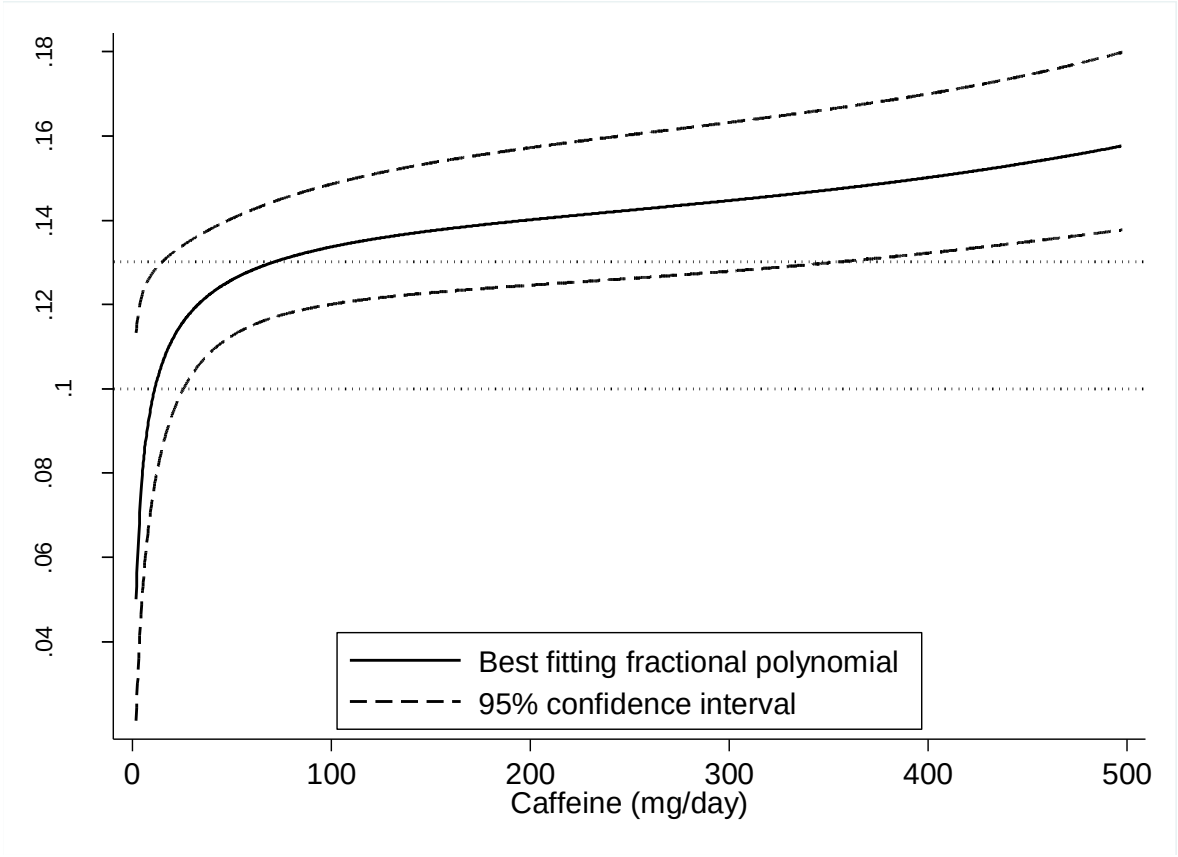


Figure 3.5.4.1. Relationship between risk of fetal growth restriction (FGR) and caffeine intake (mg/day) during pregnancy. The relation is modelled by the best-fitting second-order fractional polynomial, with 95% confidence intervals. The graph is restricted to <500 mg/day for clarity. Horizontal dotted lines mark national average risk (10%) and average risk in cohort (13%).

It is possible that the results in Figure 3.5.4.1 might be unduly influenced by a small number of women consuming high intakes of caffeine, or that the shape of the curve might be restricted by points beyond this level. To address this, we repeated the analysis excluding women consuming >300 mg/day of caffeine on average over the length of their pregnancy. This resulted in 446 women being excluded from the analysis. Figure 3.5.4.2 shows the resulting curve, fitted using the same methods, and on the same scale of caffeine intake to assist comparison.

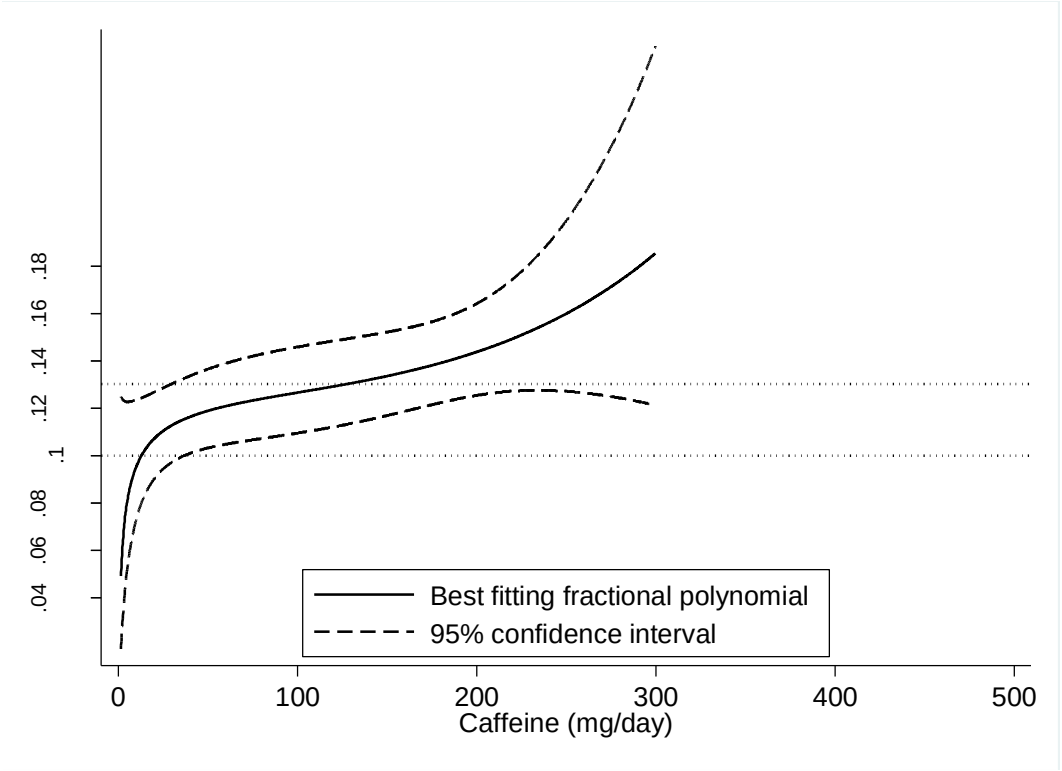


Figure 3.5.4.2. Graph based on analysis excluding women consuming >300 mg/day of caffeine. Indicated is the line of best fit, with the dashed curve being the 95% confidence interval. Dotted horizontal lines indicate average risk of FGR for our cohort (13%) and based on a sample of 50,000 from Nottingham, on which the UK growth reference curves are based (10%).

The curve indicates a continued dose-response relationship even below the current (now previous) UK recommended limit for caffeine intake of 300 mg/day. The slope appears slightly steeper than before the exclusions. At each end of the curve, confidence intervals are wide. It might also be supposed that the lower end of the curve may be restricted by individuals with unusually low intakes of caffeine. The analysis was therefore repeated excluding women consuming <20 mg/day. This resulted in a further 281 women being excluded from the analysis. Figure 3.5.4.3 shows the fitted curve based on this analysis, shown on the same axes as before, to assist comparison.

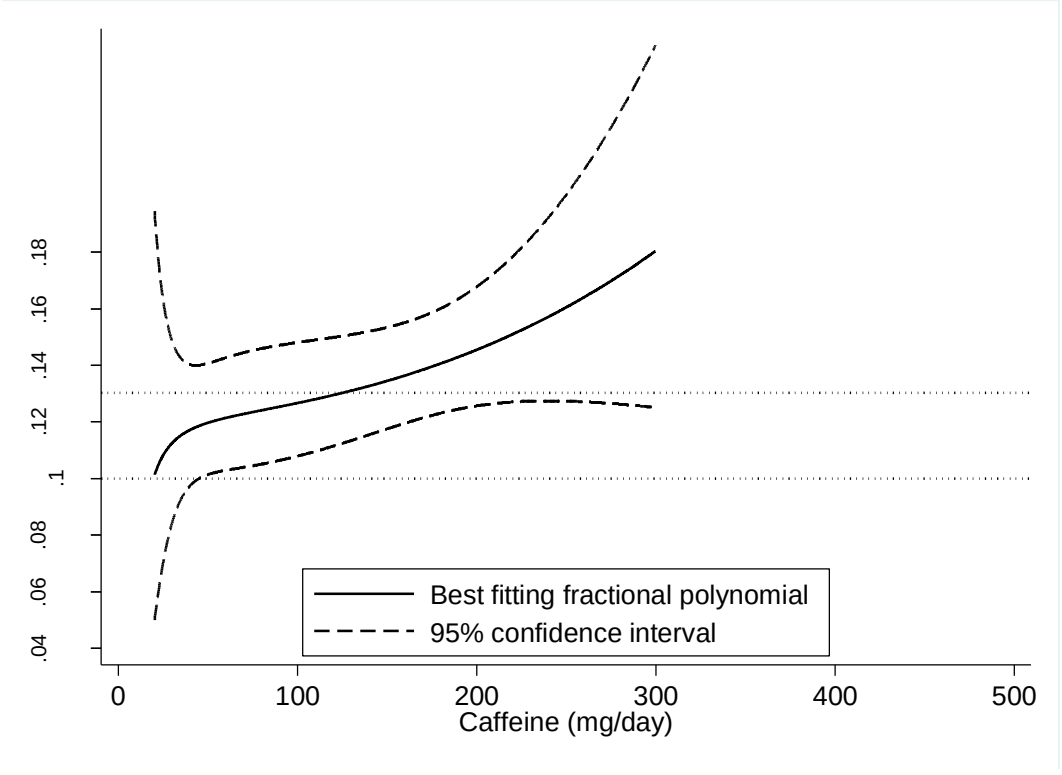


Figure 3.5.4.3. Graph based on analysis excluding women consuming either <20 mg/day or >300 mg/day of caffeine, on average over the entire pregnancy. The unbroken curve is the line of best fit, and the dashed curve is the 95% confidence interval. Dotted horizontal lines indicate average risk of FGR for our cohort (13%)

and based on a sample of 50,000 from Nottingham, on which the UK growth reference curves are based (10%).

