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Research Article

Metabolome Profiling of Normotensive and Hypertensive Rats during the Early Stages of Pregnancy

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Abstract

Hypertensive disorders during pregnancy are a leading cause of maternal and fetal mortality worldwide. During a first pregnancy, some women may develop gestational hypertension, representing a major risk to the mother and child especially when associated with preeclampsia. This pathological condition arises from a defect in placentation that is the starting point of hypertension in pregnancy. The impact of hypertension and/or pregnancy on the metabolome of rats was assessed by 1H-NMR spectroscopy. This approach revealed specific metabolic patterns in urine, plasma and tissue samples associated with hypertension and/or pregnancy. In particular, alterations in the contents of fatty acids, amino acids and glycoprotein's in plasma samples and tissue extracts were indicative of the development of a "metabolic syndrome". Also, changes in urinary creatinine already reported by other authors as potential biomarkers of an alteration in glomerular filtration due to hypertension were confirmed in our study. Finally, changes in sarcosine were also noticed, which could be seen as indicative of the invasion of cytotrophoblast during the development of the placenta. These results support the use of 1H-NMR-based metabonomics as a promising tool to provide new information on metabolic changes associated with the physiopathology of hypertension and pregnancy in rats.

ABBREVIATIONS

SHR: Spontaneously Hypertensive Rats; WKY: Wistar Kyoto; ^{1}H-NMR: Proton Nuclear Magnetic Resonance; TSP: 3-Trimethylsilyl Propionate; SARDH: Sarcosine Dehydrogenase; GNMT: Transferase-N-Methylglycine; DMG: Dimethylglycine; IUGR: Intra-uterine Growth Restriction; SGA: Small for Gestational Age

INTRODUCTION

During a first pregnancy, especially in the case of twins, some women may develop a higher transient gestational hypertension that represents a risk to the mother and fetus, especially when associated with preeclampsia (PE). This condition is related to an abnormal placentation and a poor vascularization of the placenta through the spiral arteries which is compensated by maternal hypertension and a reduction in tissue perfusion (Hladunewich M. *et al.* 2007) [1]. In addition, some authors have proposed a role of systemic inflammation in the development of preeclampsia (Sargent *et al.* 2003] [2]. In fact, during any pregnancy, such an inflammatory response may occur due to apoptotic syncytiotrophoblast debris spreading into the maternal circulation, however to a lesser extent (Borzykowski*et al.* 2006) [3]. Treatments are used to prevent serious complications of hypertension in the mother and sustain as long as possible fetal maturation. However, only induced labor and premature birth can stop the progression of preeclampsia into more severe complications.

Actually there is an increasing interest in Metbolomics application in Maternal-Fetal Medicine. This holistic approach appears to be very sensitive as a fingerprinting tool to predict the outcomes of complicated pregnancies (Kenny *et al.* 2010, Horgan *et al.* 2010, and Odibo *et al.* 2011, Bahado-Singh *et al.* 2013, Austdal *et al.* 2014 and Kuc*et al.* 2014) [4-9]. The usefulness of this new "omic" approach to improve health care in case of perinatal disorders is unfortunately still controversial as opposing data has been published by the up-above mentioned authors. The main reason for this doesn't lie in the robustness of the technology but in the complexity of those diseases. E.g. abnormal conditions

characterizing PE are numerous (hypertension, inflammation and defect in placentation) and are sometimes modified in regard of the stage of the pathology (early or late onset, (Fanoset *al.* 2013) [10], Therefore declustering such kind of pathology by the use of specific animal models still remains relevant in a validation of process and in the framework of drug therapy development and health improvement.

Several animal models of hypertension have been developed to mimic preeclampsia in rats, dogs, rabbits, or sheeps (Padjarnyet *al.* 1999) [11]. In rats, the most frequently studied models are the Dahl Salt Sensitive rats and the spontaneously hypertensive rats (SHR) (Vu *et al.* 2005, Okamoto *et al.* 1963). [12,13] SHR rats develop severe hypertension within a few months after birth and show a high incidence of hypertensive complications (Fedorovaet *al.* 2008) [14]. In that particular strain, it was demonstrated that dams with higher blood pressure take less weight during pregnancy and the body weight of fetuses and newborns is lower (Ahokaset *al.* 1990, Sadoet *al.* 2007) [15,16]. In addition, the rate of births is lower in SHR rats as compared to their corresponding normotensive Wistar Kyoto (WKY) matches. It was also reported that pregnancy had a hypotensor effect on both the WKY and SHR rat strains, with this effect being greater in SHR 20 days after conception (Peraçoliet *al.* 2001) [17]. Blood pressure behavior was dependent on the intervals between measurements and was related to animal stress. According to the same authors, the SHR strain may be considered as a model of hypovolemic chronic maternal hypertension. Another study focused on the genetic and metabolic differences between the SHR and WKY strains (Akira *et al.* 2008) (18]. The authors used a metabonomic approach based on proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy in order to characterize the urinary metabolic profile of young SHR at an early stage of hypertension as compared to WKY. Although the urine ^{1}H-NMR spectra were pretty similar in both strains, major differences were observed in the excretions of citrate, α-ketoglutarate, hippurate, creatine, and creatinine.

Interestingly, similarities between pregnancies in preeclamptic women and SHR rats exist such as a high rate of intrauterine fetal and neonatal deaths as well as newborns with low body weight. Hypertension during pregnancy can adversely affect the fetus, with an inverse correlation occurring between maternal blood pressure and litter size. On the contrary, pregnancy may influence blood pressure. Early signs of a bad association between both conditions are lacking. Thus, the objectives of the present study were to evaluate the impact of hypertension, pregnancy, and the combination of both conditions on the metabolome of hypertensive SHR rats and their corresponding normotensive WKY counterparts.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the local Ethical Committee for animal care of our Institution (University of Mons, Mons, Belgium). Normotensive Wistar Kyoto (WKY) rats were used as matches of Spontaneously Hypertensive rats (SHR). Female rats aged 8 weeks at start of the pre-test period and 11 weeks at the time of mating were used. Pre-test mean body weights were 204±5g and 195±6g for WKY and SHR rats, respectively. Animals received daily 50g of food (RN-01-20K12 Carfil Quality) and water ad libitum. Ambient temperature was kept at about 22°C in the animal room, with the light on from 7:00 am to 7:00 pm.

