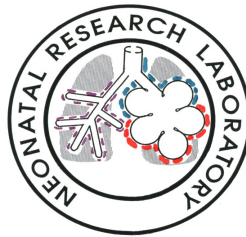# Master Work

Intrauterine growth restriction is associated with alterations of the NO/cGMP pathway in the human umbilical vein

Student: Christian Juvet
University of Lausanne

Co-tutor: Dr Anne-Christine Peyter
Neonatal Research Laboratory CHUV

Tutor: Prof Jean-François Tolsa
Clinic of Neonatology CHUV

Expert: Dr Yvan Vial, PD & MER
Department of Gynecology, Obstetrics and Genetics

Table of contents

Introduction

Intrauterine growth restriction (IUGR) is a frequent complication of pregnancy, affecting between 3 and 7% of total population (1). Even tough its definition is still under consideration, the most common criterion is a birth weight below the 10th percentile. However, a birth weight below the 5th or even the 3th percentile is also sometimes used as a criterion to define IUGR. Furthermore, another problematic point is that such a definition does not make a difference between growth-restricted babies and babies who are constitutively small. Therefore it is important to determine whether a growth curve break occurred in utero, based on prenatal ultrasonographic measurements, as this condition would prove IUGR. On the contrary, a constitutively small baby would have harmonious growth curves, based on his genetic background.

Although the mechanisms leading to IUGR remain poorly understood, its origins are classified into three groups, depending on their etiology: maternal causes (smoke, malnutrition...(2)), placental dysfunction or fetal causes (malformations, genetic syndromes, inborn errors of metabolism...). The consequence is either an extrinsic insufficiency of oxygen and nutrients supply to the fetus (maternal causes and placental dysfunction), or an intrinsic cause such as increase of needs, metabolism deficiency or restricted growth potential (fetal causes).

IUGR has been recognized to be an important cause of perinatal morbidity and mortality. Many epidemiological studies have also shown that IUGR can have an important impact on health much later in life. Indeed, a low birth weight, as it happens in IUGR, has been linked to several frequent chronic diseases in adulthood, like coronary heart disease, cardiovascular disease (3), metabolic syndrome, diabetes mellitus type II, stroke and osteoporosis (2,4). An explanation to these observations was proposed by the "Barker hypothesis", which stipulates that early development is a critical time window in which the fetus' nutrition, oxygen supply and the intrauterine environment in general, can affect the fetal gene expression, leading to a long lasting epigenetic imprint, which influences metabolism up to adulthood (5,6,7,8,9,10). Structural changes in organogenesis, which could account for a higher risk to develop chronic diseases, have also been observed in the heart, the kidney and the pancreas of growth-restricted animals (2).

In order to better understand the development of IUGR, it seems therefore important to focus on the feto-maternal exchanges, in which the placenta and the umbilical cord play a major role. The placenta is in charge of transmitting oxygen and nutrients from the mother's to the fetus' blood, and

the umbilical circulation is the only pathway for this blood to reach the fetus. Therefore, it can easily be imagined how even small changes in this system could have important consequences on the fetus' oxygen and nutrient supply.

Several studies have identified differences in placental and umbilical circulations between growth-restricted fetuses and controls. Resistance in feto-placental circulation was shown to be increased in IUGR (11). Since oxygen concentration affects the tone of chorionic plate vessels in the placenta (12), the decrease in oxygen saturation observed in IUGR umbilical blood (13) could contribute to this increased resistance. It is furthermore interesting to note that the mechanism involved in hypoxic feto-placental vasoconstriction, namely a potassium channel inhibition (14), is similar to the mechanism observed in other oxygen-sensitive tissues, like the lungs (15). Such similarities are not surprising, since the placenta and the feto-placental circulation assume in utero the role which is taken over by the lungs after birth, that is the organism's oxygen supply.

There were also several differences observed between IUGR and control umbilical vessels. Indeed, IUGR umbilical arteries were shown to have a thinner wall and a stronger contraction than control umbilical arteries (16). This may be a link to arterial hypertension observed in adulthood, but could also account for changes in the feto-placental circulation. Other in vitro studies in an ovine model of IUGR mention that IUGR umbilical arteries constrict less than controls (17). Additionally they also relax more than controls when exposed to the vasodilating agent bradykinin, but not when exposed to the nitric oxide (NO) donor sodium nitroprusside (18). This would suggest that only certain pathways are altered in IUGR umbilical arteries. But in any case, there seem to be alterations in IUGR umbilical vessels, which affect their tone and their response to vasodilating or vasoconstricting stimuli.

Since several years, a project focused on human umbilical cords in case of growth restriction is conducted under supervision of Dr A.-C. Peyter and Pr J.-F. Tolsa at the Neonatal Research Laboratory of the Clinic of Neonatology at the University Hospital CHUV in Lausanne. They hypothesized that **the decrease in oxygen and/or nutrient supply to the fetus, in case of placental insufficiency, is associated with modifications in pathways involved in the regulation of umbilical vascular tone, contributing to further impairment of the fetal-maternal exchanges and to development of IUGR.**

Pharmacological studies showed that the relaxation induced by the NO donor DEA/NO in preconstricted isolated human umbilical veins from IUGR newborns is decreased compared to controls adapted for gestational age (19).

The aim of the present project was to compare the expression of several specific proteins involved in the NO-mediated relaxation in the human umbilical vein between growth-restricted newborns and controls. In particular, we focused on the following proteins:

1) Endothelial nitric oxide synthase (eNOS), which is responsible for producing the vasodilating agent NO
2) Caveolin, a membrane protein, which regulates the membrane localization and activation of eNOS
3) Soluble guanylyl cyclase (sGC), which produces the second messenger cyclic guanosine monophosphate (cGMP) when stimulated by NO
4) Phosphodiesterase 5a (PDE5a), which degrades cGMP (20) (Illustration 1).
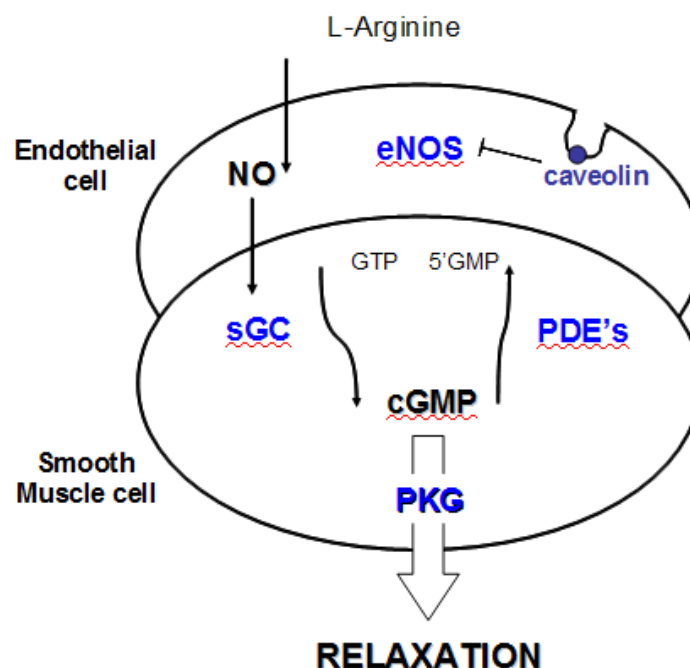

Illustration 1: Simplified schema of the NO/cGMP signaling pathway in blood vessels. NO: nitric oxide; eNOS: endothelial synthase; sGC soluble guanylate synthase; cGMP: cyclic guanosine monophosphate; PDE's: phosphodiesterases. (Courtesy of Dr Anne-Christine Peyter)

Identification of changes in specific protein expression in umbilical vessels of growth-restricted newborns could lead to identification of potential therapeutic targets to limit or prevent IUGR, but also of some biological markers to detect individuals with increased risk for developing cardiovascular disease in adulthood.