Again, the curve indicates a continued dose-response relationship even below the current (now previous) recommended limit for caffeine intake of 300mg/day. Confidence intervals are wide at each end the curve, which particularly limits the interpretation of the dose-response relationship for very low intakes of caffeine. The shape of the curve at the lower end of caffeine intakes was particularly sensitive to the choice of cut-off for excluding low intakes, because of the small number of women with very low intakes.

The associations identified in here are consistent with those suggested by other studies published since COT last considered the evidence for caffeine and birthweight.

3.5.5 Effect of caffeine upon birthweight

The amount of caffeine ingested reduced the birthweight centile by 4 (range 0 -8) for >300 mg/day compared to <100 mg/day (Table 3.5.5.1). In terms of birthweight, caffeine consumption of over 200 mg/day during pregnancy was associated with a reduction in birthweight of approximately 60-70 g, with a significant trend over categories of caffeine intake (P_{trend} = 0.004). This relationship was consistent across all three trimesters. Consumption of >100 mg/day of caffeine was associated with a reduction in birthweight of between 34-59 g in the first, 24-74 g in the second and 66-89 g in the third trimesters (Table 3.5.5.1).

In a small cohort of women who had reduced their caffeine intake from 300 mg /day prior to pregnancy to <50/day by weeks 5-12 of pregnancy (n=109) the mean birthweight of their infants was higher than that in those who maintained their

caffeine intake above 300 mg/day (n=193) (difference in birthweight=161 g, 95% CI: 24 to 297 g, p=0.02)

Table 3.5.5.1 Unadjusted and adjusted[3] linear regression for birth weight and caffeine intake (mg/day).

	Caffeine (mg/day)	Unadjusted Δ birth weight (g)	(95% CI)	P_{trend}	Adjusted Δ Birth weight (g)	(95% CI)	P_{trend}
Average over pregnancy	<100	0	-		0	-	
	100-199	-1	(-51 to 50)		-21	(-62 to 20)	
	200-299	-63	(-129 to 4)		-70	(-123 to -18)	
	300+	-144	(-221 to -66)	P<0.001	-63	(-119 to -6)	P=0.004
Weeks 1 - 12	<100	0	-		0	-	
	100-199	-6	(-58 to 45)		-34	(-76 to 8)	
	200-299	-66	(-134 to 2)		-61	(-112 to -9)	
	300+	-144	(-220 to -69)	P<0.001	-59	(-114 to -4)	P=0.009
Weeks 13 - 28	<100	0	-		0	-	
	100-199	-15	(-74 to 44)		-24	(-72 to 24)	
	200-299	-44	(-119 to 30)		-65	(-124 to -6)	
	300+	-129	(-212 to 46)	P=0.003	-74	(-138 to -10)	P=0.006
Weeks 29 - 40	<100	0	-		0	-	
	100-199	-25	(-98 to 48)		-66	(-125 to -7)	
	200-299	-61	(-154 to 31)		-69	(-141 to 3)	
	300+	-119	(-211 to -27)	P=0.009	-89	(-158 to -21)	P=0.004

[3] Adjusted estimates take account of maternal age, weight, height, ethnicity, parity, gestation, sex of the baby, amount smoked (cotinine concentration), and alcohol intake.

3.5.6 Effect of caffeine upon growth centile.

Table 3.5.6.1. Odds ratios for mean caffeine intake, in mg/kg/day, throughout pregnancy, and by trimester, and change in growth centile, adjusted for cotinine concentration and alcohol intake.

	Caffeine (mg/kg/day)	**Δ centile**	**95% CI**	**P_{trend}**
Average over pregnancy	<1.00	0	-	
	1.00-1.99	-1	(-5, 2)	
	2.00-3.99	-1	(-4, 2)	
	4.00+	-5	(-8, -1)	P = 0.02
Weeks 1 - 12	<1.00	0	-	
	1.00-1.99	-4	(-7, -1)	
	2.00-3.99	-3	(-6, 0)	
	4.00+	-5	(-9, -2)	P=0.009
Weeks 13 - 28	<1.00	0	-	
	1.00-1.99	0	(-4, 4)	
	2.00-3.99	-2	(-6, 1)	
	4.00+	-3	(-7, 1)	P=0.1
Weeks 29 - 40	<1.00	0	-	
	1.00-1.99	-4	(-8, 1)	
	2.00-3.99	-2	(-6, 2)	
	4.00+	-4	(-9, 0)	P=0.07

3.5.7.Caffeine half-life and consumption in relation to FGR.

Although only caffeine concentrations were required for calculating half-life, the CCT saliva samples were also analysed for metabolites of caffeine, at one and five hours post-caffeine challenge (Table 3.5.7.1).

CCT samples returned*		**Median salivary caffeine metabolite concentrations (IQR) ng/mL**					
Centre	n	37X		17X		137X	
		1 h	5 h	1 h	5 h	1 h	5 h
Combined	1660	351.9 (180.6-680.6)	310.2 (175.9-576.1)	185.2 (129.6-277.8)	208.3 (148.3-287.0)	1272.5 (939.1-1760.8)	839.8 (578.7-1227.7)
Leeds	509	335.4 (178.5-629.6)	307.2 (171.3-544.0)	213.0 (143.5-305.6)	231.5 (171.3-314.8)	1223.0 (917.1-1695.7)	824.2 (588.7-1213.1)
Leicester	1197	359.2 (180.2-700.4)	311.1 (175.9-592.6)	180 (129.6-266.0)	199.1 (143.5-273.1)	1296 (1477)	844.8 (575.4-1245)

Table 3.5.7.1. Mean caffeine metabolite concentrations in saliva samples obtained approximately one and five hours following caffeine challenge.

* numbers of samples varies for each metabolite

As expected, salivary concentrations of caffeine decreased significantly between 1 and 5 hours, with a concomitant increase in paraxanthine. Levels of theobromine also decreased over this period. No 17U or 13X or were detectable in any of the samples.

Using the calculated maternal caffeine half life as a proxy for clearance rate (Fig. 3.5.7.1), there was some evidence that the association between caffeine and FGR was more in women with a faster compared to a slower caffeine clearance (test for interaction, P=0.06; Table 3.5.7.2 and Figure 3.5.7.2).

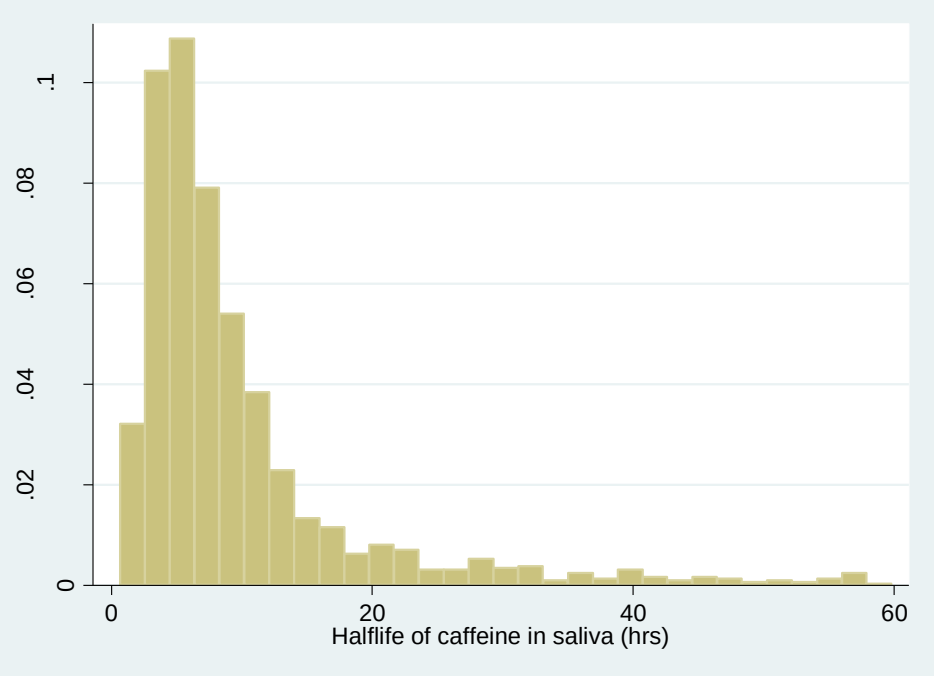


Figure 3.5.7.1 Distribution of salivary caffeine half-life in pregnant women (n = 1538).

Table 3.5.7.2 Stratification of the OR for FGR by caffeine half-life (proxy for clearance; shorter half life = faster clearance and longer half life = slower clearance) and intake, taking account of maternal age, weight, height, ethnicity, parity, gestation, child's gender, and adjusted for amount smoked (cotinine concentration) and alcohol intake.