Metabonomic study

Two groups of rats were used: the first group included six SHR rats and the second group consisted of six matching normotensive WKY rats. The animals were individually placed in metabolic cages for daily urine collection. Body weight as well as food and water consumptions were also recorded daily to assess animal welfare. During the pre-test period, urines were collected at 24h-intervals for 5 consecutive days in order to observe the metabolic changes between normotensive and hypertensive rats in non-pregnant females. Then, each female was placed in the presence of a breeding male and underwent daily vaginal smears to detect the presence or absence of sperm. The day when the vaginal smear got positive was arbitrarily assigned as study Day 0. Mated females were next individually placed in conventional cages for a 5-Day period corresponding to the time requested for implantation. On the 5th day, rats were again transferred in metabolic cages for 5 consecutive days. At the end of the experiment, the animals were euthanized by decapitation after gas anesthesia. All the experiments were conducted at the same time to avoid environmental bias at the exception of a small time offset due to mating (2 days).

Urine fractions were collected every 24 hours intervals in 50 ml Falcon tubes containing 500 µL of a 1 mM sodium azide solution on refrigerated rack (4°C) from time 0 to 120h. Sodium azide and low temperature were used in combination to minimize the risk of bacterial contamination. Urine samples were centrifuged at 13,000 g for 10 minutes and 5 ml of supernatant was put in a freezer at -80°C. On the day of ^{1}H-NMR analysis, 400 µl of each sample were added to 200 µl of phosphate (0.2 M Na_2HPO_4/0.04 M NaH_2PO4) buffer in an 80/20 (V: V) mixture in H_2O / D_2O. This mixture was next centrifuged at 13,000 g for 10 minutes and 600 µl of the supernatant were mixed with 50 µl of a 1 mM deuterated TSP (sodium 3-trimethylsilyl propionate-2, 2, 3, 3-D4) solution prepared in D_2O. TSP was used as external reference for spectral calibration.

Plasma samples from the SHR rats and WKY were collected after euthanasia in heparinized tubes. The plasma samples were stored at –80°C until NMR analysis. Before NMR analysis, the frozen plasma samples were thawed at room temperature. 200 µL of the plasma was mixed with 400 µL of deuterated phosphate buffer. This mixture was next centrifuged at 13,000 g for 10 minutes and 600 µl of the supernatant were mixed with 50 µl of the above described TSP solution.

At necropsy, implantation sites were removed, freeze-clamped, and immediately stored at -80°. This strategy was preferred to the proper isolation of the placenta or the embryo to avoid any tissue alteration due to a longer duration for necropsy. Before NMR analysis, implantation sites were grounded in a mortar under liquid nitrogen until obtaining a fine powder. Then, 2 ml of perchloric acid 7% were added to the powder which was left at room temperature until complete defrosting. Then, water was added to reach the same volume in all samples and

centrifuged at 10,000 g for 10 minutes. PH of the supernatants was adjusting between 7.1 and 7.5 with 5N KOH. The solution was again centrifuged at 15,000g for 5 minutes and the supernatant was freeze-dried. Then, the powder was collected and suspended in 600µl of deuterated phosphate buffer. Finally, 50 µl of the TSP solution were added.

^{1}H-NMR spectroscopy

550 µl of each prepared urine sample were placed in a 5 mm diameter NMR tube and analyzed by ^{1}H-NMR spectroscopy at 400 MHz on a Bruker Avance spectrometer equipped with an automatic sampler. Solvent suppression of residual water signals was achieved via the NOESYPRESAT pulse sequence, in which the residual water peak was irradiated during the relaxation delay (2.0s) and during the mixing time (0.1 s). ^{1}H-NMR spectra were collected using 64 scans containing 32768 data points at a spectral width of 6410.256 Hz with an acquisition time of 2.55 s.

550 µl of each prepared plasma sample were placed in a 5 mm diameter NMR tube and analyzed by ^{1}H-NMR spectroscopy at 500 MHz on a Bruker Avance spectrometer equipped with an automatic sampler. Proteins contribution to the signal was reduced by the use of CPMG pulse sequence, in which the residual water peak was irradiated during the relaxation delay (1.0s) and during the mixing time (0.1 s). ^{1}H-NMR spectra were collected using 64 scans containing 14998 data points at a spectral width of 10330.578 Hz with an acquisition time of 0.73 s.

550 µl of each prepared acid extract were placed in a 5 mm diameter NMR tube and analyzed by ^{1}H-NMR spectroscopy at 500 MHz on a Bruker Avance spectrometer equipped with an automatic sampler. Solvent suppression of residual water signals was achieved via the NOESYPRESAT pulse sequence, in which the residual water peak was irradiated during the relaxation delay (3.0s) and during the mixing time (0.1 s). ^{1}H-NMR spectra were collected using 128 scans containing 54832 data points at a spectral width of 10330.578 Hz with an acquisition time of 2.65s.

Representative samples were also analyzed by ^{1}H-NMR spectroscopy at 700 MHz on a Bruker Avance II spectrometer equipped with an automatic sampler to enhance metabolite identification. ^{1}H NMR sprectrum was acquired using the NOESYPRESAT experiment using gradient selection, whereby residual HOD signal was suppressed by presaturation for 2.22s during the repetition delay between scans and during the mixing time of 50ms. 16 numbers of scans were collected to produce the final spectrum represented by 32768 data points at 21008.404 Hz spectral width.

Treatment of ^{1}H-NMR spectra and multivariate analysis

Each ^{1}H-NMR spectrum was processed using the MestreNova software (V5.3.0-4536, Mestrelab Research SL). Briefly, this treatment consisted in baseline correction using the "full auto/ whittaker smoother" application and phase correction using first the automated "metabonomics" routine proposed by the software and manually improved when necessary. TSP chemical shift was set to 0 ppm and used as external reference for the assignment of the chemical shifts of other resonances. The spectra were then normalized to the reference with a peak integral arbitrarily set at a value of 100. Finally, the spectra were binned by dividing the spectral window from 0.08 to 10 ppm by 0.04 ppm steps. The area under the curve of the 248 resulting spectral subregions were calculated and the values transferred to a spreadsheet (Excel, Microsoft Office) in which each row represented a chemical shift and each column the corresponding integral values of all sub regions obtained after binning each individual spectrum. The regions of the spectrum corresponding to water (4.5 to 5 ppm) and urea (5.5 to 6 ppm) resonances were excluded. Then the area under the curve of each descriptor (buckets) was divided by the total area under the corresponding spectrum in order to solve possible dilution effects considering urine samples