In this project, we decided to focus on the umbilical vein, since this is the only vessel through which oxygen and nutrients are brought from the placenta to the fetus. Therefore, an impaired function of the umbilical vein affecting its diameter would have a direct influence on the fetus' oxygen and nutrient supply. We limited our investigations to female newborns, because pharmacological studies performed on isolated umbilical veins of newborn boys failed to show any difference in NO-induced relaxation between IUGR and controls (19). Moreover, IUGR is more frequent in female fetuses (21,22).

Material and methods

Umbilical cords were collected from the maternity ward of the Department of Obstetrics and Gynecology, University Hospital of Lausanne immediately after birth. Exclusion criteria were

- Maternal positive HIV, hepatitis A, B or C serology or unknown serological status
- Preeclampsia complicated pregnancy
- Newborns with malformations or genetic syndromes
- Multiple pregnancy
- Umbilical cord with anatomic variations in form of other status than two arteries and one vein
- Birth weight over the 90th percentile

The control group and the IUGR group were defined as follows. Neonates with a birth weight between the 10th and 90th percentile (adapted for gestational age) were attributed to the control group. Newborns with a birth weight below the 10th percentile were considered as IUGR. When available, prenatal data were collected to determine whether a break in the intrauterine growth curve occurred, in order to confirm IUGR status, versus a constitutively low birth weight.

Because previous studies showed that vascular reactivity varies along the umbilical cord (23), we decided to focus on the segment closer to the fetus. The umbilical cord was cut as close as possible to the fetus and a ~10-cm segment was put into a deoxygenated physiological solution and kept at 4°C until dissection. This "fetal" segment was then used as followed: the portion near the fetus was dissected and used for pharmacological testing of the vessels. The following portion was dissected to separate the vein and the arteries from the Wharton's jelly in order to perform RNA and protein expression analyses. Finally a 1-cm segment was harvested for histological analyses.

Western Blot

Umbilical veins were crushed using a cryogenic mortar, and homogenized in a lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, 5 µg/ml pepstatin, 3 µg/ml aprotinin, 10 µg/ml leupeptin, 0.1 mM AEBSF, 1 mM sodium vanadate, 50 mM NaF, 20 mM CHAPS). After a 10min centrifugation (3'000 x g, 4°C) to discard insoluble material, total protein concentration in tissue homogenates was quantified using BCA protein assay (Pierce). For each group (IUGR and control), samples from 40 patients were randomly distributed in four pools, by mixing an equal amount of proteins (100µg) from every sample. This allows to average individual variability inside every pool and to focus on the main differences between IUGR and controls.

Conditions used for Western Blotting experiments are summarized in Table 1.

Protein	Amount of protein (µg)	Acrylamide gel concentration	Primary antibody concentration	Primary antibody make
eNOS	60	7.5%	1/200	BD Transduction Laboratories
Caveolin	20/40	7.5%/12.5%	1/4000	BD Transduction Laboratories
sGC	40	7.5%	1/500	Cayman
PDE5a	40	7.5%	1/250	Santa Cruz
Actin	-	-	1/250	Sigma

Table 1: Conditions used to detect the studied proteins by Western Blot

Migration was performed during 35 minutes at 200V, followed by transfer during 2 hours at 100V. Then, the membrane was blocked overnight in a solution containing casein. The membrane was then washed in TBST and incubated 1 hour with the primary antibody diluted in a casein solution.

After another wash with TBST, the membrane was incubated 30min in a 1/2000 biotinylated anti-rabbit secondary antibody solution, re-washed in TBST and incubated 30min with avidin-peroxydase (Vectastain). After a last wash, the membrane was incubated 5min with ECL (Thermo Scientific) and images were taken with a LAS-4000 image reader (Fujifilm). Exposure time varied between different membranes, even for the same protein, depending on the intensity of the signal.

Then, actin content was assessed on each membrane to control for protein amount, using the same protocol as above, but with a primary antibody concentration of 1/250 and using a classical peroxidase-linked secondary antibody at a concentration of 1/10'000 instead of a biotinylated secondary antibody. Since actin expression is relatively constant in a given tissue, it can be used to normalize intensity observed for the protein of interest, to correct for potential variations in the protein amount charged on the gel, due for example to possible imprecision in pipeting or protein quantification.

The images were analyzed using the ImageJ software to determine the intensity of each band. The band intensity of the protein of interest was divided by the intensity of the corresponding actin band to normalize the results. For each protein of interest, at least four Western Blots were performed. To normalize for differences between membranes, like exposure time or efficiency of immunoblotting, this ratio was not expressed as an absolute value, but relatively to the protein/actin ratio of a predetermined "reference pool" chosen among the control pools.

For each pool, the average protein content of 4 or 6 membranes was calculated. Then the average protein content was calculated for the IUGR group and the control group, as well as their standard deviation. These two averages were then compared by a Student's t test.

Immunohistochemistry

The umbilical cord's segment used for histological analysis was dehydrated by successive baths in increasing concentrations of ethanol, then incubated in xylene and finally paraffin to obtain paraffin blocks. Sections of 5μm were cut using a microtome and fixed onto microscope slides. Immunostaining was performed on histological sections to determine tissular localization of the tested proteins, namely eNOS, sGC and PDE5a.

Slides carrying the sections were washed in xylene, decreasing concentrations of ethanol and finally in water in order to eliminate paraffin and rehydrate the tissues. Slides were then incubated in boiling citrate buffer for 3x5 minutes in order to unmask antigenic sites. After washing (all washes were performed in a PBS 0.5% Triton X-100 solution), slides were incubated with 1.5% goat serum diluted in PBS. Since the secondary antibody was produced in goat, this step was performed to block all sites, which could have a non-specific affinity for the secondary antibody and therefore give rise to a non-specific signal, yielding a strong background staining. After a brief wash, slides were then covered overnight with the primary antibody diluted in PBS buffer. The concentration of the different antibodies were as follows:

- eNOS   1/200 (BD Transduction Laboratories)
- sGC   1/100 (Cayman)
- PDE5a   1/100 (Santa Cruz)

After a new wash, the slides were incubated for 1 hour with the secondary biotinylated anti-rabbit antibody diluted in a PBS buffer, followed by another wash and a 1 hour incubation with avidin-peroxydase (Vectastain). The slides were washed again and then incubated for 30 min with Vector Red (Vectastain). After a last wash, slides were dehydrated by successive baths in increasing concentrations of ethanol and finally xylene, before fixation with Eukitt® resin. The slides were visualized with a fluorescence microscope (Leica).