Half-life	Caffeine (mg/day)	OR	(95% CI)	P_{trend}
≤ median	<100	1	-	
(Faster clearance)	100-199	1.6	(0.9 to 3.0)	
(n=774)	200-299	2.4	(1.3 to 4.4)	
	300+	1.7	(0.9 to 3.3)	P=0.02
> median	<100	1	-	
(Slower clearance)	100-199	1.1	(0.6 to 1.7)	
(n=764)	200-299	0.6	(0.3 to1.3)	
	300+	1.5	(0.7 to 2.9)	P=0.8
Test for interaction:	$P_{interaction}$ = 0.06			

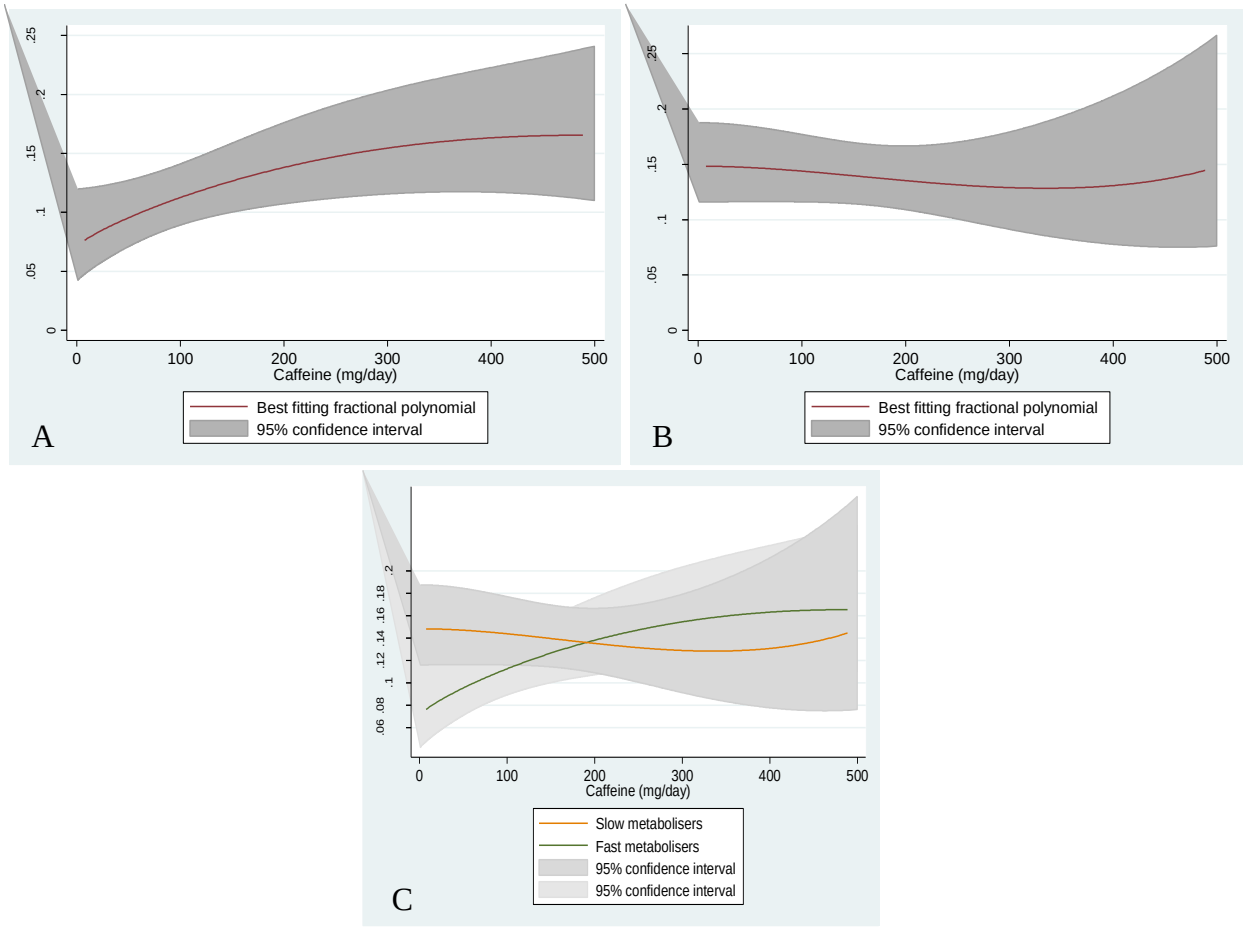


Figure 3.5.7.2 Caffeine half-life interaction plots: (A) Half-life < median (faster metabolisers; (B) half-life > median (slower metabolisers); and (C) combined.

There were no clear patterns for either salivary caffeine, or metabolites (paraxanthine, theobromine, theophylline), in saliva samples obtained at booking, and risk of fetal growth restriction (Table 3.5.7.3 and Figure 3.5.7.3).

Table 3.5.7.3 Salivary caffeine, paraxanthine, theobromine, and theophylline at booking and FGR (adjusting for salivary cotinine concentration and alcohol intake).

	n	**Metabolite (ng/mL)**	**OR**	**95 % CI**	**p-trend**
	1568	**Caffeine**			
Quartiles		< 193	1	-	
		193-503	1.3	(0.9, 1.9)	
		504-1136	1.1	(0.7, 1.6)	
		1137+	1.1	(0.7, 1.7)	P = 0.9
	1418	**Paraxanthine**			
		< 84	1	-	
		84-180	1.2	(0.8, 1.8)	
		181-293	1.3	(0.9, 2.0)	
		294+	1.1	(0.7, 1.7)	P = 0.6
	1737	**Theobromine**			
		< 229	1	-	
		229-481	0.9	(0.6, 1.4)	
		482-939	0.9	(0.6, 1.3)	
		940+	1.0	(0.7, 1.5)	P = 0.9
	1063	**Theophylline**			
		< 25	1	-	
		25-49	1.4	(0.9, 2.4)	
		50-99	1.1	(0.7, 1.7)	
		100+	1.4	(0.9, 2.2)	P = 0.2

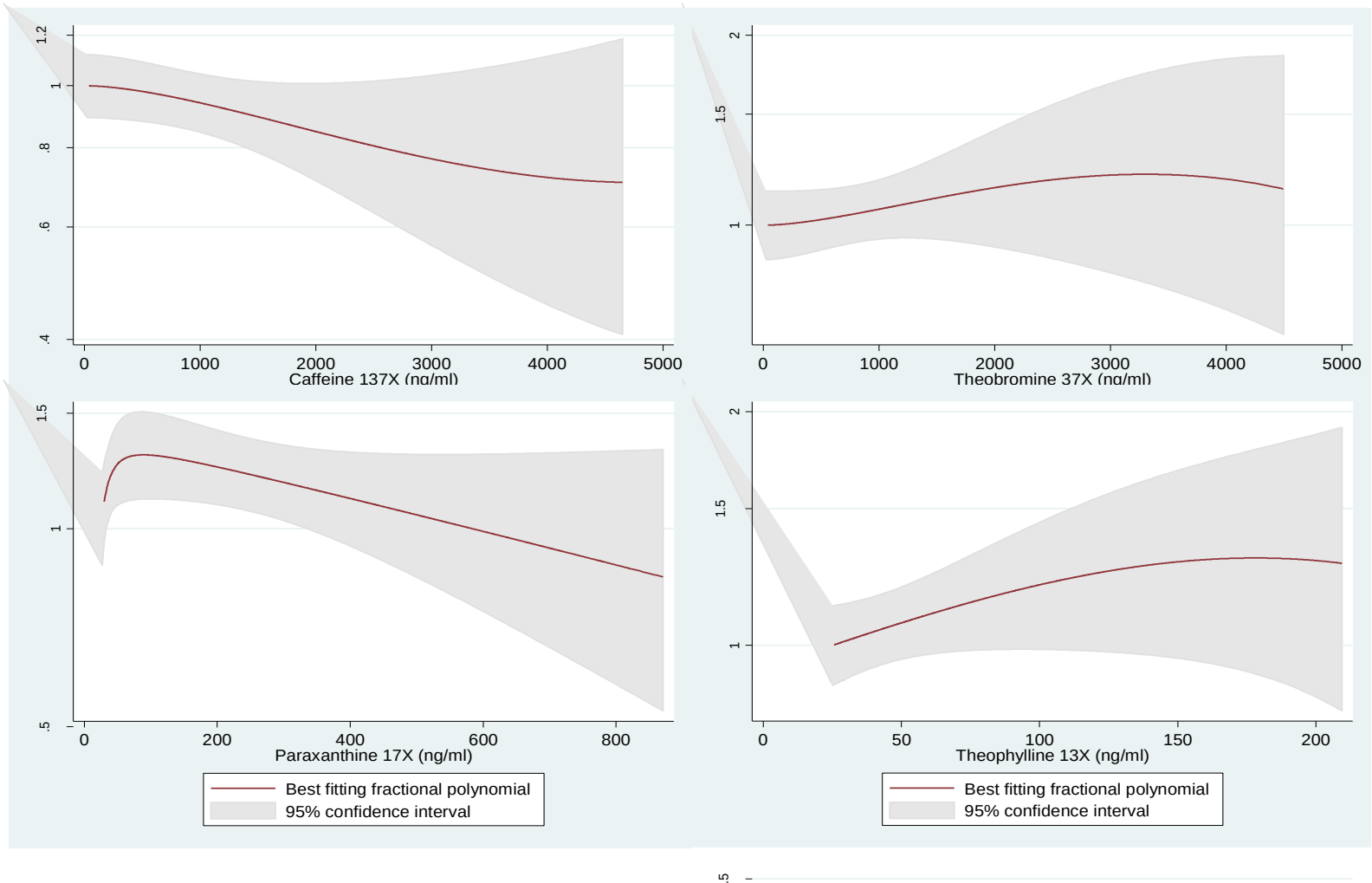


Figure 3.5.7.3. Relationship between risk of fetal growth restriction (FGR) and salivary concentration of caffeine, or metabolite (ng/mL) in a sample obtained at booking. The relationship is modelled by the best-fitting second-order fractional polynomial, with 95% confidence intervals.

When examining the ratio of caffeine and metabolites, there did not appear to be any clear patterns, or statistically significant trends, although the ratios of paraxanthine/caffeine and paraxanthine/theophylline both showed a non-significant positive trend (Table 3.5.7.4).