The data table was next imported in SIMCA-P+ 11.5 (Soft Independent Modeling of Class Analogy, Umetrics, Sweden) for multivariate analysis purposes. After mean-centering of the observations (positioning the origin of the axes at the midpoint of the dataset) without scaling, multivariate analysis was conducted. Principal component analysis (PCA) and Partial least square discriminant analysis (PLS-DA) were carried out to discriminate the metabolic patterns between hypertensive and normotensive rats (pregnant or not). The quality and reliability of the models were described by R^2 and Q^2 parameters. The goodness of fit parameter R^2 represents the explained variation in the data and the goodness of prediction parameter Q^2 uses cross-validation to estimate the predictive ability of the model. In designing metabonomic experiments, selection of a specific method of multivariate analysis (PCA or PLS-DA) is to be guided by the experimental data and objectives. For exploratory studies where the differences between groups of interest are unknown or unpredictable, the choice of the PCA provides a first informative overview on the data structure and relationships between groups. Even when the data structure is predictable, the use of unsupervised methods such as PCA is useful to corroborate this. Ideally, the results of PCA are used to formulate an initial biological inference. The main reason for this is due to the fact that the observed separations in a score plot only occur when the intra-group variability is smaller than the inter-group variability; while the separation of groups by PLS –DA analysis may be coincidental (Worley 2013, Putri*et al.* 2013). [19,20] This leads us to describe the variability of the dataset by PLS-DA analysis instead of PCA only if groups of interest have a cumbersome overlapped distribution in a PCA score plot. The distribution of the variables was displayed on plots (loadings-plots) in order to check what NMR descriptors were responsible for the separation or gathering of the observations. The resonances arising from metabolites were identified from their chemical shifts from TSP and from their respective multiplicity in regards of available databases and from a thorough review of the literature. Differences observed between groups were evaluated using Wilcoxon rank sum tests in R software version 2.15.0 to compare normalized buckets between groups of interest.

RESULTS AND DISCUSSION

In thisstudy, urine samples were collected every 24 hours on refrigerated and automated metabolic cages and in the presence of sodium azide for 5 consecutive days. This experimental procedure that avoids bacterial contamination is based on recommendations by the COMET project for best collection and

preparation of bio fluids samples for NMR-based metabonomics evaluation (Lindon *et al.* 2003) [20]. Both aliphatic and aromatic regions of a ^{1}H-NMR spectrum of urine collected in such conditions from a hypertensive pregnant SHR rat are shown in (Figure 1a). The resonances arising from urine metabolites were identified from their chemical shifts from TSP (set at 0 ppm) and from their respective multiplicity. The scores plot obtained from the PCA of the reduced spectral data (Figure 1b) showed a clear separation between the different groups of animals. Obviously, the discrimination of urine samples along the first principal component direction could be attributed to the pregnant state of the females, while the separation along the second principal component was due to the presence or absence of hypertension. It has to be pointed out that some individuals initially anticipated as pregnant on the basis of a positive smear but finally found not pregnant, were redistributed to the appropriate group before starting the analysis. In order to better characterize the metabolic perturbations allowing such separations, multivariate data analysis (PCA or PLS-DA) was applied pair wise to matching groups.

The results obtained to characterize the contribution of hypertension and pregnancy to this observation is detailed hereafter.

Hypertensive vs normotensive pregnant females

The scores plot resulting from the PCA of the reduced ^{1}H-NMR spectral data (Figure 2a) showed a nice separation between urine samples collected from pregnant hypertensive SHR females as compared to their control matches (pregnant normotensive WKY rats). The corresponding loadings plot (Figure 2b) clearly displays the resonances responsible for the separation of both groups. Based on their chemical shifts and multiplicity, those resonances were attributed to the corresponding urine metabolites. Table 1 gathers the metabolites presenting a lower or higher concentration in the urine samples collected from hypertensive rats. The most important differences could be attributed to lower urine levels of taurine, succinate, α-ketoglutarate, dimethylglycine, creatinine, sugars, amino acids, and N-acetyl-glycoproteins. In the same time, other metabolites were more excreted in case of hypertension: hippurate, allantoin, malonate, glucuronic acid, fumarate, tryptophane and some unindentified peaks reported by (Akira et al. 2008) as fairly variable in male SHR rats.

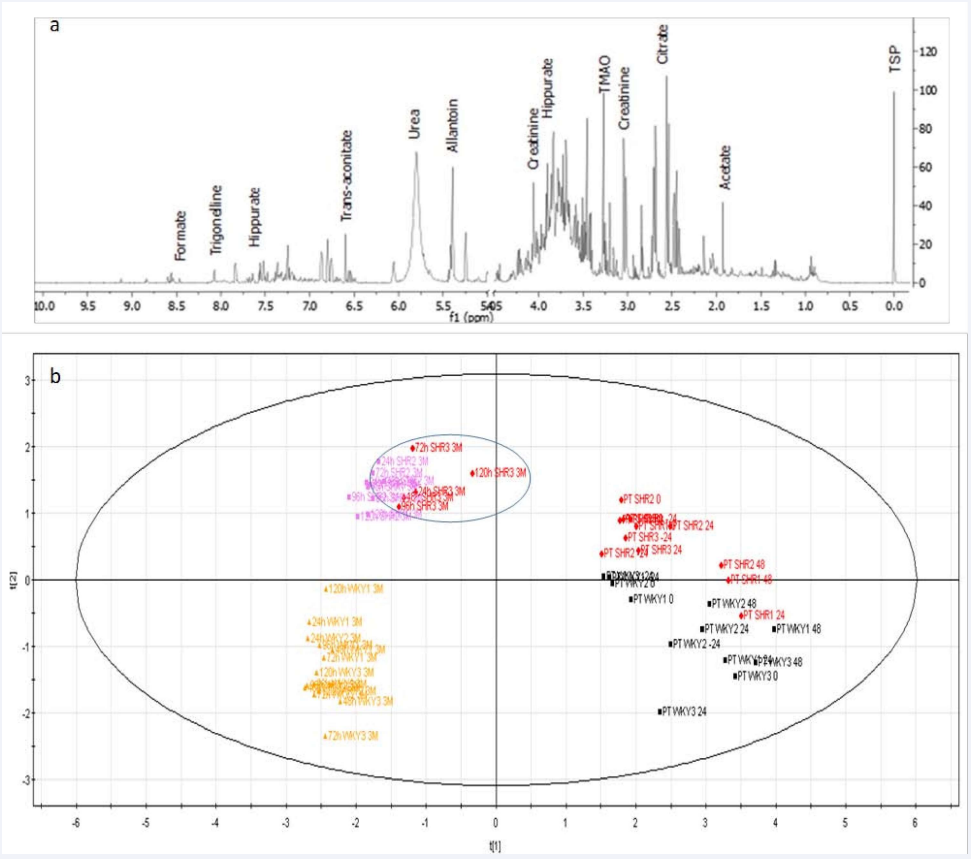

Figure 1 Proton NMR spectrum(700 MHz)of urine of pregnant hypertensive SHR rats (a) and PCA scores plot showing the distribution of the reduced 1H-NMR spectral data of urine samples collected over time from : non-pregnant normotensive WKY rats (black squares), non-pregnant hypertensive SHR rats (red diamonds), pregnant normotensive WKY rats (orange triangles), or pregnant hypertensive SHR rats (pink squares); the model parameters were: R2Xcum = 0.8906, R2X1 = 0.5269, R2X2 = 0.1410, Q2cum = 0.6602 and Hotel ling's T2: 0.95.