Results

The demographic data related to the population studied in the present report are summarized in Table 2. Gestational age at birth, as well as maternal age are similar in the IUGR and the control group. Birth weight, size, head circumference, placental weight and umbilical cord diameter were significantly lower in the IUGR group compared to controls. Furthermore, an intrauterine growth curve break was confirmed by the prenatal data in about 70% of the individuals of our IUGR group, therefore confirming IUGR status, versus a constitutively small birth weight.

GIRLS	CTRL	IUGR	p-value
Number of patients included	40	40	
Gestational age (weeks)	39.6 ± 0.9	39.4 ± 1.0	0.2309
Birth weight (g)	3366 ± 285	2617 ± 242 *	<0.0001
Size (cm)	49.4 ± 1.6	46.4 ± 1.7 *	<0.0001
Head circumference (cm)	34.6 ± 1.1	32.7 ± 1.1 *	<0.0001
Placental weight (g)	638 ± 135	452 ± 75 *	<0.0001
Umbilical cord diameter (mm)	12.2 ± 2.3	10.9 ± 2.4 *	0.0104
Maternal age (years)	31.6 ± 5.8	30.5 ± 5.0	0.3551

Table 2: *Demographic data of newborn girls providing the umbilical cords used for the Western Blots. Data are expressed as mean ± SD. * Statistical difference between both groups (p< 0.05, Mann-Whitney test).*

The results obtained in the Western Blotting and immunostaining experiments are presented below for each protein of interest.

1) eNOS

Western Blot

Four Western Blots were performed for eNOS. The ratio between eNOS band intensity and actin band intensity was determined for each pool and expressed relatively to the ratio of pool C on each of the four membranes (Fig.1). The average value of the four membranes was calculated for each pool. With these values, the mean and the standard deviation were calculated for the control group (four pools) and the IUGR group (four pools). The calculated mean protein content, normalized by actin, was about 2.5 times higher in IUGR pools than in controls ($p < 0.05$) (Fig.2).
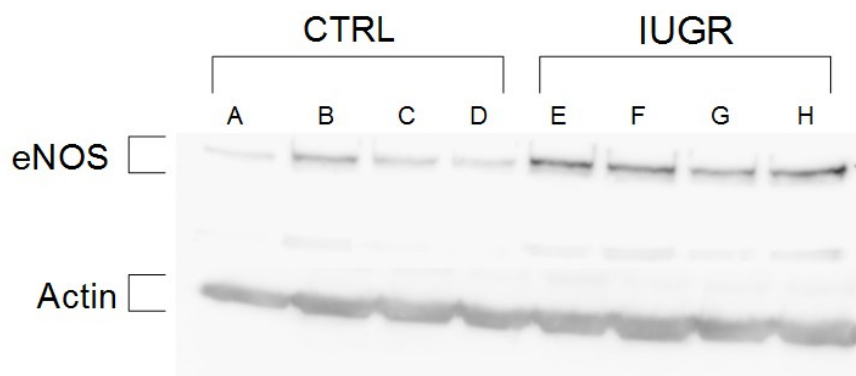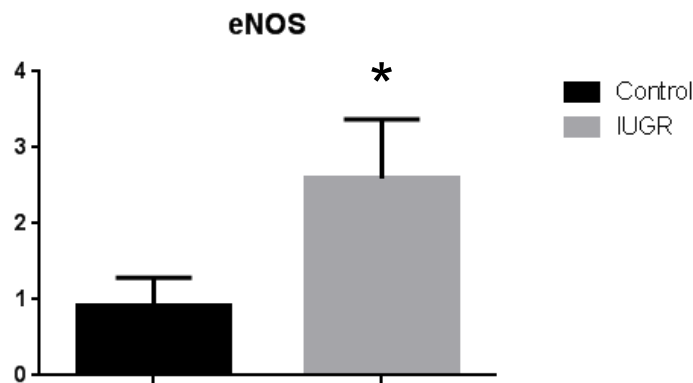

Figure 1: eNOS Western Blot with 60μg of proteins and 1/200 primary antibody concentration

Figure 2: Relative eNOS protein expression in human umbilical veins of growth-restricted and control newborn girls. Data represent mean ± SD (n=4 pools of 10 patients for each group). * A significant difference was found between control and IUGR group (p<0.05, unpaired Student's t test).

Immunohistochemistry

eNOS immunostaining was performed on six histological sections, respectively three controls and three IUGR. Images were taken with a magnification of 100X. eNOS immunostaining was not only observed in the endothelium, but also in the smooth muscle and Wharton's jelly. Although signal intensity was slightly variable between sections of a same group, there was no significant difference observed in intensity or distribution of fluorescence in the arteries or in the veins between both groups. However, there was a much higher signal in Wharton's jelly of the control group than in the Wharton's jelly of the IUGR group.

Figure 3 shows vessels and Wharton's jelly of two sections, respectively one from a control umbilical cord and one from an IUGR umbilical cord. These two sections are representative of most sections from each group.

Figure 3:

Images A and B show eNOS distribution in umbilical vein, respectively in a control section (A) and in an IUGR section (B).
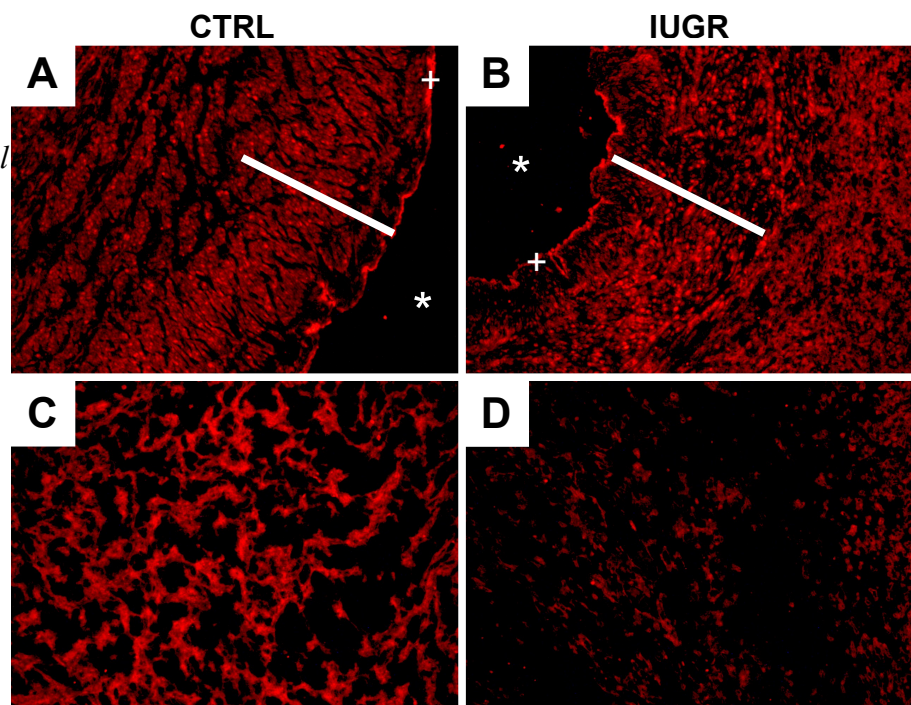
Images C and D show eNOS distribution in the Wharton's jelly, respectively in a control section (C) and in an IUGR section (D)

Sections were incubated overnight with a 1/200 primary antibody solution.