Table 3.5.7.4. Relationship between the ratio of (i) paraxanthine and caffeine; (ii) theophylline and caffeine; (iii) 37x and caffeine; (iv) paraxanthine and theophylline; (v) 17x/17x+37x+13x; (vi) 13x/17x+37x+13x; (vii) 17x/37x; and (viii) 13x/37x, in saliva obtained at booking, and risk of FGR (adjusted for salivary cotinine concentration and self-reported alcohol intake).

	n	**Metabolites**	**OR**	**95 % CI**	**p-trend**
	1375	paraxanthine/caffeine			
Quartiles		< 0.168	1	-	
		0.169-0.290	1.2	(0.7, 1.8)	
		0.291-0.541	1.6	(1.0, 2.4)	
		0.542+	1.2	(0.7, 1.8)	P = 0.3
	1052	Theophylline/caffeine			
		< 0.040	1	-	
		0.040-0.074	0.7	(0.5, 1.2)	
		0.074-0.170	1.0	(0.7, 1.7)	
		0.171+	0.7	(0.6, 1.6)	P = 0.7
	1544	37x/caffeine			
		< 0.376	1	-	
		0.376-0.920	1.1	(0.7, 1.6)	
		0.921-2.567	1.0	(0.6, 1.4)	
		2.568+	1.0	(0.7, 1.5)	P = 1.0
	-	paraxanthine/theophylline			
		< 0.168	1	-	
		0.169-0.290	1.5	(0.9, 2.5)	
		0.291-0.541	1.6	(1.0, 2.6)	
		0.542+	1.5	(0.9, 2.5)	P = 0.1
	1375	17x/17x+37x+13x			
		Q1	1.0	-	
		Q2	1.0	(0.7, 1.5)	
		Q3	1.0	(0.6, 1.5)	
		Q4	1.1	(0.7, 1.6)	P = 0.8
	1375	37x/17x+37x+13x			
		Q1	1.0	-	
		Q2	0.9	(0.6, 1.4)	
		Q3	1.0	(0.6, 1.5)	
		Q4	1.0	(0.6, 1.5)	P = 0.9
		13x/17x+37x+13x			
		Q1	1.0	-	
		Q2	1.3	(0.9, 1.9)	
		Q3	0.9	(1.3, 2.4)	
		Q4	0.9	(0.6, 1.4)	P = 0.4
		17x/37x			
		< 0.168	1.0	-	
		0.168-0.290	1.1	(0.7, 1.6)	
		0.291-0.541	0.9	(0.6, 1.4)	
		0.542+	1.2	(0.8, 1.8)	P = 0.7
		13x/37x			
		< 0.168	1.0	-	
		0.168-0.290	1.0	(0.7, 1.7)	
		0.291-0.541	0.9	(0.6, 1.4)	

0.542+	1.0	(0.6, 1.6)	P = 0.9

3.5.8 Effect of smoking upon birthweight

Based on cotinine concentrations, active smokers had nearly double the odds of FGR compared to non-smokers, adjusting for the same confounders as in earlier models (OR=1.9; 95% CI, 1.4 to 2.6; P<0.001). Considering birth weight as the outcome measure, babies born to active smokers were 178 g (95% CI, 127 to 230) lighter than those born to non-smokers (P<0.001). Furthermore, nausea in the first trimester was reported by 81% of the population and adjusting for nausea did not alter the results (see Section 3.5.9).

3.5.9 Implications of nausea in pregnancy

Table 3.5.9.1 Nausea and FGR

Pregnancy outcome	**Total n**	**With nausea (%)**
FGA	335	270 (81)
AGA	2175	1772 (81)
Total	2510	2042 (81)

Table 3.5.9.2 Nausea and mean caffeine intake over pregnancy (mg/kg/day).

Caffeine (mg/kg/day)	**Total n**	**With nausea (%)**
<1.00	771	636
1.00-1.99	550	466 (85)
2.00-3.99	695	552 (79)
4.00+	459	361 (79)
Total	2475	2015 (81)

Table 3.5.9.3. Nausea, mean caffeine intake (mg/kg/day) in first trimester, and risk of FGR, after adjustment for cotinine concentration and alcohol intake.

Group	**Caffeine intake (mg/kg/day)**	**OR**	**95% CI**	**p-trend**
Nausea	<1.00	1	-	
(n=2042)	1.00-1.99	1.5	(1.0, 2.2)	
	2.00-3.99	1.5	(1.0, 2.1)	
	4.00+	1.7	(1.1, 2.5)	P=0.02
No nausea	<1.00	1	-	
(n=472)	1.00-1.99	0.3	(0.1, 1.0)	
	2.00-3.99	1.0	(1.0, 2.3)	
	4.00+	1.1	(0.5, 2.4)	P=0.4

There was some evidence for an interaction between nausea in 1st trimester, caffeine consumption and FGR, specifically, caffeine consumption was associated with a worse outcome for women experiencing nausea in first trimester, compared to those

not experiencing nausea. This confirms previous research. However, 80% of women do experience nausea in the 1st trimester

3.5.10 Sensitivity analyses

The conclusions relating to caffeine and birthweight are robust. Sensitivity analyses were undertaken using different definitions of low birthweight (g and GROW package). Analyses were repeated for caffeine exposure described as mg/day or as a dose mg/kg/day. High risk pregnancies were excluded, the results for the low risk births showed a similar increasing risk with increasing caffeine intake (Table 3.5.10.1). Primiparous women had similar results to multiparous subjects in terms of increasing risk with increasing caffeine (Table 3.5.10.2). There was also no statistically significant difference between results at the different centres (Table 3.5.10.3).

Table 3.5.10.1. Sensitivity to parity: relationship between caffeine intake and FGR (adjusting for salivary cotinine concentration and alcohol intake).

Group	**Caffeine (mg/kg/day)**	**OR**	**95%, CI**	**p-trend**
All	<1.00	1.0	-	
(n = 2635)	1.00-1.99	1.2	(0.9, 1.7)	
	2.00-3.99-	1.4	(1.0, 1.9)	
	4.00+	1.5	(1.1, 2.2)	P = 0.02
Primips	<1.00	1.0	-	
(n = 1228)	1.00-1.99	1.3	(0.8, 2.0)	
	2.00-3.99-	1.4	(0.9, 2.1)	
	4.00+	1.8	(1.1, 3.0)	P = 0.02

Table 3.5.10.2. Sensitivity to exclusion criteria: relationship between caffeine intake and FGR (adjusting for salivary cotinine concentration and alcohol intake).

Group	**Caffeine (mg/kg/day)**	**OR**	**95%, CI**	**p-trend**
All	<1.00	1.0	-	
(n = 2635)	1.00-1.99	1.2	(0.9, 1.7)	
	2.00-3.99-	1.4	(1.0, 1.9)	
	4.00+	1.5	(1.1, 2.2)	P = 0.02
Low risk	<1.00	1.0	-	
(n = 1228)	1.00-1.99	1.2	(0.8, 1.8)	
	2.00-3.99-	1.4	(1.0, 2.0)	
	4.00+	1.6	(1.1, 2.4)	P = 0.01

Table 3.5.10.3. Sensitivity to recruitment centre: relationship between caffeine intake and FGR (adjusting for salivary cotinine concentration and alcohol intake).

Group	Caffeine (mg/kg/day)	OR	95%, CI	p-trend
Leeds	<1.00	1.0	-	
(n = 1298)	1.00-1.99	1.4	(0.8, 2.3)	
	2.00-3.99-	1.3	(0.8, 2.1)	
	4.00+	1.8	(1.1, 3.1)	P = 0.03
Leicester	<1.00	1.0	-	
(n = 1337)	1.00-1.99	1.1	(0.7, 1.8)	
	2.00-3.99-	1.5	(1.0, 2.3)	
	4.00+	1.2	(0.7, 2.0)	P = 0.3

4.0 DISCUSSION

4.1 CAT validation

Caffeine intakes

The mean caffeine intakes from both the CAT and diary were lower than intakes previously reported by pregnant women in the UK (204 mg/day for a 60 kg woman) [53]. However, since this previous study [53], a report has been published by the Food Standards Agency (FSA) advising pregnant women to limit caffeine consumption to less than 300 mg/day [10], and this may have decreased pregnant women's caffeine intakes in the UK. It is also possible that some women may have decreased or under-reported their caffeine intake as a result of taking part in this study.

The CAT is a detailed assessment of caffeine intake which is straightforward to complete. In contrast, ten women did not provide detailed information on caffeine intake particularly relating to brand level information in the comparison method, the food diary, even though they were instructed to do so. This may have contributed to the overall difference in estimated caffeine intakes between the CAT and the diary. There was a greater difference between caffeine intakes from the CAT and diaries from women who did not provide complete brand information compared to women who did provide this level of information in the diaries. Another contributor to difference in estimates of caffeine intakes between the two methods could be the different time periods assessed by the two tools. The diary assessed food and drink consumption over the three days whereas the CAT assessed recalled intakes over an 8 week period. Despite the difference in estimates, there was still an adequate agreement between the two methods.

The level of agreement between the caffeine intakes from the CAT and diary was greatest among women who were in their earliest gestational weeks. This may be because the CAT which was administered in this test assessed caffeine intake early in pregnancy (weeks 5-12). Women later in pregnancy reported higher caffeine intakes in the CAT, although only 4 women were in the second half of pregnancy (over 20 weeks), nevertheless, recall bias may have been introduced as these women may have been reporting caffeine intakes similar to their current intakes rather than intakes between weeks 5-12 of pregnancy. This is plausible, as caffeine intake may be lower in the first trimester due to nausea or intentional avoidance.