(b). Note that samples circled in blue were collected from one female (rat SHR3) presenting a positive smear at the onset of the experiment turned out to be non-pregnant at necropsy.

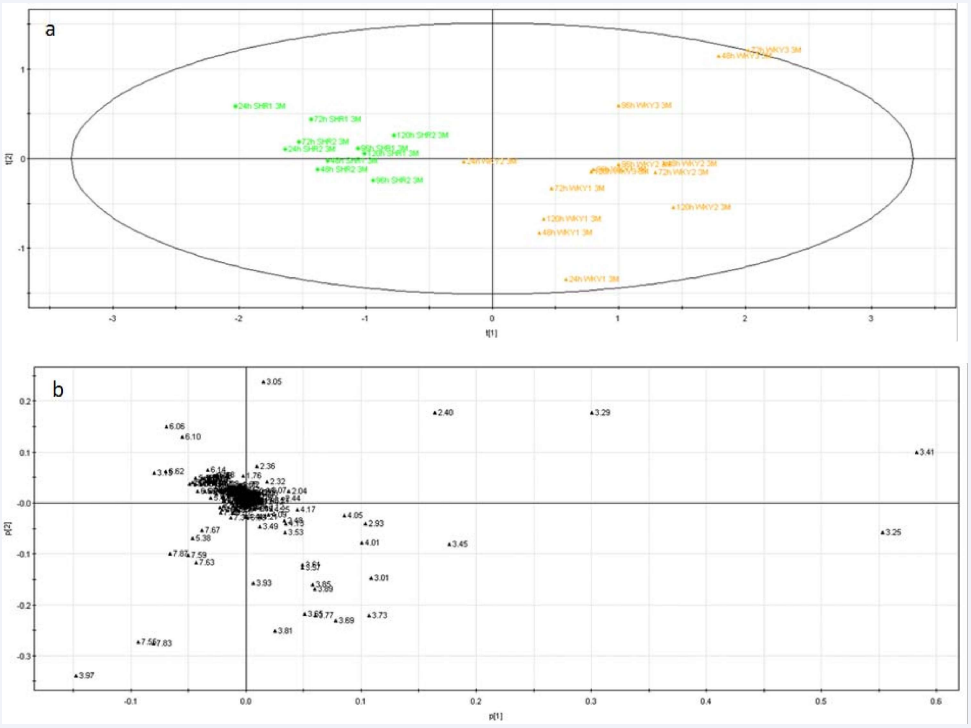

Figure 2 PCAscores plot (a)of 1H-NMR spectral data of urine samples collected over time and showing a clear separation between pregnant hypertensive SHR females (green symbol)as compared to pregnant normotensive WKY rats (orange triangles) and PCA loadings plot (b) showing the spectral resonances responsible for the separation of groups in the scores plot presented in a. Each point corresponds to the median chemical shift of a particular descriptor (spectral subregion after data reduction). The model parameters were: R2Xcum = 0.9331, R2X1 = 0.6496, R2X2 = 0.1338, Q2cum = 0.6831 and Hotelling's T2: 0.95.

Table 1: List of urine metabolites identified as most discriminating between pregnant hypertensive SHR rats and their control matches (pregnant normotensive WKY rats).

Increased level in the pregnant hypertensive SHR rats compared to their control matches	Decreased level in the pregnant hypertensive SHR rats compared to their control matches
Hippurate	Taurine **
Allantoin	Succinate**
Malonate**	α-ketoglutarate
Glucuronic acid **	DMG**
Fumarate **	Fructose (β-pyranose) **
Tryptophane **	Creatinine
Unknown (6.62 / 6.66 / 6.70, multiplet) **	Glucose**
Unknown (6.06 ppm, singulet) **	Amino-acids**
Unknown (7.02 ppm, multiplet) **	Fructose (β-furanose)
Unknown (6.26 ppm, multiplet) **	N-acetyl-glycoproteins
Unknown (6.74 ppm, multiplet) **	

* $P \leq 0.05$, ** $P \leq 0.01$ in the exact Wilcoxon rank-sum test (no adjustment for multiple testing)
Increased or decreased excretions were assessed in regard of both multivariate analysis and statistical comparison of normalized buckets attributed to a specific metabolite.

Hypertensive versus normotensive non-pregnant females

The scores plot obtained from the PLS-DA of the reduced ^{1}H-NMR spectral data of urine samples collected over time showed a clear separation between non-pregnant hypertensive SHR and their non-pregnant normotensive WKY matches (Figure 3a). At necropsy, one female presenting a positive smear at the onset of the experiment turned out to be non-pregnant (rat SHR3 circled in blue in Figure 3a). Although quite isolated on the scores plot, it remained nevertheless matched with the group of non-pregnant SHR (circled in red), demonstrating consistency in the results. The corresponding loadings plot (Figure 3b) showed the resonances responsible for the separation of both groups. Based on their chemical shifts and multiplicity, those resonances were attributed to urine metabolites which are listed in Table 2. When urines from non-pregnant hypertensive SHR rats were compared to their control matches (non-pregnant normotensive WKY rats),