*: Lumen
+: Endothelium
I: External circular muscular layer

2) Caveolin

Western Blot

Six Western Blots were performed for caveolin. Four of them in a 12% acrylamide gel with 20µg proteins from each pool, and two of them in a 7.5% acrylamide gel with 40µg proteins from each pool. The ratio between caveolin band intensity and actin band intensity was determined for each pool and expressed relatively to the ratio of pool C on each of the six membranes (Fig.4). The average value of the six membranes was calculated for each pool. With these values, the mean and the standard deviation were calculated for the control group (four pools) and the IUGR group (four pools). The calculated mean protein content, normalized by actin, was very similar between the IUGR group and the control group (p=0.85) (Fig.5).
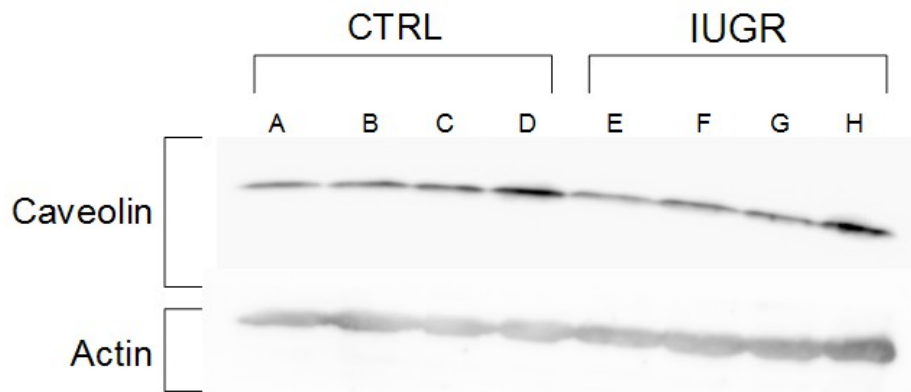

Figure 4: Caveolin Western Blot with 40µg of proteins and 1/4'000 primary antibody concentration

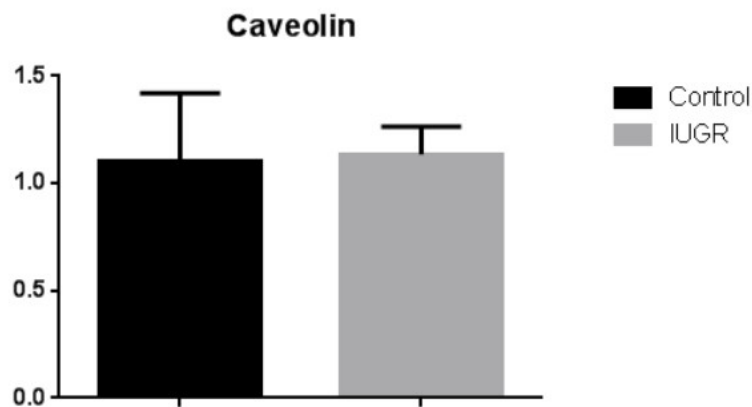*Figure 5:* Relative caveolin protein expression in human umbilical veins of growth-restricted and control newborn girls. Data represent mean ± SD (n=4 pools of 10 patients for each group). No significant difference was found between both groups.

3) sGC

Western Blot

Six Western Blots were performed for sGC. The ratio between sGC band intensity and actin band intensity was determined for each pool and expressed relatively to the ratio of pool A on each of the six membranes (Fig.6). The average value of the six membranes was calculated for each pool. With these values, the mean and the standard deviation were calculated for the control group (four pools) and the IUGR group (four pools). The calculated mean protein content, normalized by actin, was about twice as high in the IUGR group as in the control group ($p<0.05$) (Fig.7).
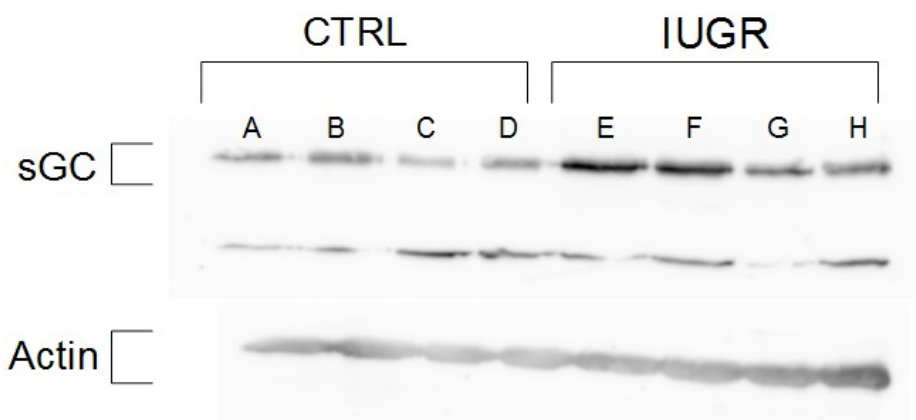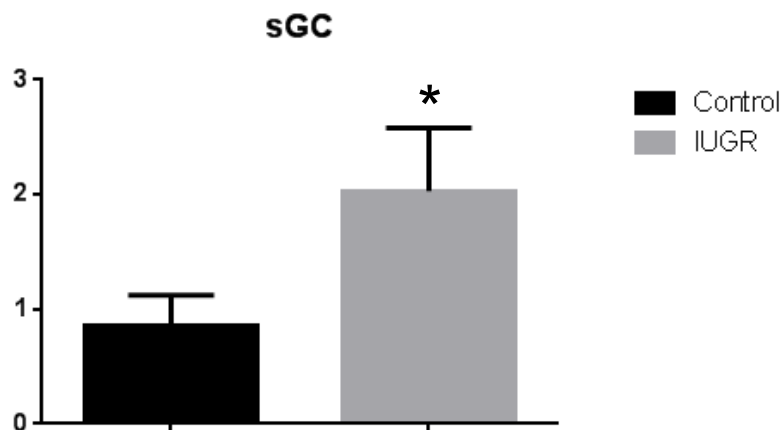

Figure 6: sGC Western Blot with 40μg of proteins and 1/500 primary antibody concentration

Figure 7: Relative sGC protein expression in human umbilical veins of growth-restricted and control newborn girls. Data represent mean ± SD (n=4 pools of 10 patients for each group). * A significant difference was found between control and IUGR group ($p<0.05$, unpaired Student's t test).

Immunohistochemistry

sGC immunostaining was performed on ten histological sections, respectively five controls and five IUGR. Images were taken with a magnification of 100X. Signal was found in the endothelium, the smooth muscle, and weakly in the Wharton's jelly (Fig.8). Signal intensity appeared to vary between sections of a same group and therefore no difference could be observed in intensity or distribution between the IUGR and the control group.