Saliva samples

In general, salivary caffeine and paraxanthine concentrations agreed with and reflected each other closely. It is also clear that the saliva concentrations generally responded to the caffeine intake recorded. However, for some of the women, the saliva measures did not appear to increase after reported caffeine intake. This may be due to error in completing the diary, or due to differences in metabolism and clearance between women resulting in lower concentrations at the time samples were collected. Caffeine is metabolised by the cytochrome P450 family of enzymes in the liver, in particular the major enzyme being CYP1A2 [54] with metabolites produced through demethylation and oxidation. Paraxanthine is a primary metabolite produced by demethylation of the N^3 position methyl group. Caffeine has a half life of about 3 – 7 hours unless the rate of action is affected by genetic and /or environmental factors, for example, CYP1A2 activity is decreased by female sex hormones during pregnancy or treatment with oral contraceptive [55].There is wide variation in CYP1A2 messenger RNA expression; up to 40-fold variation has been described [56, 57]. Genetic

polymorphisms of the CYP1A2 gene, smoking char-grilled foods, Brassica vegetables and prescription medicines also affect the rate of caffeine metabolism. Saliva measures were chosen in preference to blood and urine due in part to the ease of obtaining the samples and the lower invasiveness of the measure for the subjects. Plasma and saliva clearance of caffeine are highly correlated [58]. Newton *et al.* [59] concluded that salivary caffeine levels probably reflect the unbound plasma caffeine concentration and therefore can be used to estimate the pharmacokinetic parameters of the drug. They estimated that the overall saliva/plasma concentration ratio was 0.74 ± 0.08. Other evidence has suggested that the complex metabolism of caffeine together with different parameters controlling the renal clearance of each metabolite, makes the use of urinary metabolic ratios an inaccurate probe in populations [60].

For the saliva measures, the between-sample variation was greater than the between-woman and between-day variation. This is expected as the serum and saliva concentration of caffeine varies widely in response to recent caffeine intake. However, because of its longer half-life, paraxanthine concentrations will fluctuate less throughout the day and may be a better measure of caffeine intake. However in this study, the between-sample variation of saliva caffeine was lower than the between-sample variation of saliva paraxanthine. Despite this relatively large variability, in general, both saliva measures adequately agreed with both the CAT and diary. As expected, the greatest level of agreement between the saliva measures and assessment of caffeine intake was found between seven saliva samples collected on day one and actual caffeine intake on that day, illustrating that a one-day diary is a good snap shot of actual caffeine intake. Several measurements of salivary caffeine and paraxanthine over a day are far more likely to reflect intake than a single measurement. Both caffeine and paraxanthine have relatively short half lives and concentrations in saliva

(or plasma) change markedly over a day reflecting recent consumption. Given that caffeine intake over the day is episodic, repeat measurements of salivary caffeine and paraxanthine are more likely to record this pattern of consumption that a single measurement at one time point.

A moderate, yet lower agreement was found between the saliva measures and the more habitual intake calculated from the mean caffeine intake of the three days reported in the diary. Even though the CAT reflects longer-term habitual caffeine intake than the 3-day diary, the agreement between the CAT and saliva measures was only marginally less than that between the 3-day diary and saliva measures.

General comments

Despite using different methodologies, both the CAT and diary have emerged as being equally good at assessing caffeine intake. However, it is the CAT that provides a practical, yet detailed, and therefore more accurate assessment of long-term habitual caffeine intake.

Of the women recruited, only 38 % completed the study, which could be due to the demands of completing a three-day food and drink diary and collecting nine saliva samples and monitoring caffeine intake over two days. It is important to consider that this sample of women may not be representative of the total pregnant population as approximately one third of the sample were employed in managerial and professional occupations.

As is evident from this study, assessing long-term caffeine intake using food and drink diaries is not only impractical, but it is also likely to omit detail such as brand information. In this study it was apparent that when such information was omitted from diaries, estimated caffeine intakes were on average 27 mg/day lower

than intakes from the more detailed CAT – which is approximately equivalent to half a cup of tea. This suggests that the use of average values for sources of caffeine intake may under-estimate caffeine intakes. A further source of error could be introduced by not considering strength of tea or coffee as commonly consumed. Different preparatory approaches to making tea or coffee can lead to variations in caffeine content [61]. In the CAT, we did ask women to record the strength of tea and coffee they prepared and to state whether it was weak, medium or strong. We did not use this information, however, since there was limited published data information available on variation in caffeine content by brand and preparation method. For instant coffee we did record and use in our analysis whether level or heaped teaspoons of dry coffee were used. Our previous experience of assessing diet has indicated that individual perceptions will vary and thus we could introduce more measurement error by using more subjective records. In addition, brewing times for cafetiere prepared coffee makes little difference to caffeine content of the brewed drink[4].

Repeated saliva measures may also be a useful measure of caffeine exposure. However, even if caffeine intake was accurately assessed, there exist inter-individual differences in metabolism that will influence spot saliva measurements. This may be especially relevant when assessing effects of caffeine exposure on pregnancy outcome, as caffeine metabolism decreases throughout pregnancy [62].

4.2 Main study

This is one of the largest prospective studies investigating the association of caffeine and birth weight. Although only 20% of those invited took part, we do not feel that this low response rate lessens the validity of our data, as the association of

[4] Kadja, (personal communication)

caffeine with birth weight should not be different from that in the general population especially as various confounders were taken into consideration. In addition, examination of our maternity databases indicated that the population studied was similar to that of the units as a whole.

4.3 Principal Findings

Caffeine consumption during pregnancy almost halved in early pregnancy (250 mg/day pre-pregnancy to 150 mg/day in the first trimester), a finding similar to previous reports [63]. Interestingly, intakes of caffeine rose in the third trimester unlike alcohol intake which remained lower than pre-pregnancy throughout. Maintaining a lower caffeine intake throughout pregnancy might be important. A study by Vik *et al*. [19] found that The risk of SGA birth was nearly doubled if the mother had a high rather than a low caffeine intake in the third trimester [odds ratio (OR) 1.8; 95% confidence intervals (CI) 1.2, 2.5].

The mean caffeine intake throughout pregnancy was much lower (159 mg/day) than the limit of 300 mg/day recommended by the UK government's Food Standards Agency [10] and in the USA [25]. The caffeine intake was validated by comparison with a food diary and repeated exposure estimates from saliva [50] and we believe that, for the first time this reflects a true picture of total caffeine intake by woman during pregnancy. Over 60% of the caffeine consumed was from tea and only 14% from coffee. Weng *et al* reported that coffee was the sole source of caffeine in 19% of their pregnant cohort, and 44% consumed caffeine from combined coffee and non-coffee sources [64]. Since 26% of caffeine in our cohort was neither from coffee nor tea, studies which concentrated on coffee and tea alone would have grossly underestimated caffeine intake.

Caffeine and birthweight

Several studies have concluded that caffeine intake of more than 300 mg/day is associated with either low birth weight or FGR [12, 13, 65]. Our study confirms these findings but further defines the nature of the association. We could find no level of intake at which there was no association with increased risk of FGR, and this risk was maintained throughout pregnancy. The size of association for caffeine in relation to increased risk of FGR was of a similar size to that for alcohol intake in pregnant women in this study. Although, the overall size of the reduction in birth weight maybe seen as small, an extra weight of 60-70 g could make a difference in the perinatal morbidity and mortality in an already compromised fetus. The steep decline in risk associated with caffeine intakes of less than 30 mg/day may be attributable to unmeasured confounding. Furthermore, women who consume little or no caffeine may be generally more health-conscious, than those who consume slightly more, and the effect may be one for which we have been unable to assess or adjust.

An interesting observation in our study was the strong association between caffeine intake and birth weight across all of the trimesters. However, from these results we cannot define a critical time window for any maximal effect. This clearly warrants further investigation.

A major strength of our study is that we have objectively quantified caffeine from all known sources. Our findings further emphasize the weaknesses of studies where caffeine intake was equated to that of coffee alone. Furthermore, in this study we demonstrated that average caffeine consumption of >100 mg/day, after adjusting for smoking status and alcohol intake, was associated with a reduction in birth weight of 34-59 g in the first, 24-74 g in the second and 66-89 g in the third trimesters.

Similar results were seen by Bracken *et al*. in a prospective study of 2291 pregnant women in the USA, where mean birth weight was reduced by 28 g per 100 mg/day of caffeine consumption (95% CI -0.10 to -0.46, P=0.0001), but the risk for FGR was unchanged (OR=0.96) [61]. This could be explained by methodological differences in the studies. In a Danish cohort of 1207 women drinking at least 3 cups of coffee per day before 20 weeks, who were randomised to receive either caffeinated or decaffeinated instant coffee, there were no statistically significant difference in birth weight between the two groups after adjustment for parity, gestational age at birth and smoking (95% CI -40 to 73; P=0.57) [66]. Compared to our study, the women were recruited in the second half of pregnancy, thereby the association of first trimester caffeine intake was not assessed and also there was no biochemical confirmation of compliance in the randomised groups. In addition, Bicalho and Filho in a case-control study (Brazil) have also reported no association between caffeine consumption and low birth weight [67], after adjusting for confounding variables.

Some of the variation in previously reported associations between caffeine and pregnancy outcomes may reflect the unmeasured effect modification of inter-individual differences in caffeine metabolism. The degree to which the fetus is exposed to caffeine and its metabolites, which pass freely across the placenta, is dependent upon maternal CYP1A2 enzyme activity because of the absence of hepatic expression in the fetus. In the present study, we complemented a detailed assessment of caffeine intake with a measure of caffeine metabolism and observed a tendency towards a greater association of caffeine with FGR amongst women with faster caffeine clearance. Caffeine is primarily metabolised in the human liver by CYP1A2 to paraxanthine in most individuals [68] but there is little data about metabolism in pregnant women. In our study caffeine was metabolised to paraxanthine, theobromine

and theophylline, with theobromine present in highest concentration in majority of the women. As we were unable to measure the rate of formation or subsequent metabolism of these primary metabolites, we cannot attribute the association with fetal growth to any single metabolite. The association we observed may be due to caffeine itself and/or to one or a combination of the metabolites. Klebanoff *et al*. [69] reported a positive association between maternal paraxanthine concentration in the third trimester with an increased risk of having a small-for-gestational age infant among women who smoked. In another study, the highest concentrations of paraxanthine were associated with an increased risk of spontaneous abortion [8]. Recently, higher cord blood paraxanthine concentrations have been shown to be associated with an increased risk of intrauterine growth restriction after adjustment for caffeine levels, implying an effect of CYP1A2 activity rather than absolute levels of paraxanthine [45]. Whilst the current study does not enable us to conclude whether it is caffeine or its metabolite(s) that are responsible for the observed association with FGR, the data do indicate the importance of further consideration of the role of CYP1A2 activity and caffeine metabolites.