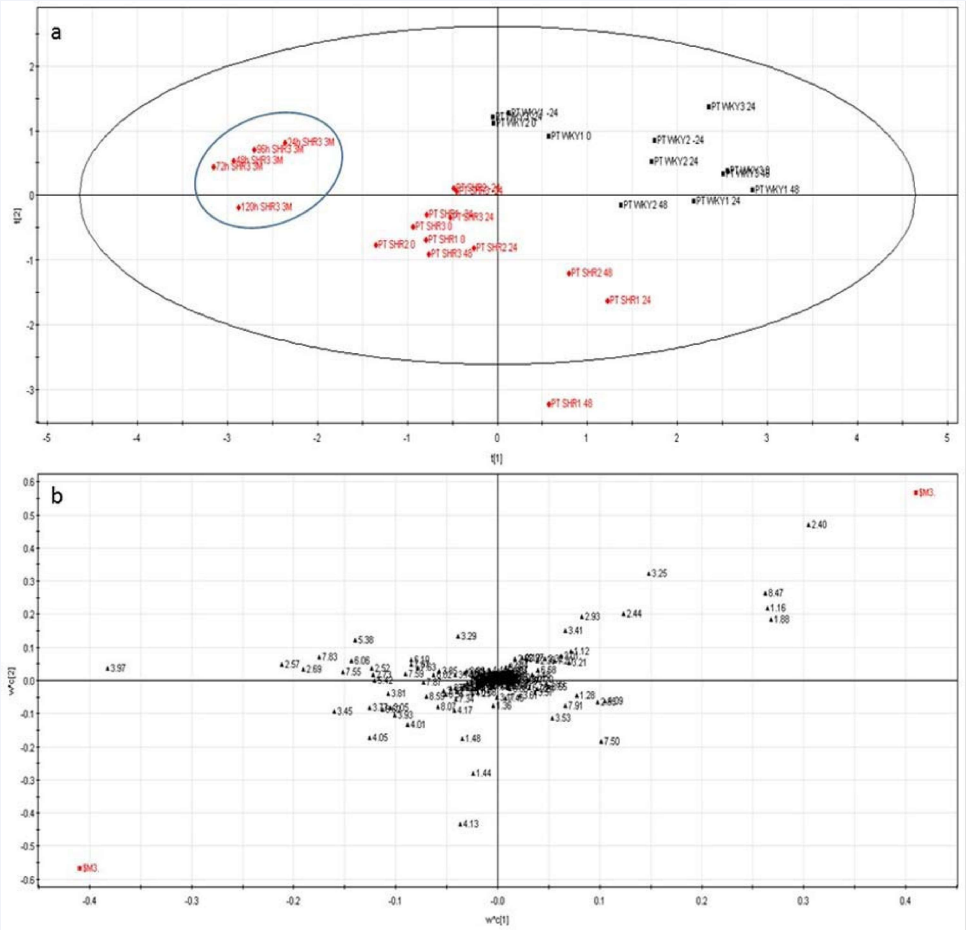

Figure 3 PLS-DA scores plot (a) of the reduced 1H-NMR spectral data of urine samples collected over time from non-pregnant hypertensive SHR rats (red diamonds)and their non-pregnant normotensive WKY matches (black squares) and PLS-DA loadings plot (b) showing the spectral resonances responsible for the separation of groups in the scores plot presented in a. Each point corresponds to the median chemical shift of a particular descriptor (spectral subregion after data reduction). Note that samples circled in blue were collected from one female (rat SHR3) presenting a positive smear at the onset of the experiment turned out to be non-pregnant at necropsy. The model parameters were: R2Xcum = 0.8109, R2X1 = 0.4017, R2X2 = 0.1211, Q2cum = 0.6478 and Hotelling's T2: 0.95.

Table 2: List of urine metabolites identified as most discriminating between non-pregnant hypertensive SHR rats and their control matches (non-pregnant normotensive WKY rats).

Increased level in the non-pregnant hypertensive SHR rats	Decreased level in the non-pregnant hypertensive SHR rats
Hippurate*	Taurine**
Alanine	Succinate**
Creatinine*	Formate**
Acetoacetate	Propylene glycol**
Citrate*	Citrulline
Dimethylamine *	Carnitine **
Allantoin	DMG
Amino acids	Choline*
Creatine**	β-hydroxyisobutyrate **
Trans - Aconitate **	Unknown (1.12 ppm, quadruplet)
Guanine **	
Trigonelline**	
Unknown (4.17 ppm, singulet)*	
Unknown (6.06 ppm, singulet)*	

* $P \leq 0.05$, ** $P \leq 0.01$ in the exact Wilcoxon rank-sum test (no adjustment for multiple testing)
Increased or decreased excretions were assessed in regard of both multivariate analysis and statistical comparison of normalized buckets attributed to a specific metabolite.

the most prominent changes were lower urine levels of taurine, succinate, citrulline, carnitine, together with increased urinary excretions of creatine, trans-aconitate, guanine and trigonel line. The results obtained to characterize the contribution of pregnancy to the metabolic alterations observed in the collected urines are summarized in Table 3 and resulted from the comparisons of non pregnant vs pregnant rats (both SHR and WKY; (Figure 4 and 5), respectively).

At the end of the experiment, animals were euthanized, plasma samples were collected and implantation sites were excised and freeze-clamped for further acidic extraction. Their metabolic fingerprints were both manually analyzed according to the "peak to peak" method, meaning that ^{1}H-NMR spectra were systematically compared from two different conditions because it appears that the goodness of prediction of their multivariate analysis was too low ($R^2 - Q^2 > 0.3$). Spectral differences in peaks intensities were considered as increased / decreased level of the corresponding metabolites when comparing both conditions. The method is detailed in the figure 6.

Implantation sites excised from hypertensive SHR females expressed a metabolic signature clearly different from those removed from normotensive WKY. The main changes in case of hypertension are listed in Table 4 and correspond i.e. to decreased levels of guanidoacetate, myo-inositol, serine, proline,

Table 3: List of urine metabolites identified as most discriminating between non-pregnant and pregnant rats.

Increased level in the pregnant normotensive WKY rats compared to non-pregnant normotensive WKY rats	**Decreased level in the pregnant normotensive WKY rats compared to non-pregnant normotensive WKY rats**
Acetoacetate**	Alanine**
Glucose**	α-hydroxyisobutyrate**
α-ketoglutarate**	α-hydroxybutyrate**
Arginine**	Cis-aconitate
Citrate**	Creatinine**
Choline	Formate**
DMG	Glycerol**
Hippurate**	Glycine
Histidine**	Succinate
Methionine** Myo-inositol**	Tyrosine Unknown (7.50 ppm, multiplet)
Sarcosine**	
Trigonelline**	
Unknown (3.89 ppm, multiplet)**	
Unknown (6.06 ppm, singulet)**	
Unknown (6.58 ppm, singulet)**	
Unknown (8.03 ppm, multiplet) **	
Increased level in the pregnant hypertensive SHR rats compared to non-pregnant hypertensive SHR rats	**Decreased level in the pregnant hypertensive SHR rats compared to non-pregnant hypertensive SHR rats**
Allantoin**	Alanine**
Betaine*	α-hydroxyisobutyrate**
Citrate**	α-hydroxybutyrate**
Creatinine**	Formate**
Fumarate**	Fructose**
Glucose	Glycerol**
Hippurate**	Unknown (4.17 ppm, multiplet) **
Malonate**	Unknown (7.50 ppm, multiplet) **
Methionine**	
Sarcosine**	
Taurine**	
4-aminohippurate**	
Unknown (6.06 ppm, singulet)	

* $P \leq 0.05$, ** $P \leq 0.01$ in the exact Wilcoxon rank-sum test (no adjustment for multiple testing). Increased or decreased excretions were assessed in regard of both multivariate analysis and statistical comparison of normalized buckets attributed to a specific metabolite.