Figure 8:

Images A and B show sGC distribution in umbilical vein, respectively in a control section (A) and in an IUGR section (GB.
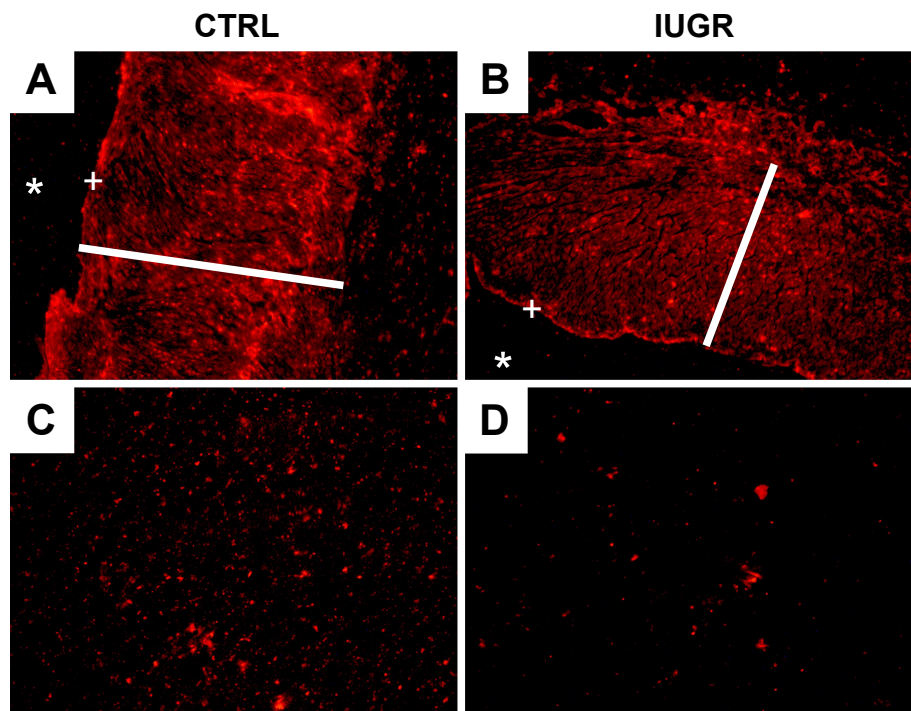
Images C and D show sGC distribution in the Wharton's jelly, respectively in a control section (C) and in an IUGR section (D)

Sections were incubated overnight with a 1/100 primary antibody solution.

*: Lumen
+: Endothelium
*I: External circular muscular layer*

4) PDE5a

Western Blot

Six Western Blots were performed for PDE5a. The ratio between PDE5a band intensity and actin band intensity was determined for each pool and expressed relatively to the ratio of pool B on each of the six membranes (Fig.9). The average value of the six membranes was calculated for each pool. With these values, the mean and the standard deviation were calculated for the control group (four pools) and the IUGR group (four pools). One membrane was excluded because of its inhomogeneous actin bands, of which some were almost absent, probably because of technical problems during transfer or blotting. The calculated mean protein content, normalized by actin, was significantly lower in IUGR group compared to controls ($p<0.05$) (Fig.10). However, these results should be considered with caution, as a lack of reproducibility in staining between membranes led to an important variability of ratios of a same pool between the different membranes.

Figure 9: PDE5a Western Blot with 40μg of proteins and 1/250 primary antibody concentration
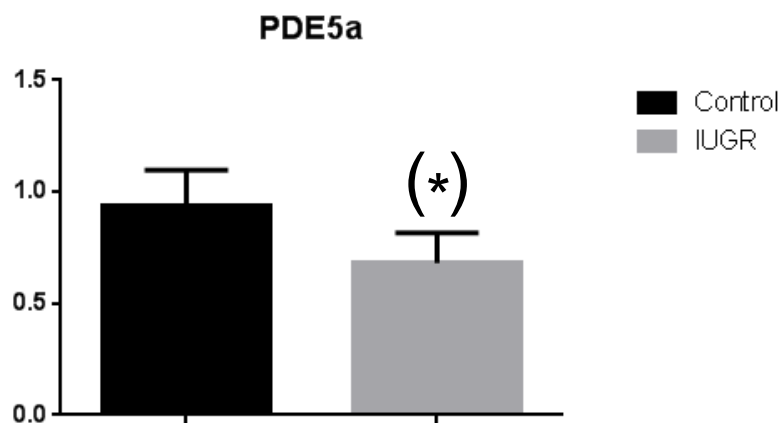
*Figure 10: Relative PDE5a protein expression in human umbilical veins of growth-restricted and control newborn girls. Data represent mean ± SD (n=4 pools of 10 patients for each group). * A significant difference was found between both groups (p<0.05, unpaired Student's t test)*

Immunohistochemistry

PDE5a immunostaining was performed on ten histological sections, respectively five controls and five IUGR. Images were taken with a magnification of 100X. Signal was found in the endothelium and the smooth muscle, but was almost absent in the Wharton's jelly (Fig11). The signal in some sections was very weak. Therefore it was difficult to observe differences between both groups. However we observed a difference between the two arteries of a same section in the control group. Indeed in 4/5 sections in the control group, one of the arteries had a stronger signal than the other. The one exception was a section on which none of the two arteries was marked.

Figure 11:

Images A and B show PDE5a distribution in umbilical vein, respectively in a control section (A) and in an IUGR section (B).
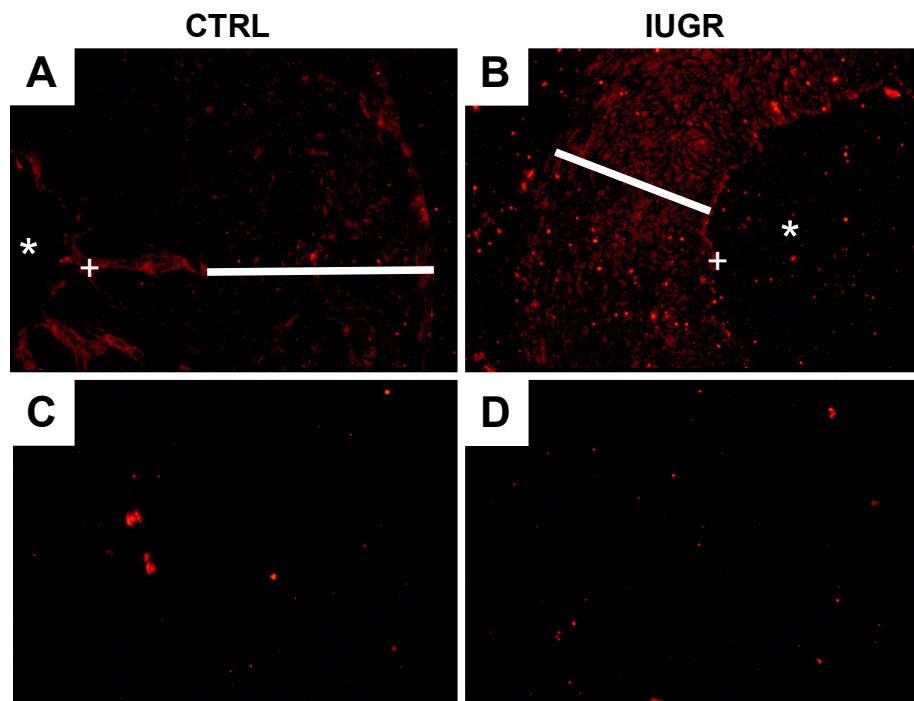
Images C and D show PDE5a distribution in the Wharton's jelly, respectively in a control section (D) and in an IUGR section (H)

Section were incubated overnight with a 1/100 primary antibody solution.