5.0 SUMMARY CONCLUSIONS

This large prospective cohort study has demonstrated that maternal caffeine intake is associated with an increased risk of fetal growth restriction. The threshold at which this risk is significantly higher is not well characterised. Although several retrospective and studies with design limitations have referred to thresholds of 150, 200 and 300 mg/day, these have been determined indirectly from the estimated caffeine intake derived mainly from reported coffee and tea intake. As we have shown in this study, there are several other sources of caffeine and these must be taken into account when estimating the risk of adverse pregnancy outcome with caffeine intake. We suggest that a more sensible advice for women contemplating pregnancy is to reduce their caffeine intake from all sources prior to conception. Once pregnancy is confirmed, every effort should be made to stop caffeine consumption, or to markedly reduce it, as this data confirms that the association of fetal growth restriction with caffeine is reduced for those consuming less than 100 mg/day.

Further research is needed to study CYP1A2 activity and caffeine metabolites in relation to its possible influence on the feto-placental unit and explore any relevant mechanistic pathways.

6.0 PUBLICATIONS ARISING

Published

Cooke, MS., Kirk, S., Cade, J., Evans, MD., Greenwood, D., Konje, J., Lunec, J., Simpson, N., Walker, J. and Wild, C. (2003) Caffeine's role in pregnancy outcome – a complex picture? *British Medical Journal*, (rapid response) http://bmj.com/cgi/eletters/326/7386/420#30614.

Boylan SM, Cade JE, Kirk SFL, White KLM, Shires S, Hay AWM, Simpson NAB and Wild CP. The development of a caffeine assessment tool (CAT) for use in pregnancy. *Proc Nutr Soc*. 2004;63:74A.

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Theses

S. Boylan. PhD Thesis. Caffeine intake and metabolism during pregnancy. University of Leeds. 2006.

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Acknowledgements

We would like to thank Professor Gordon Gibson, Dr Fred Kadlubar, and Professor Mark Klebanoff, for their useful comments during the study. The Leicester team wishes to acknowledge Vilas Misty, Clare Lawrence, Bhavin Daudia and the Department of Chemical Pathology, University Hospitals of Leicester NHS Trust for sample handling and processing.

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Appendix I - Example CAT

3 CAFFEINE ASSESSMENT TOOL

29 – 36 WEEKS

This study is about caffeine intake during pregnancy. The first part of the questionnaire is in 2 sections to cover your intake of certain foods and drinks over:

Weeks 29-36 of this pregnancy (i.e. from the 29th week of your pregnancy until your 36th week of pregnancy inclusive).

The second part of this questionnaire asks you about your alcohol intake, smoking habits, drug use, symptoms of pregnancy, work and physical activity.

Please answer every question. If you are uncertain about how to answer a question then do the best you can. It may seem quite long but it should be easy to work your way through. If you have any problems or queries while completing this questionnaire, please feel free to discuss them with the researcher.

Once you have completed this questionnaire, please return it to the researcher. Your answers will be treated as strictly confidential and will be used only for medical research.

Please tick or fill in the details required on each page as appropriate.

SECTIONS A: YOUR INTAKE OF FOODS AND DRINKS

The next few pages contain a list of foods and drinks. Only foods and drinks that we are interested in are included.

If you are unsure about a particular brand or amount, please put a '?' in the appropriate space in the table.

For the questions on *coffee, tea, hot chocolate, cola, and energy drinks* you need to do the following:

- Write brand details in the space provided
- Tick the 'decaf' box if you had a decaffeinated drink
- Write the **number** of each type of drink you had under the right heading – either per day **or** per week **or** per month.
- Tick the last box 'None' if you don't drink the item.

1.1 EXAMPLE

2,3,4. Weeks 29-36 of this pregnancy *

DRINK	*BRAND*	Decaf (√)	How many cups/mugs?			None (√)
			Per day	Per week	Per month	
COFFEE						
Instant (home)	e.g. Nescafe Gold Blend			4		
	e.g. Maxwell House	√	2			

**** Please note: the top of each page states which period of pregnancy each table is referring to.***

1.1.1.1 1.1.1.2DRINK	1.1.1.2.1.1.1.1.1 1.1.1.2.1.1.1.1.2 BRAND	Decaf (√)	1.1.1.3How many cups/mugs? 1.1.1.3.1.1.1.1.1 1.1.1.3.1.1.1.1.2 Per day	 1.1.1.3.1.1.1.1.3 1.1.1.3.1.1.1.1.4 Per week
1.1.1.3.1.1.1.1.5 COFFEE				
Instant (home)			1	2
			1	2
Instant (away) e.g. at work, a friend's house, coffee shop			1	2
			1	2
Filter/cafetiere (home)			1	2
			1	2
Filter/cafetiere (away) e.g. at work, a friends house			1	2
			1	2
Filter/cafetiere (bought) e.g. coffee shop, Starbucks			1	2
			1	2
Espresso shot (home/work)			1	2
Espresso sachet (home/work) e.g. latte, mochaccino, cappuccino etc.			1	2
Espresso shot (bought)			1	2
Espresso (bought) e.g. latte, mochaccino, cappuccino etc.			1	2
1.1.1.3.1.1.1.1.6 HOT CHOCOLATE				
Hot chocolate (home/away/bought)			1	2
			1	2
TEA (including herbal/fruit teas)				
Tea bags (home/away/bought)			1	2
			1	2
			1	2
			1	2
Tea leaves (home/away/bought)			1	2
			1	2
Iced tea (bottle)			1	2
Instant tea (home/away)			1	2

MISCELLANEOUS DRINKS				
Cola (not Dr Pepper)			1	2
			1	2
			1	2
Energy drinks			1	2
			1	2

Continued overleaf

2.3.2 Weeks 29-36 of this pregnancy

Please put a (√) on every line

1.1.1.4 **1.1.1.4.1.1.1.1.1**	**1.1.1.4.1.1.1.1.2** **1.1.1.4.1.1.1.1.3** **Never**	Less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once per day	2-3 per day	4-5 per day	6+ per day
OTHER SOFT DRINKS										
Dr Pepper Can	0	1	2	3	4	5	6	7	8	9
Bottle	0	1	2	3	4	5	6	7	8	9
Glass	0	1	2	3	4	5	6	7	8	9
Dr Pepper Diet Can	0	1	2	3	4	5	6	7	8	9
Bottle	0	1	2	3	4	5	6	7	8	9
Glass	0	1	2	3	4	5	6	7	8	9
Ginzinga Can	0	1	2	3	4	5	6	7	8	9
Iron-Bru Can	0	1	2	3	4	5	6	7	8	9
Bottle	0	1	2	3	4	5	6	7	8	9
Glass	0	1	2	3	4	5	6	7	8	9
Iron-Bru Diet Can	0	1	2	3	4	5	6	7	8	9
Bottle	0	1	2	3	4	5	6	7	8	9
Glass	0	1	2	3	4	5	6	7	8	9
Lucozade Bottle	0	1	2	3	4	5	6	7	8	9
Glass	0	1	2	3	4	5	6	7	8	9
Lucozade NRG	0	1	2	3	4	5	6	7	8	9
Mountain Dew Can	0	1	2	3	4	5	6	7	8	9
Mountain Dew Diet Can	0	1	2	3	4	5	6	7	8	9
Schizan Can	0	1	2	3	4	5	6	7	8	9
Schizan Diet Can	0	1	2	3	4	5	6	7	8	9
ALCOHOL										
Wine (glass)	0	1	2	3	4	5	6	7	8	9
Beer, Lager, Stout (half pint)	0	1	2	3	4	5	6	7	8	9
Cider (half pint)	0	1	2	3	4	5	6	7	8	9
Port, Sherry, Liqueurs (glass)	0	1	2	3	4	5	6	7	8	9
Vodka Kick (VK), WKD	0	1	2	3	4	5	6	7	8	9

Continued overleaf

Spirits, e.g. Whisky, Gin, Vodka (single measure)	0	1	2	3	4	5	6	7	8	9
CHOCOLATE (One portion e.g. one bar, one packet of sweets, one biscuit, one piece of chocolate cake)										
Energy bar e.g. Boost	0	1	2	3	4	5	6	7	8	9
Milk chocolate *coated* bars e.g. Twix, Mars, Rolo, Kit-Kat — Full size bars	0	1	2	3	4	5	6	7	8	9
Milk chocolate *coated* bars e.g. Twix, Mars, Rolo, Kit-Kat — Mini bars	0	1	2	3	4	5	6	7	8	9

Please put a (√) on every line

1.1.1.5 **1.1.1.5.1.1.1.1.1**	**1.1.1.5.1.1.1.1.2** **1.1.1.5.1.1.1.1.3** **Never**	Less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once per day	2-3 per day	4-5 per day	6+ per day
CHOCOLATE **(One portion e.g. one bar, one packet of sweets, one biscuit, one piece of chocolate cake)**										
Milk chocolate bars e.g. Dairy milk	Full size bars	0	1	2	3	4	5	6	7	8	9
	Mini bars	0	1	2	3	4	5	6	7	8	9
Plain chocolate *coated* bars e.g. Bounty	Full size bars	0	1	2	3	4	5	6	7	8	9
	Mini bars	0	1	2	3	4	5	6	7	8	9
Plain chocolate bars e.g. Bournville		0	1	2	3	4	5	6	7	8	9
Chocolate coated biscuits e.g. Viscount, Hob Nobs		0	1	2	3	4	5	6	7	8	9
Chocolate brownie, cake		0	1	2	3	4	5	6	7	8	9
Chocolate/coffee yoghurt		0	1	2	3	4	5	6	7	8	9
Chocolate croissant		0	1	2	3	4	5	6	7	8	9
Chocolate mousse/ice-cream		0	1	2	3	4	5	6	7	8	9
Chocolate milk (shake) glass		0	1	2	3	4	5	6	7	8	9
OTHER FOODS										
Barbecued foods (one portion)		0	1	2	3	4	5	6	7	8	9
Grapefruit (including juice)		0	1	2	3	4	5	6	7	8	9
Cruciferous vegetables *		0	1	2	3	4	5	6	7	8	9
Oily fish †		0	1	2	3	4	5	6	7	8	9

* Cruciferous vegetables = broccoli, brussel sprouts, cabbage, cauliflower, parsnips, turnips, watercress, rutabagas, daikon radish, kale, kohlrabi, mustard greens.