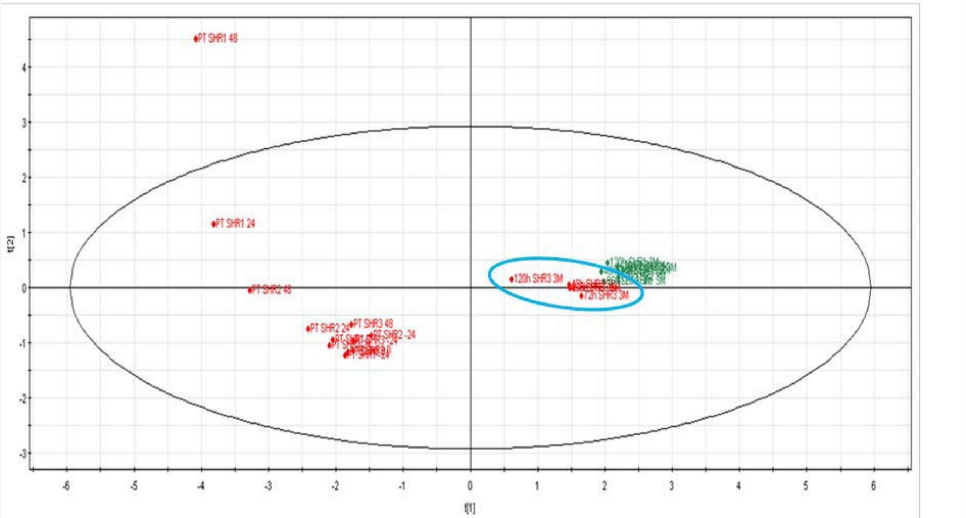

Figure 4 PCAscores plot of 1H-NMR spectral data of urine samples collected over time and showing a clear separation between pregnant hypertensive SHR rats (green symbol)as compared to non pregnant SHR females (red symbol). Note that samples circled in blue were collected from one female (rat SHR3) presenting a positive smear at the onset of the experiment turned out to be non-pregnant at necropsy. The model parameters were: R2Xcum = 0.8027, R2X1 = 0.6674, R2X2 = 0.0891, Q2cum = 0.5961 and Hotelling's T2: 0.95.

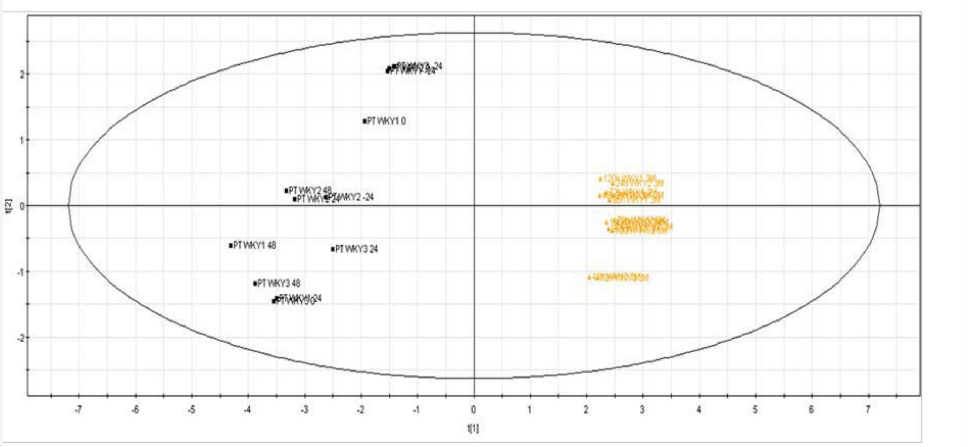

Figure 5 PCAscores plot of 1H-NMR spectral data of urine samples collected over time and showing a clear separation between pregnant WKY rats (orange symbol)as compared to non pregnant WKY females (black symbol) The model parameters were: R2Xcum = 0.7942, R2X1 = 0.6506, R2X2 = 0.0852, Q2cum = 0.5809and Hotelling's T2: 0.95.

trimethylamine-*N*-oxide, glycerol, succinate, and citrate. Other metabolites were more concentrated in the extractions of implantation sites from hypertensive rats: betaine, lactate, creatinine, α-glucose, N-acetyl-glycoproteins, creatine, glutamine, leucine, and alanine.

Finally, plasma samples were also analyzed by ^{1}H-NMR spectroscopy. In case of hypertension, the most noticeable changes in pregnant females (Table 5) were decreased plasma levels of methylamines, sarcosine, and tyrosine in case of hypertension, together with higher levels of α-hydroxyl-*n*-valerate, valine, lipids (VLDL), alanine, glycoprotein's, succinate, citrate, fatty acids, glucose, and glycine.

DISCUSSION

This study aimed at evaluating the metabolic changes accompanying the development of hypertension and the impact of pregnancy on the metabolic profiles. The work was performed in animal's spontaneously developing hypertension (SHR rats) and in their normotensive control matches (WKY rats).

The main methodology used was based on the metabonomics evaluation, allowing a non-invasive exploration of the temporal metabolic changes in urine and plasma samples, as well as in implantation sites (embryos, placenta and uterine wall) collected 10 days after mating. This methodology was used to establish specific patterns of the biochemical perturbations caused by hypertension and/or pregnancy. This work was intended to discovering potential markers of perinatal disorders associated with hypertension as for example in case of preeclampsia.

Spontaneously hypertensive rats (SHR) do develop hypertension between the 7th and 15th weeks of age to reach

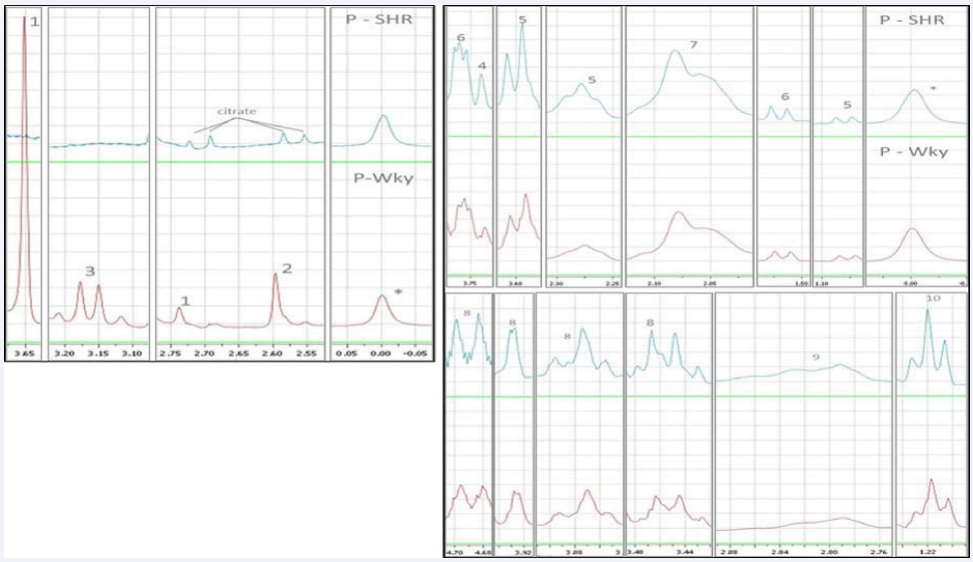

Figure 6 500MHz CPMG 1H-NMR spectra of rat blood plasma showing dramatic changes between pregnant SHR rats (P-SHR) and pregnant WKY rats (P-Wky) models in regions attributed to sarcosine (1), methylamine (2), tyrosine (dubious, 3), glycine (4), valine (5), alanine (6), glycoproteins (7), glucose (8), free fatty acids (9), VLDL (10) and TSP (*).