*: Lumen
+: Endothelium
I: External circular muscular layer

Discussion

This project aimed to better understand modifications occurring in the regulation of umbilical circulation in growth-restricted neonates. As pharmacological experiments showed a decreased NO-induced relaxation in isolated umbilical veins of newborn girls with IUGR, we investigated the expression of several proteins implicated in the NO/cGMP-mediated relaxation.

The first tested protein was the endothelial NO synthase (eNOS) which produces the vasorelaxant agent NO. Our results showed that eNOS protein content was about 2,5 times higher in IUGR veins than in control veins.

This increase in eNOS protein content would contribute to facilitate relaxation, as the production of NO would be increased. This result is nevertheless compatible with the preliminary pharmacological experiments which showed a decreased NO-induced relaxation in IUGR veins compared to controls, since in these experiments, isolated veins were exposed to cumulative doses of a NO donor in the presence of a NOS inhibitor. They therefore give no information on the quantity of NO produced in IUGR or control veins.

eNOS immunostaining was not only found in the endothelium, but also in the smooth muscle and in the Wharton's jelly of umbilical veins. A similar distribution is reported by Mauro et al (24), who described eNOS immunostaining in the endothelium and in the Wharton's jelly, but did not mention a signal in the smooth muscle.

Remarkably, the only difference observed between histological sections from IUGR and control group was not the increased protein content in IUGR veins revealed by the Western Blot, but an increased immunostaining signal in the Wharton's jelly of the IUGR group. Nevertheless, this observation should be considered with caution as immunostaining is not an appropriate method to assess protein content of a tissue. Therefore these observations should absolutely be confirmed by Western Blot before being considered as interpretable.

Immunostaining did not allow to determine the signal distribution in the Wharton's jelly, since the signal was too weak to show images at a higher magnification.

There are some controversies in the literature on eNOS protein content and activity in the human umbilical vein in IUGR. Some report a reduced eNOS protein content and activity in human umbilical vein endothelial cells (HUVEC) (25,26), while others point out an increased eNOS protein content and activity for infants with birth weights below the 25th percentile, this effect being maximal between the 3th and the 10th percentile, a population which is considered as IUGR in our study (27). Furthermore, some other studies report an increased eNOS protein content in IUGR HUVEC, but a decreased activity of this protein, resulting in lower cGMP concentrations (28). Nevertheless, these experiments considered only endothelial cells. Our immunostaining experiments showed that eNOS is also present in the smooth muscle. Therefore our Western Blot's results cannot directly be compared to those of other studies. It would however be interesting to assess whether this eNOS increase is predominant in the smooth muscle or in the endothelial cells. A reliable method would be a cell culture of endothelium and smooth muscle cells from IUGR and control veins, to assess by Western Blot in which tissue eNOS protein content is increased.

Moreover, eNOS activity can be regulated by many ways, such as activating or inhibiting phophorylations, or intracellular calcium (29). Thus, the measured protein content does not provide information about the *in-vivo* activity modulation of eNOS.

Furthermore, other isoforms of NOS, like inductible NOS or neural NOS, should be tested, since these isoforms can be expressed in several different tissues. These isoforms could replace each other in pathological condition (30). Indeed, iNOS expression has been shown to be present in HUVEC and its expression seems to be increased in IUGR (24,27).

To assess the real NO production of eNOS, subjected to its different regulations, eNOS activity should be measured with a blocking, if possible, of the other NOS isoforms. However, this is technically difficult because of the high instability and reactivity of gaseous NO. Another way to evaluate eNOS activity would be to determine by Western Blot the proportion of activated eNOS protein, which is phosphorylated on a specific serine residue (29).

It is also important to notice that independently of its production, NO quantity can be altered by the presence of reactive oxygen species (ROS), as they degrade NO. Hypoxic conditions, as they are present in the feto-placental circulation of a growth-restricted fetus, are known to favor formation of such ROS in the mitochondria (31,32). Considering that mitochondria undergo several alterations in IUGR (33,34,35), it could be conceivable that these changes have an influence on the presence of

ROS.

The second protein we studied was caveolin, a small membrane protein involved in the formation of caveolae. Caveolae correspond to invaginations of the plasma membrane, present for example in endothelial cells, which concentrate numerous membrane proteins implicated in cellular trafficking and signaling, like eNOS. In these caveolae, eNOS is bound, and so inhibited by caveolin until a rise in intracellular calcium levels allows the calcium/calmodulin complex to displace caveolin and to free eNOS from its repression (29). Therefore a change in caveolin expression could influence the activity of eNOS and hence affect vasoreactivity. The Western Blots in our experiments showed no difference in caveolin protein content between IUGR and control umbilical veins. This observation was reproducible across all our membranes.

The third protein we investigated was sGC, which, when stimulated by NO, produces cGMP and thus promotes smooth muscle relaxation and so vasodilatation. sGC protein content was found to be approximately twice as high in IUGR umbilical veins compared to controls. Since this difference occurs to be consistently observed throughout all our six membranes, this result appears to be reliable. Such an increase should favor the NO-induced relaxation in the IUGR veins.

This result is surprising because pharmacological studies showed a decreased NO-induced relaxation in isolated umbilical veins of IUGR newborn girls. However, there are different ways to explain these apparently contradictory results.

First, as discussed for eNOS, protein activity can be modulated by several factors (36) so that increased protein content does not necessarily result in higher protein activity. Interestingly, several molecules proposed to reduce sGC activity, as hydrogen peroxide or superoxide, are ROS, which could be increased in IUGR as discussed above. An appropriate method to have an overview of sGC activity would be to measure the cGMP production by ELISA, in the presence of an inhibitor of phosphodiesterases (PDE), which degrade cGMP.

Second, the decreased NO-induced relaxation observed in the pharmacological studies could be due to downstream alterations of the NO/cGMP pathway, for example at the level of PKG or below.
In this case, the observed sGC increase would be a counteracting mechanism to compensate for this alteration.

Third, a parallel increase of the cGMP degrading enzymes PDEs would also be conceivable, counteracting the higher cGMP production by sGC and resulting in an unchanged or even decreased cGMP amount.

It is interesting to note that Steinert et al found no significant change in sGC protein content between HUVEC from babies born preterm or from a preeclampsia affected mother, and controls (37). Nevertheless, babies born preterm are not necessarily growth-restricted, and even though preeclampsia is a major cause of IUGR, no indication of birth-weight was precised. Moreover, only endothelial cells were studied while we studied the whole vein.

Immunohistochemistry did not point out the difference shown by the Western Blot. Signal was found in the endothelium and the smooth muscle but no real difference between IUGR and control sections could be observed. It is interesting to notice that signal intensity was somewhat variable between the five sections of a same group. Such variations could be due either to technical problems or more likely to interpersonal variability. Indeed in the Western Blot we tested pools composed of umbilical veins homogenates from ten different individuals, whereas in the immunostaining experiments each section corresponds to one individual.