† Oily fish = salmon, tuna (fresh, not tinned), herring, kipper, mackerel, marlin, pilchards, sprats, swordfish.

Continued overleaf

Study no:	DOB:	Initials:

CONFIDENTIAL

The CARE Study

Background Questionnaire

This study is about caffeine intake during pregnancy. This questionnaire is designed to find out more about you. There are questions about your weight, employment, education, current pregnancy, family history, illness, dental history and diet. Please answer every question.

Your answers will be treated as strictly confidential and will be used only for medical research.

2 SECTION A INFORMATION ABOUT YOU

Please tick the most appropriate answers or fill out the details required as appropriate on both sides of each page.

D D / M M / Y Y Y Y

1. What is your date of birth? **.... ....**

Feet Inches cm Don't know

2. What is your height? **.....** **.....** **or ..... or .....**

3. What was your weight just before you became pregnant?

2.1 Stones Pounds Kilogrammes Don't know

..... **or** **.....** **or** ....

4. How much did you weigh at birth?

Grams Pounds Ounces Don't know

..... **or** **.....** **.....** **or** **.....**

5. Approximately how many weeks pregnant was your mother when you were born?

..... Don't know **.....**

6. How many weeks pregnant are you? **.....**

7. What is your ethnic origin? (Please tick against **one option only**)

White	□1	Pakistani	□6
Bangladeshi	□2	Black – Carribbean	□7
Indian	□3	Black – other	□8
Chinese	□4	Japanese	□9

EMPLOYMENT:

8. Have you ever had a paid job? Yes □1 No □2

9. Has your partner ever had a paid job?

Yes □1 No □2 Not applicable □3

*(if **both** you and your partner never had a paid job, please go to question 15)*

Please complete questions 10 to 13 for you and/or your partner:

10. Are/were you or your partner…

	You	Your partner
An employee?	□1	□2
Self-employed with employees?	□3	□4
Self-employed/freelance without employees?	□6	□5

(go to question 13 if you or your partner are without employees)

11. *For employees:* indicate below how many people work (worked) for your or your partner's employer.

For self-employed: indicate below how many people you and/or your partner employ (employed). Go to question 12 when you have completed this question.

	Your partner	You
1 to 24	□1	□1
25 or more	□3	□4

13. Please tick one box for you and one for your partner (if applicable) to show which **best** describes the sort of work you and/or your partner do/does. (If you/your partner are **not working now**, please tick a box to show what you did in your last job).

	You	Your partner
Modern professional occupations: *such as:* teacher, nurse, physiotherapist, social worker, welfare officer, artist, musician, police officer (sergeant or above), software designer	□1	□2
Clerical and intermediate occupations: *such as:* secretary, personal assistant, clerical worker, office clerk, call centre agent, nursing auxiliary, nursery nurse	□3	□4
Senior managers or administrators: (usually responsible for planning, organising and co-ordinating work and for finance) *such as:* finance manager, chief executive	□5	□6
Technical and craft occupations: *such as:* motor mechanic, fitter, inspector, plumber, printer, tool maker, electrician, gardener, train driver	□7	□8
Semi-routine manual and service occupations: *such as:* postal worker, machine operative, security guard, caretaker, farm worker, catering assistant, receptionist, sales assistant	□9	□10
Routine manual and service occupations: *such as:* HGV driver, van driver, cleaner, porter, packer, sewing machinist, messenger, labourer, waiter/waitress, bar staff	□11	□12
Middle or junior managers: *such as:* office manager, retail manager, bank manager, restaurant manager, warehouse manager, publican	□13	□14

14. How would you describe **your** work? If you are not currently working please describe your most recent job.

Mostly sitting ☐1
Mostly standing ☐2
Mostly moving about ☐3
Mixture of the above ☐4

EDUCATION:

15. How old were you when you finished your full time education? ☐☐ years old.

16. Do you have any of the following qualifications? *Tick all applicable.*

2.3.1 **CSE** ☐**1**

GCE "O" Level ☐2

GCSE ☐3

"A" level, Highers ☐4

Teaching diploma, HNC ☐5

City & Guilds ☐6

Degree ☐7

Other ☐8 please describe: _____

Not applicable ☐9 _____

2.3.1.1 SECTION B GENERAL HEALTH

17. Has a doctor ever told you that you have, or have had any of the following conditions? Please tick all that apply and if known, give the year at which each condition was first diagnosed.

	Yes	No	Year of diagnosis
Hypertension	□1	□2	.....
Gestational hypertension	□1	□2	.....
Gestational diabetes	□1	□2	.....
Insulin dependent diabetes	□1	□2	.....
Non-insulin dependent diabetes	□1	□2	.....
SLE (Systemic Lupus Erythematosus)	□1	□2	.....
Anorexia Nervosa	□1	□2	.....
Bulimia Nervosa	□1	□2	.....
Any other long-term illness	□1	□2	.....

If yes, please write the type of illness here: _____

20. a) What method of contraception did you last use?

Combined Pill □1 IUD □2 Hormonal IUD □3
Cutaneous implant □4 Subcutaneous injection □5 Condom □6
Diaphragm □7 Interrupted coitus □8 Rhythm method □9
Mini Pill □10 Oral contraceptives □11
Other □12 please describe _____
None □13

b) When did you stop using contraception?

M M / Y Y Y Y
....

21. Is this your first pregnancy? Yes □ 1 No □2

If no, please record details of previous pregnancies in the table below:

Pregnancy no.	**Week of pregnancy e.g 40th**	**Delivery Date DD/MM/YYYY**	**Sex of baby**	**Birth weight** *(please state in grams or in pounds & ounces)*		
				Grams	**Pounds**	**Ounces**
1						
2						
3						
4						
5						
6						
7						
8						

22. Have you had any miscarriages? Yes □1 No □2 111

If yes, please state how many miscarriages you have had and the stage of pregnancy it occurred:

Number of miscarriages: **. . . . . .**

2.3.1.1.1 Miscarriage 1: Weeks gestation **. . . . . ..**

Miscarriage 2: Weeks gestation **. . . . . .**

2.3.1.1.2 Miscarriage 3: Weeks gestation **. . . . .**

Miscarriage 4: Weeks gestation **. . . . . .**

Miscarriage 5: Weeks gestation **. . . . . .**

2.3.1.2 SECTION D CURRENT PREGNANCY

23 a) Please state the day of the first day of bleeding of your last menstrual period:

D D / M M / Y Y Y Y

. . . .

b) How sure are you of this date?

Sure □1 Fairly sure □2 Not sure □3

c) Please state the average number of days between the first day of each period (monthly cycle):

. . . . . .

d) Please state your estimated due date:

D D / M M Y Y Y Y

. . . . .

SECTION E FAMILY HISTORY

Your mother:

24. Were any of your sisters and/or brothers born before 37 weeks of pregnancy or at least 3 weeks early? *Include any brothers or sisters who were premature and died soon after they were born*.

Yes □1 No □2 No brothers/sisters □3 Don't know □4

25. Did your mother have any miscarriages?

Yes □1 No □2 Don't Know □3

If yes, how many miscarriages did she have? **. . . . . .**

26. Did your mother smoke when she was pregnant with you/your brothers/sisters?

Pregnant with you: Yes □1 No □2 Don't know □3

Pregnant with brothers/sisters: Yes □1 No □2 Don't know □3 Not applicable □4

Your sister(s):

27. Do you have any sisters? Yes □1 No □2

If yes;

a) have any of your sisters ever been pregnant?

Yes □1 No □2

	Yes	No	Don't know
b) had a miscarriage?	□1	□2	□3
c) given birth to a baby before 37 weeks gestation? *(or at least 3 weeks early)*	□1	□2	□3

28. Did your sister smoke when she was pregnant?

Appendix III – Statistical analysis plan

Primary outcome measure
Fetal Growth Restriction (FGR). Based on growth centiles. Binary variable. [note this variable is already adjusted for maternal height, weight, ethnicity, parity, baby's gender, gestation (days)].

Secondary outcome measures

Low absolute birthweight. Binary variable (≤2.5kg / >2.5kg).*

Birthweight should be analysed as incrementals rather than a binary variable, e.g. <2.5 kg, 2.5 kg – 4.99 kg, etc.

Degree of FGR. Growth centile z-score. Continuous variable.
Birthweight. Grams. Continuous variable.

Pre-term labour. Binary variable.*
Gestation (days). Continuous variable.

Late miscarriage. Binary.
Still birth. Binary.
Essential hypertension. Binary.**
Gestational hypertension. Binary.**
Pre-eclampsia. Binary.**

Growth velocity from scans [Leicester only]

*We seek clarification from the Steering Group on the definition of this variable.
Pre-term labour falls into two categories.
Pre-term is defined as labour occurring at 37 weeks plus 6 days and below.
Pre-term labour requiring medical intervention is defined as 33 weeks plus 6 days and below.
**We seek clarification from the Steering Group that we have drawn appropriate distinction between these outcomes, and how they should be identified from the database.
Essential hypertension covers individuals who were hypertensive prior to pregnancy.
Gestational hypertension refers to pregnancy-induced hypertension, without the presence of protein in the urine.