Table 4: List of metabolites identified in the extracts of conceptus as most discriminating between pregnant hypertensive SHR rats and their control matches (pregnant normotensive WKY rats).

Increased level in the pregnant hypertensive SHR rats compared to their control matches	Decreased level in the pregnant hypertensive SHR rats compared to their control matches
Glutamine	Serine
Alanine	Proline
Leucine	Succinate
Betaine	Citrate
Glycoproteins	Guanidoacetate
Creatine	Fructose (β-furanose)
Creatinine	Myo-inositol
Lactate	Glycerol
Phosphorylethanolamine	TMAO
Unknown (0.92 ppm, multiplet)	Unknown (2.77 ppm, dublet)
Unknown (1.28 ppm, undefined)	
Unknown (5.34 ppm, multiplet)	

Increased or decreased levels were assessed according to the "peak by peak" method, meaning that 1H-NMR spectra were systematically compared from two different conditions.

a plateau in blood pressure of about 200 mmHg. This pressure is also associated with other characteristics encountered in preeclampsia, such as abnormally elevated plasma levels of cardiac steroids, proteinuria and obstetric complications (premature birth and growth retardation) (LaMarca*et al.* 2006, Sargent *et al.* 2003) [21,2].

The results generated in this animal model allowed us to identify some specific metabolic profiles (either from urine, plasma, or implantation sites), which were characteristic of hypertension and/or pregnancy and possibly, by extension, of preeclampsia.

Regarding the plasma metabolic changes during pregnancy, the peak-to-peak comparison of spectra obtained from plasma samples collected from pregnant hypertensive vs normotensive females (Table 5) highlighted a lower concentration of sarcosine in the plasma samples from pregnant SHR rats. Sarcosine is an N-methylated derivative of glycine which is produced when glycine N-methyl transferase (GNMT) methylates glycine

Table 5: List of plasma metabolites identified as most discriminating between pregnant hypertensive SHR rats and their control matches (pregnant normotensive WKY rats).

Increased level in the pregnant hypertensive SHR rats compared to their control matches	Decreased level in the pregnant hypertensive SHR rats compared to their control matches
Valine	Methylamine
Alanine	Sarcosine
Glycine	Tyrosine
Glucose	
Fructose (β-pyranose)	
Glycoproteins	
Citrate	
Acetate	
Succinate	
"Free fattyacids"	
"Amino-acids"	
Unknown (3.45 ppm, multiplet)	

Increased or decreased levels were assessed according to the "peak by peak" method, meaning that 1H-NMR spectra were systematically compared from two different conditions.

and is converted back to glycine by sarcosine dehydrogenase (SARDH). It is a natural amino acid found in muscles and other body tissues. Recent studies (Sreekumar *et al.* 2009, Khan *et al.* 2013) [23,24] focused on the role of sarcosine in the progression of prostate cancer. Sarcosine was identified as a metabolite whose concentration is directly related to cancer progression and metastasis production. Indeed, experiments suggested that the level of sarcosine was higher in invasive cell lines from prostate cancer when compared to benign prostate epithelial cells. When GNMT was inactivated, there was a slowdown in the invasion process in prostate cancer. Furthermore, both the addition of exogenous sarcosine and the inactivation of SARDH induce an invasive phenotype in benign prostate epithelial cells. Altogether, those results suggested that sarcosine could be a potentially important metabolic intermediate in the invasion of cancer cells and a marker of tumor aggressiveness.

Extrapolating from those data, one could hypothesize that sarcosine may also play a role in the invasion of cytotrophoblast cells during the development of the placenta. Our results indicate a decrease in the plasma sarcosine in SHR rats (confirmed by a higher excretion in the urines of pregnant SHR as shown in Table 3), which could explain the lack of invasion as one potential cause of the IUGR characterizing the pregnancies of SHR rats (Sado*et al.* 2007) [16].

Contrarily to sarcosine, some metabolites are more concentrated in plasma collected from pregnant SHR, as for instances valine, alanine, glycine, fatty acids, glycoproteins and the glucose. In addition to being at least partly (glycine) linked to the fate of sarcosine, the metabolism of amino acids is of paramount importance in the close relationship between females and fetuses during pregnancy. Indeed, the maternal organism must fulfill the growing demand of the fetus by providing all the necessary nutrients. The amino acids then cross the placenta to incorporate fetal tissues. In addition, fetal requirements increase exponentially, forcing the mother to cope with this situation by increasing the availability of amino acids. There is also an active process of concentration of amino acids by the placenta, which explains why their distribution to the fetus varies among individuals due to the ability of placenta cells to fuel the fetal protein biosynthesis process (Palou *et al.* 1977) [25].In parallel, during the initial phase of pregnancy, an increase of glucose is also necessary to ensure the needs of fetal growth. This adds an additional and considerable pressure on maternal resources. Our results indicate an increase of maternal plasma amino acids in hypertensive rats, which consequently causes fetal distress. The same observation is made for glucose. Indeed, in case of preeclampsia, not only the efficiency of the placenta is reduced due to poor cytotrophoblast invasion, but there is also a vasoconstriction of afferent arterioles. Both effects converge towards a reduction in the absorption of nutrients by the fetus and indirectly induce their accumulation in maternal blood. However, this hypothesis seems inconsistent with the results obtained from the extracts of implantation sites. An additional supply of glucose is provided by the gluconeogenic pathway fueled by the excess of plasma alanine (Palou *et al.* 1977) [25].

It also appears that these changes can be correlated with the "metabolic syndrome." According to the diagnostic criteria of the "World Health Organization" or "National Cholesterol Education Program," the "metabolic syndrome" affects more than 15 to 25% of the adult population in the Western world and is characterized by the combination of multiple risk factors for diabetes, cardiovascular disease, insulin resistance, dyslipidemia and hypertension (Buresova*et al.* 2006)[26].