The fourth and last tested protein was PDE5a. Phosphodiesterases (PDEs) are a group of enzymes degrading cyclic nucleotide second messengers, like cGMP or cAMP. The specificity for one or the other of these two molecules depends on the PDE subfamily. We decided to focus on PDE5a, because the PDE5 subfamily has a high affinity for cGMP and thus plays an important role in smooth muscle.

Our Western Blot's results showed a slight decrease in PDE5a protein content in the IUGR group compared to controls. Nevertheless, this result should be considered with great caution as reproducibility between different membranes was problematic. Consequently, p-value was only little below 0.05 (p=0.0435) and only when certain pools where chosen as reference pools.

The encountered technical problems could be due to some properties of the used antibody. Another antibody against the same protein should be tested to see whether it would give more reproducible results. Nevertheless the results, even though it should be emphasized that they are not that reliable, will be analyzed and discussed in the following lines. Further experiments using for example another antibody, or qPCR to measure mRNA content, should contribute to confirm or infirm them.

The observed slight decrease in PDE5a protein content in IUGR group compared to controls does not explain the observations made in the pharmacological experiments. Indeed a lower PDE5a protein content would have pro-relaxing effects, since it would increase the half-life of the pro-relaxing second messenger cGMP. But, as it is the case for the other tested proteins, PDE5a activity can be modulated in different ways. Indeed, it has been shown that PDE5 activity is highly increased when cGMP is bound. These observations were made in vitro, and as PDE5a has a very high affinity for cGMP, it is likely that PDE5a is fully activated by cGMP in vivo. There is also evidence that PDE5a phosphorylation by PKG can stabilize cGMP binding and therefore yield higher activity, resulting in a negative feedback loop. It is furthermore interesting to note that cGMP can also modulate other PDE subfamilies, which are more specific to cAMP. By this way, cGMP could affect cAMP level in the cell, which is also implicated in the smooth muscle tone regulation (38).

Thus, to have a more complete view of PDEs presence and activity in the cells, other isoforms and other subfamilies should be tested, as they could compensate for the PDE5a deficit. To evaluate the contribution of each subfamily, cGMP degradation could be measured using a fluorescent ELISA in the presence of selective inhibitors of a different PDEs.

Furthermore it would be interesting to assess changes in cAMP quantity, which would also have an influence on smooth muscle tone.

Interpretation of immunohistological results was complicated for PDE5a, as many sections had an extremely low signal, especially in the IUGR group. This could be due to the lower PDE5a protein content in the IUGR sections, but as the used antibody is probably problematic, as mentioned before, this seems to be a much more likely cause of the staining problems. Again, it would be interesting to repeat this experiment with another anti PDE5s antibody, and maybe a positive control to assess whether the absence of signal is due to technical problems or little protein content. Because of this very poor staining, no conclusion can be drawn regarding the possible differences of distribution or intensity between IUGR and controls.

In summary, our results show that in growth-restricted female human umbilical vein, eNOS and sGC protein content is increased, while PDE5a protein content seems to be decreased compared to controls. Caveolin protein content was unchanged between the two groups. Immunostaining did not

point out a difference in distribution of our tested proteins between IUGR and control groups, but suggest that eNOS content in the IUGR Wharton's jelly could be increased. Both observations should absolutely be confirmed by Western Blot, as immunostaining is a qualitative method to assess signal localization, but not a quantitative method allowing to reliably assess protein content. This is also the most likely reason why our immunostaining data does not correlate with our Western Blot's result.

In general, it is important to note that a higher specific protein content does not necessarily result in a higher activity, since the different proteins can be regulated in different ways, as discussed above for each protein. But interestingly, all the protein expression alterations we observed should favor NO/cGMP-mediated relaxation by increasing the cGMP level in the smooth muscle cell. However, this is not what was observed in the pharmacological experiments, which showed a decreased NO-induced relaxation. It is therefore likely that these pro-relaxing alterations are a response to compensate for a decreased relaxation ability of the IUGR veins, probably due to other alterations elsewhere in the pathway.

Further experiments will therefore have to assess the consequence of the observed protein content alterations on cGMP level. This second messenger was reported to be decreased in IUGR HUVEC (28) but increased in preeclampsia HUVEC (37). It would be interesting to assess cGMP level in the smooth muscle where it can yield relaxation. Therefore separate cell cultures of endothelium and smooth muscle cells would be useful. These could also be used to assess whether the alterations we described above are present predominantly in the endothelium, in the smooth muscle or in both of these tissues. Other isoforms of the tested proteins should be tested too, as these could replace each other and assume their function, and thus affect cGMP levels. Moreover, other potential alterations further downstream in the pathway should be investigated. The first of them would be PKG which, when activated by cGMP, affects intracellular calcium levels and membrane polarisation and thus promotes relaxation. It would also be interesting to assess possible changes in myosin light chain kinase phosphorylation, secondary to PKA activity regulated by cAMP level, which is dependent, among others, of PDE activity. Other vasorelaxation-inducing pathways, as the prostaglandin or the EDHF pathways, should be investigated too.

Furthermore it would be interesting to assess if such alterations of relaxation pathways are also present in the umbilical arteries of growth-restricted individuals, giving an insight of possible general changes in the organism's vessels, which could contribute to development of cardiovascular

disease much later in life. Umbilical vessels from male newborn boys should be tested too. Additionally, the contraction pathways should also be tested in umbilical veins and arteries. Alteration in structure, composition and protein expression in the Wharton's jelly could also have an influence on the reactivity of umbilical vessels and should therefore be tested.

Acknowledgements

Bibliography

1. Romo A, Carceller R, Tobajas J. Intrauterine growth retardation (IUGR): epidemiology and etiology. Pediatr Endocrinol Rev. 2009 Feb;6 Suppl 3:332-6.

2. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med. 2008 Jul 3;359(1):61-73

3. Barker DJ. In Utero Programming of Cardiovascular Disease. Theriogenology 53, nº 2 (15 janvier 2000): 555-574.

4. Godfrey KM, Barker DJ. Fetal nutrition and adult disease. Am J Clin Nutr. 2000 May; 71(5 Suppl):1344S-52S.

5. Dyer JS, Rosenfeld CR. Metabolic imprinting by prenatal, perinatal, and postnatal overnutrition: a review. Semin Reprod Med. 2011 May;29(3):266-76

6. Barker DJ. The fetal origins of diseases of old age. Eur J Clin Nutr. 1992 Oct;46 Suppl 3:S3-9

7. Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. N Engl J Med. 1976 Aug 12;295(7):349-53.

8. Thompson RF, Einstein FH. Epigenetic basis for fetal origins of age-related disease. J Womens Health (Larchmt). 2010 Mar;19(3):581-7.

9. Gluckman PD, Hanson MA. Living with the Past: Evolution, Development, and Patterns of Disease. Science 17 September 2004: Vol. 305 no. 5691 pp. 1733-1736

10. Waterland RA, Garza C. Potential mechanisms of metabolic imprinting that lead to chronic disease. American journal of clinical nutririon 1999 Feb;69(2):179-97.