Primary exposures
Caffeine intake. Mean over pregnancy.*

*We seek clarification from the Steering Group on the definition of this variable re: whether this should be mg/day or mg/kg/day.
Both analyses would be useful where possible. However, data on the women's weight during pregnancy may be lacking.

Secondary exposures

Caffeine intake. Mean during development phase.*
Caffeine intake. Mean during growth phase.*

*We seek clarification from the Steering Group on the definition of this variable re: the gestation that each represents.
It is preferable to look at exposure during the different trimesters of pregnancy – i.e. weeks 1-12, 13-28 and 29-delivery.
We also ask the Steering Group to specifically consider whether there are any other exposures for the Statistician's Group to consider.
Exposure to coffee, tea, coke etc. rather than caffeine content
Caffeine metabolites, caffeine in saliva.
It will be useful to analyse exposure in relation to half-life for caffeine metabolism.

Sample size
1500 women will be recruited both in Leeds and Leicester, with about 10% having Fetal Growth Restricted (FGR) infants. This number will give 80% power to detect a 13% increase in odds of having a FGR infant with an increase in caffeine intake equivalent to one cup of average instant coffee per day for a 60kg woman (i.e. an odds ratio of 2.0 comparing non-caffeine consumers with those consuming 7.2 mg/kg/day – equivalent to 5½ cups of average instant coffee per day). Alternatively, on the basis that approximately 10% of the women studied will fall into the 'high' caffeine exposure category, 1500 pregnancies would be required in order to have 80% power, at the 5% level of statistical significance, to detect a two-fold increase in the proportion of FGR babies. This would equate to an overall average birthweight reduction of 10%. Also, assuming that the mean metabolic ratio for CYP1A2 during early pregnancy is 6.8 (SD=2.3); during late pregnancy it is 10.4 (4.5) and that this follows a normal distribution, we would have good power to detect modest influences of phenotypes on outcomes (eg. 90% power to detect an odds ratio for having a low birthweight baby of 1.1 for every 1 point change in metabolic ratio for CYP1A2 after pregnancy).

Combining the samples from the two study sites will give a final sample size of 3,000 women. With double the sample size we will be able to detect a difference between low birth weight and normal groups of ¾ cup of average instant coffee (compared to 1 cup with half the sample at each site).

Response rates

- We agreed that the final report should contain information about the response rates, exclusions, and completion rates, where possible.
- We agreed that this would be useful to present separately for each site, as well as combined.
- We agreed that the following table would be useful:

	Leeds	Leicester	Combined
Number of women approached			
Number of women giving consent to participation			
Number eligible for inclusion			
Number excluded			
Reason for exclusion: (taken from database and protocol) Maternal age Social reasons Geography Multiple pregnancy Interpreter required Assisted conception Miscarriage Out of dates Medical Psychiatric Bio-Hazard Asylum seekers Substance abuse Previous FGR infant Previously included in study Previous pre-eclampsia Previous pre-term labour Previous gestational diabetes Previous hypertension Current hypertension Other			
Number of women returning completed questionnaires: 1-4 weeks 5-12 weeks 13-20 weeks 21-28 weeks 29-40 weeks			
Number of women eligible for caffeine challenge			
Number of women completing caffeine challenge			
Number of women withdrawn from study: Termination Still birth Neo-natal death			

Miscarriage			
Number of live births			
Number of FGR infants			
Number of infants ≤2.5kg			

Descriptive statistics

	Leeds		Leicester		Combined	
	Fetal Growth Restriction (≤10th centile)					
	Yes	No	Yes	No	Yes	No
Mother's age (yrs) <25 25-29 30-34 35+						
Mother's pre-pregnant weight (kg) <55 55-64 65-74 75-84 85+						
Mother's height (cm) <155 155-164 165-174 175+						
Mother's body mass index (kg/m^2) <20 20-24 25-29 30+						
Caffeine intake during development stage <100 100-199 200-299 300+						
Caffeine intake during growth stage <100 100-199 200-299 300+						
Half-life of caffeine clearance (hours) <5						

5-10 10-20 20+						
Alcohol intake (ml/day) None <20 20-50 50-100 100+						
Current self-reported smoker at start of pregnancy						
Cigarettes per day amongst current smokers at start of pregnancy						
Current nicotine replacement user						
Cotinine status (ng/ml)* Non-smoker (<1ng/ml) Passive smoker (1-15ng/ml) Current smoker (> 15ng/ml)						
Ex-smoker						
Self-reported exposure to others' regular smoking						
Currently in paid work						
Socio-economic classification managerial and professional intermediate occupation small account workers lower and supervisory routine and semi-routine						
Highest education qualification None O-level A-level Degree level or above						
Parity 0 1 2+						
Previous FGR infant						
Diabetes No Gestational Pregestational						
Hypertension No						

Gestational Essential Pre-eclampsia						
Child's gender Male Female						
Gestation (wks) <38 38-40 41+						
Birth weight (g) <2500 2500-2999 3000-3499 3500-3999 4000+						

Modelling

We agreed:

- Logistic regression will be used for binary outcomes adjusting for confounders.
- Linear regression will be used for continuous outcomes adjusting for confounders.
- To include unadjusted analyses in a secondary, descriptive capacity.
- To include centre (Leeds / Leicester) as a stratifying variable within the analysis, using Stata's "svy" commands. This recognises the separate sampling procedures used.

- We agreed to leave outliers and influential observations in the model unless values are clearly incorrect. Sensitivity analysis will be used to investigate the robustness of the model to individual observations.

- We agreed that the Statisticians' Group would decide on the form that caffeine variables would be in the modelling on the basis of the data.

This decision will be made on the basis of linearising the association, giving best model fit, residual and added variable plots, simplicity of use, presentation, and transparency. Consideration will be given to using the untransformed continuous variable, log-transformation, fractional polynomials, and categorisation. Our current expectation is that log-transformation will be beneficial, but that fractional polynomials, whilst useful for informing the decision, would not aid interpretation. Categorisation into several categories should provide a simple way of presenting data, though may lead to loss of information and power, especially when assessing effect modification. Categorisation would be used if the form of the relationship could not be linearised.

Standard method for assessing model fit and validity (e.g. residual plots) would be used for the linear regression. Our expectation is that no transformation of outcomes will be necessary.

Potential confounders

For all outcomes: maternal age, smoking (yes/no from cotinine), how much smoked (amongst current smokers), partner who smokes (yes/no), alcohol intake over time period, total energy intake.*

In addition, for outcomes other than FGR, maternal height, weight, ethnicity, parity, baby's gender, gestation (days) would be considered as potential confounders. Care will be taken to consider possible causal pathways so that over-adjustment is avoided.

The Statistician's Group discussed inclusion of current employment status, socio-economic classification, and highest educational qualification as potential confounders. Whilst social class may well be associated with both birthweight and caffeine intake, care will be taken to consider possible causal pathways so that over-adjustment is avoided. Analyses will be repeated with and without adjustment for these variables to assess robustness of the model to the decision.

*Advice is sought from the Steering Group on drugs that may be associated with FGR for Statisticians to consider including in models.
It will be necessary to retrospectively look at information what drugs the women were taking during pregnancy.

Effect modification
Interaction terms will be used to formally test the question "Does phenotype modify any effect of caffeine?". Phenotype will be measured by the half-life of caffeine clearance from saliva. Main effects will be included in the model as well as the caffeine by half-life interaction term, though interpretation of the main effects in the presence of interaction will only be done with caution.

- We agreed that the Statisticians' Group would decide on the form that half-life would be in the modelling on the basis of the data.

This decision will be made on the basis of linearising the association, giving best model fit, residual and added variable plots, simplicity of use, presentation, and transparency. Consideration will be given to using the untransformed continuous variable, log-transformation, fractional polynomials, and categorisation. Our current expectation is that log-transformation will be beneficial, but that fractional polynomials, whilst useful for informing the decision, would not aid interpretation. Categorisation into several categories should provide a simple way of presenting data, though may lead to loss of information and power, especially when assessing effect modification. Categorisation would be used if the form of the relationship could not be linearised.

We discussed the dangers of extreme values of caffeine intake or half-life having undue influence on the interaction of two continuous variables, and the possible beneficial effects of the presumed log-transformation.

- We agreed to test the sensitivity of our model, and in particular the estimate of interaction, to categorising caffeine intake and half-life.

The Statisticians' Group noted that the caffeine challenge was only performed on women who were consuming caffeine. This may mean that non-consumers are excluded from the analysis of effect modification by phenotype.*

*We draw the Steering Group's attention to this and seek clarification if this was the intended approach.
Although not all women completed a caffeine challenge, the challenge was performed by women with a range of caffeine intakes.

Exclusions and Sensitivity analyses
Anyone previously entered into the study will be excluded to ensure independent observations.

As agreed at the previous Statisticians' Group, the main analysis will include all women recruited at each site.

Additional sensitivity analyses will be performed as follows:

1. *Sensitivity analysis 1*: exclude high risk women (previous FGR infant, previous pre-eclampsia, previous pre-term labour, previous gestational diabetes (IDDM), previous gestational hypertension, current gestational hypertension with or without medication, women on medication for inflammatory diseases.
2. *Sensitivity analysis 2*: primips only.

The purpose of these sensitivity analyses will be to investigate the influence of the inclusion and exclusion criteria on the overall conclusions. To assist with this comparison, results will be stratified by site and criteria as follows:

	Leeds	Leicester
Primips		
Multips - lower risk		
- higher risk		

Presentation
- It was agreed that, in terms of presentation, use of categories was clearest.

- It was agreed that, to illustrate the effect modification, presenting the effect (if any) of caffeine by subgroups was clearest.