Pregnancy creates an environment similar to the metabolic syndrome (Simmons, 2006) [27], including the development of insulin resistance, increased blood glucose and triglycerides levels and high blood pressure. In addition to being a risk factor for developing cardiovascular complications and diabetes, metabolic syndrome during pregnancy can be harmful to the fetus. Gestational diabetes as well as preeclampsia can be considered as expressions of this syndrome during pregnancy. Indeed, the latter is directly associated with future development of the syndrome by abdominal obesity, an accentuation of dyslipidemia, metabolic disorders of insulin and glucose during and after pregnancy. Thus, women presenting a metabolic syndrome, at least those having high triglycerides, obesity and hypertension, may be more prone to develop preeclampsia (Simmons 2006)[27].

In our study, the ^{1}H-NMR analysis of plasma samples from SHR revealed increased levels of circulating fatty acids, suggesting the possible development of the dyslipidemic symptom encountered in the "metabolic syndrome", as described above. This in turn contributes to the development of insulin resistance. Circulating fatty acids reduce glucose uptake in muscle by a competitive process for the oxidation phase. The free fatty acids are oxidized in priority, then leading to increased production of acetyl CoA that in turn inhibits the enzymes of glycolysis. Muscular energy is supplied primarily by the oxidation of free fatty acids and the muscle glycogen stock remains intact. In summary, storage and use of glucose in muscle is reduced while in the liver, there is a stimulation of gluconeogenesis. All this helps to raise blood sugar. Interestingly, high plasma levels of amino acids and glycoproteins have been reported in the "metabolic syndrome" (Kamaura *et al.* 2010) [28].

Regarding the acid extracts of implantation sites (embryos, placenta and uterine wall – Table 4), we identified lower levels of serine, succinate, citrate and myo-inositol in gravid females. A reduction in plasma serine level is typically associated with pregnancy (Kalhan *et al.* 2003) [29]. This reduction, also noticed in implantation sites may have multiple origins. A decrease in serine concentration can occur when the blood sugar rises, as encountered in the metabolic syndrome. It can also be caused by a low perfusion of the placenta and resulting exchange problems. Overall, the lower availability of serine affects fetal development, particularly because of its role in the biosynthesis of nucleotides. Decreases in succinate and citrate in implantation sites from SHR most likely reflect a lower energy production in the fetus, possibly consequent to mild hypoxia caused by maternal vasoconstriction. This could be a cause of fetal distress typically associated with growth retardation in cases of gestational hypertension and preeclampsia. Finally, it was demonstrated that myo-inositol uptake and content, phosphoinositide turnover, and inositol phosphate production are reduced in aortic vasculature of gravid rats. Indeed, the basal rate of incorporation of ^{3}H-myo-inositol and ^{3}H-glycerol into phosphoinositides of aortae from pregnant rats

in vitro was significantly reduced, when compared with vessels from virgin animals (Conrad *et al.* 1991) [30]. It also turned out that myoinositol supplementation improves insulin resistance in patients with gestational diabetes (Corrado*et al.* 2011) [31]. This makes it possible to establish a link with one of the manifestations of the "metabolic syndrome", which is accentuated by the reduction of myo-inositol. By contrast, glutamine, alanine, leucine and glycoproteins were found in excess in acid extracts of implantation sites in SHR. Also observed in the plasma samples, these increases in amino acids and glycoproteins could possibly be related to the "metabolic syndrome" described above.

Finally, the main metabolic changes measured in the urine samples of pregnant SHR (Table 1) consisted in a decrease in creatinine concentration accompanied by an increase in tryptophan. Creatinine is an endogenous substance produced during muscle catabolism. It is excreted primarily in the urine. It is mainly used to evaluate glomerular filtration without incurring significant tubular reabsorption. Creatinine is therefore a good indicator of the renal function. As already reported by others (Leoncini *et al.* 2004, Akira *et al.* 2008) [32,18], the reduction in creatinine excretion observed in SHR could reflect an impairment of the renal function caused by hypertension. In preeclampsia, there is an increase in plasma cardiac steroids (LaMarca *et al.* 2006) [22] responsible of a vasoconstrictor effect that reduces the glomerular filtration rate and, therefore, also decreases the excretion of amino acids and glucose. On the other hand, the urinary excretion of tryptophan was found increased in SHR. Tryptophan, as a key player of protein synthesis, may play an important role in the differentiation, development and cell growth in the fetus. Although poorly understood, its involvement in the recovery of a normal glomerular filtration is not in doubt. Indeed, injections or dietary supplement of tryptophan restores a normal glomerular filtration rate in people with chronic kidney disease (Martinsons *et al.* 2003) [33].

Comparing our results to previous works dedicated to complicated pregnancies and metabonomics highlights the complexity of such pathologies. Even if similar observations than in the urines of pregnant SHR rats have already been reported in the urines of preeclamptic women (lower concentration of glucose, higher concentration of trigonelline and alanine; (Austdal *et al.*, 2014) [8], opposite results are also reported in our work (decreased concentration of creatinine and higher concentration of both malonate and hippurate in the urines of pregnant SHR rats – Table 1). Interestingly, we also reported here similar urinary changes of those reported by the above mentioned author but when comparing urinary fingerprint of non-pregnant SHR rats to their control matches; e.g. increased urinary excretion of dimethylamine and decreased excretion of formate and DMG. This may be helpful to determine the hypertension contribution to the specific urinary fingerprint of preeclamptic women but also the expression of preexisting hypertension in some preeclamptic women. Previous metabolic investigations on plasma or serum of preeclamptic women (Kenny *et al.,* 2010; Odibo *et al.*, 2011 and Bahado-Singh *et al.* 2013) [4,6,7] revealed similarities with what we observed in the plasma of pregnant SHR rats; e.g. increased level of valine, alanine, glucose, citrate, acetate and α-hydroxy-N-valerate. On the other hand, (Turner *et al.* 2008; Autdal *et al.* 2014 and Kuc *et al.* 2014) [34,8, 9] reported changes in plasmatic amines that we were not able to highlight in SHR rats (e.g. increased level of tyrosine and decreased levels of taurine, glycylglycine and asparagine as a specific feature of PE). Elevated creatine, creatinine and phosphorylethanolamine as well as decreased concentration of myo-inositol we highlight in the implantation sites of SHR rats (Table 4) were similar to changes in the first urine of newborns suffering of IUGR (Dessi *et al.*, 2011)[35] as well as in placental-conditionned medium of SGA pregnancies (Kenny *et al.*, 2010)[4].

CONCLUSION

The coexistence of both similarities and differences between our results and recent clinical investigations based on metabolomics approaches is very interesting because it will help to target a single disease (in our study the contribution of hypertension) in syndromes such as preeclampsia and IUGR. Even if further investigations, i.e. using *in vitro* models such as placental cell cultures, will be helpful to validate our current results, our study support the use of ^{1}H-NMR-based metabonomics as a promising tool to provide new information on metabolic changes associated with the physiopathology of hypertension and pregnancy in rats but not only.

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