11. Wareing M, Greenwood SL, Fyfe GK, Baker PN. Reactivity of human placental chorionic plate vessels from pregnancies complicated by intrauterine growth restriction (IUGR). Biol Reprod. 2006 Oct;75(4):518-23.

12. Wareing M, Greenwood SL, Baker PN. Reactivity of human placental chorionic plate vessels is modified by level of oxygenation: differences between arteries and veins. Placenta 2006 Jan;27(1):42-8.

13. Lackman F, Capewell V, Gagnon R, Richardson B. Fetal umbilical cord oxygen values and birth to placental weight ratio in relation to size at birth. Am J Obstet Gynecol. 2001 Sep;185(3):674-82.

14. Hampl V, Bíbová J, Straník Z, Wu X, Michelakis ED, Hashimoto K, Archer SL. Hypoxic fetoplacental vasoconstriction in humans is mediated by potassium channel inhibition. Am J Physiol Heart Circ Physiol. 2002 Dec;283(6):H2440-9. Epub 2002 Aug 22.

15. Marino M, Bény JL, Peyter AC, Bychkov R, Diaceri G, Tolsa JF. Perinatal hypoxia triggers alterations in K+ channels of adult pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol. 2007 Nov;293(5):L1171-82.

16. Burkhardt T, Matter CM, Lohmann C, Cai H, Lüscher TF, Zisch AH, Beinder E. Decreased umbilical artery compliance and igf-I plasma levels in infants with intrauterine growth restriction - implications for fetal programming of hypertension. Placenta 2009 Feb;30(2):136-41.

17. Lee G, Oka M, Statwick J, Arroyo J, Galan H. Vasoconstriction in umbilical arteries at term affected by meclofenamate and nitro-L-arginine in an ovine model of intrauterine growth restriction. Am J Obstet Gynecol. Volume 204, Issue 1, Supplement, January 2011, Pages S165–S166

18. Lee G, Oka M, Statwick J, Arroyo J, Galan H. Bradykinin-induced relaxation is enhanced in isolated umbilical arteries from an ovine model of IUGR at term. Am J Obstet Gynecol. Volume 204, Issue 1, Supplement, January 2011, Pages S165–S166

19. Peyter AC, Delhaes F, Baud D, Vial Y, Diaceri G, Hohlfeld P, Tolsa JF. Intrauterine growth restriction is associated with structural and functional alterations in human umbilical vein. Submitted, 2014

20. Santos-Silva AJ, Cairrão E, Morgado M, Alvarez E, Verde I. PDE4 and PDE5 regulate cyclic nucleotides relaxing effects in human umbilical arteries. Eur J Pharmacol. 2008 Mar 17;582(1-3):102-9.

21. Melamed N, Yogev Y, Glezerman M. Fetal gender and pregnancy outcome. J Matern Fetal Neonatal Med. 2010 Apr;23(4):338-44.

22. Radulescu L, Ferechide D, Popa F. The importance of fetal gender in intrauterine growth restriction. J Med Life 2013 Mar 15;6(1):38-9.

23. Gordon L, Joo JH, Andronikos R, Ollikainen M, Wallace EM, Umstad MP, Permezel M, Oshlack A, Morley R, Carlin JB, Saffery R, Smyth GK, Craig JM. Expression discordance of monozygotic twins at birth: effect of intrauterine environment and a possible mechanism for fetal programming. Epigenetics 2011 May;6(5):579-92.

24. Mauro A, Buscemi M, Provenzano S, Gerbino A. Human umbilical cord expresses several vasoactive peptides involved in the local regulation of vascular tone: protein and gene expression of Orphanin, Oxytocin, ANP, eNOS and iNOS. Folia Histochemica et Cytobiologica 2011;49(2):211-8.

25. Krause B J, Carrasco-Wong I, Caniuguir A, Carvajal J, Farías M, Casanello P. Endothelial eNOS/arginase imbalance contributes to vascular dysfunction in IUGR umbilical and placental vessels. Placenta 34, n° 1 (janvier 2013): 20-28.

26. Casanello P, Krause B, Torres E, Gallardo V, González M, Prieto C, Escudero C, Farías M, Sobrevia L. Reduced l-Arginine Transport and Nitric Oxide Synthesis in Human Umbilical Vein Endothelial Cells from Intrauterine Growth Restriction Pregnancies is Not Further Altered by Hypoxia. Placenta 30, n° 7 (juillet 2009): 625-633.

27. Pisaneschi S, Strigini FA, Sanchez AM, Begliuomini S, Casarosa E, Ripoli A, Ghirri P, Boldrini A, Fink B, Genazzani AR, Coceani F, Simoncini T. Compensatory feto-placental upregulation of the nitric oxide system during fetal growth restriction. PLoS One 2012;7(9):e45294.

28. Casanello P, Sobrevia L. Intrauterine growth retardation is associated with reduced activity and expression of the cationic amino acid transport systems y+/hCAT-1 and y+/hCAT-2B and lower activity of nitric oxide synthase in human umbilical vein endothelial cells. Circulation Research 2002 Jul 26;91(2):127-34.

29. Rafikov R, Fonseca FV, Kumar S, Pardo D, Darragh C, Elms S, Fulton D, Black SM. eNOS activation and NO function: Structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity. J Endocrinol. 2011 Sep;210(3):271-84.

30. Talapka P, Bódi N, Battonyai I, Fekete E, Bagyánszki M. Subcellular distribution of nitric oxide synthase isoforms in the rat duodenum. World J Gastroenterol. 2011 Feb 28;17(8):1026-9.

31. Lluis JM, Buricchi F, Chiarugi P, Morales A, Fernandez-Checa JC. Dual role of mitochondrial reactive oxygen species in hypoxia signaling: activation of nuclear factor-{kappa}B via c-SRC and oxidant-dependent cell death. Cancer Res. 2007 Aug 1;67(15):7368-77.

32. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J Biol Chem. 1998 Jul 17;273(29):18092-8.

33. Thompson JA, Regnault TR. In utero origins of adult insulin resistance and vascular dysfunction. Semin Reprod Med. 2011 May;29(3):211-24.

34. Chang YL, Wang CN, Wei PC, Peng HH, Chao AS, Chang SD, Cheng PJ, Wang TH. Mitochondrial activation in the growth-restricted fetus of monochorionic twins. Fertil Steril. 2013 Jul;100(1):241-6.e1-2.

35. Liu J, Chen D, Yao Y, Yu B, Mao X, He J, Huang Z, Zheng P. Intrauterine growth retardation increases the susceptibility of pigs to high-fat diet-induced mitochondrial dysfunction in skeletal muscle. PLoS One 2012 ;7(4):e34835.

36. Derbyshire ER, Marletta MA. Structure and regulation of soluble guanylate cyclase. Annual Review of Biochemistry 2012;81:533-59.

37. Steinert JR, Wyatt AW, Poston L, Jacob R, Mann GE. Preeclampsia is associated with altered Ca2+ regulation and NO production in human fetal venous endothelial cells. Faseb Journal 2002 May;16(7):721-3.

38. Kass DA, Champion HC, Beavo JA. Phosphodiesterase type 5: expanding roles in cardiovascular regulation. Circulation research 2007 Nov 26;101(11):1084-95.