Autophagy and human parturition: evaluation of LC3 expression in placenta from spontaneous or medically induced onset of labor.

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Abstract

Induction of labor is one of the most used procedure in obstetrics, performed to achieve vaginal delivery through cervical ripening and stimulation of uterine contractions. We investigated on the impact of induction of labor upon placental autophagy, a catabolic pathway activated in response to alteration of the physiological intracellular conditions. We collected 28 singleton placentas at the time of uncomplicated term vaginal delivery (7 spontaneous onset of labor, 21 induced labor). Autophagy was evaluated by immunohistochemistry and immunoblotting. No significant difference in the autophagy expression was found between spontaneous or induced onset of labor. We found an inverse relationship between autophagy expression and the maternal pre-pregnancy body mass index, irrespective of the mode of labor onset. This results could be related to the nutritional maternal habits before and throughout pregnancy rather than rapid metabolic changes during labor.

Key words: autophagy, placenta, labor, LC3, HIF, CRF, body mass index

Introduction

Autophagy is an inducible, intracellular catabolic pathway by which organelles or portion of cytoplasm are sequestered in autophagosomes, a double-membrane vesicles that fuse with lysosome to allow material breakdown and recycling [1].

In uncomplicated term pregnancies we have previously shown that autophagy is increased in placentas from cesarean when compared to vaginal delivery [2]; other studies have demonstrated higher levels of autophagy in pregnancies complicated by preeclampsia [3], intrauterine growth restriction [4, 5, 6] or both [4], when compared to normal pregnancies. In these studies placentas were collected at the time of elective cesarean section in both uncomplicated and complicated pregnancies.

Induction o labor is a procedure widely used in obstetrics, even though a number of common indications to induction have insufficient evidence to guide practice [7]. Nevertheless, as the procedure in the majority of cases leads to vaginal delivery, in the United States it has been estimated that approximately 1 in 4 women are inducted for maternal and/or fetal benefit [8]. Pharmacological induction of labor is an iatrogenic interruption of the uterine quiescence; autophagy is a process that respond to environmental changes and hormonal stimuli [9]. However, thus far, the impact of induction of labor upon placental autophagy has not been investigated even though placental autophagy itself is attracting the interest of researchers for its possible implications in maternal fetal medicine. For this reason we underwent this study with the aim to investigate on the relationship between autophagy and induction of labor; our hypothesis was that placental autophagy could be increased in cases of pharmacological induction, therefore we evaluated the expression of autophagy markers in term placentas from vaginal deliveries after spontaneous or induced labor.

2. Materials and methods

2.1 Cases selection and sample collection

28 normal shaped, singleton placentas were obtained at the time of uncomplicated term vaginal delivery from non-smoking women with uneventful pregnancies. Seven placentas were from women with spontaneous onset of labor [group SP], while 21 placentas were collected from women with induction of labor performed according to the Bishop score: 7 cases with prostaglandin E2 only [group PG], 7 with oxytocin only [group OX], 7 with prostaglandin E2 followed by oxytocin [group PO]. Cases selection was made matching patients for maternal and fetal characteristics: maternal age, pre-pregnancy body mass index and neonatal birth-weight were similar between the four groups.

No woman received medications during pregnancy and/or epidural analgesia during labor.

In each woman we measured: the time to delivery; umbilical arterial blood gases (pO_2, pCO_2), pH, base excess, lactate and glucose concentration from a doubly clamped portion of the cord with a Radiometer ABL 700 Analyzer; the weight of the placenta after trimming of the fetal membranes and umbilical cord and after removal of obvious blood clots; the longest diameter of the surface (D1) and its perpendicular diameter (D2), measured with a plastic ruler placed on the fetal surface; from these two measurements we calculated the placental surface area, assuming an elliptical surface, with the formula: D1 x D2 x $\pi/4$; we also calculated the ratio between fetal and placental weight in grams, as the F/P ratio.

Samples from grossly unremarkable placental parenchyma were collected immediately after delivery: full-thickness sections were selected and stored in 10% formalin solution for further immunohistochemical investigation; samples midway between the chorionic and basal plates were washed in phosphate-buffered saline solution to clear maternal blood, immediately frozen in liquid nitrogen, and stored at -80°C for further processing of protein extraction and western blotting. Each

104 placental section was sampled randomly in a site midway between the cord insertion and the
105 periphery.

106 *2.2 Investigated markers*

107 Placental autophagy expression was investigated utilizing *Microtubule-associated protein light*
108 *chain 3 (LC3)*. LC3 is the mammalian homologue of yeast Atg8 and intervenes in the late stage of
109 autophagosome formation, particularly LC3-II, the membrane bound autophagic vescicle-associate
110 form, that represents the phosphatidylethanolamine conjugated product of LC3-I that is obtained
111 after LC3 activation [10]. For its role during the autophagosome genesis, LC3-II is commonly used
112 as a specific marker of autophagy [11].

113 Placental *Corticotropin-releasing hormone (CRF)* secretion is a marker of the timing of human
114 parturition and delivery [12, 13]: placental expression of CRF and its relationship with LC3-II were
115 investigated to detect any changes of autophagy expression related to placental hormonal changes in
116 spontaneous or induced labor.

117 Placental *Hypoxia Inducible Factors (HIF) -1α,* is a transcription factor regulating the cellular
118 response to hypoxia [14]: the expression of HIF-1α and its relationship with LC3-II were assessed
119 to verify whether induction of labor might increase the level of placental hypoxia and, in turn affect
120 autophagy.

121 *2.3 Immunohistochemistry*

122 Immunohistochemical studies were carried out on 4 μm thick tissue sections from formalin-fixed
123 paraffin-embedded tissues samples, using a Novolynk Polymer Detection System (Novocastra
124 Laboratories) with primary rabbit polyclonal anti-LC3 antibody (NB100-2220, Novus Biologicals),
125 rabbit polyclonal anti-CRF antibody (CRF, FL-196, Santa Cruz Biotechnology) and rabbit
126 polyclonal anti-HIF-1α antibody (HIF-1α, H-206, Santa Cruz Biotechnology).

127 Sections were deparaffinized in bioclear for 20 minutes than washed twice in ethanol. Antigen-
128 retrieval bath containing 0,25 mM di EDTA at pH 8 for 30 minutes at 95C° was used for CRF and

HIF-1α whereas bath containing 9 mM sodium citrate at pH 6.0 for 30 min at 95°C was used for LC3. Endogenous peroxidase activity was quenched with 3% H_2O_2 in distilled H_2O. Staining was performed with diaminobenzidine and fast red as a chromogen. For LC3, CRF and HIF-1α staining, the primary antibody was applied at the dilution of 1:500, 1:75 and 1:75 respectively and incubated overnight at 4°C. Slides with absence of the primary antibody were included as negative controls. Slides were immunostained in the same batch, to ensure identical condition for comparison.

2.4 Western blotting

The 14000xg supernatant from homogenized samples was diluted with loading buffer, boiled and stored at -20°C. 50 µg of proteins were separated on 15% or 6% polyacrylamide gels (depending on the molecular weight of the markers studied), and transferred onto nitrocellulose.

LC3-I (cytosolic form,18 kDa) and LC3-II (membrane bound form, 16 kDa) were identified using primary rabbit monoclonal (LC3B antibody, Cell Signaling, dilution 1:1000).

CRF (25 kDa) was identified using primary rabbit polyclonal antibody (CRF, FL-196 antibody, Santa Cruz Biotechnology, dilution 1:500).

HIF-1α (120 kDa) was identified using primary rabbit polyclonal antibody (HIF-1α, H-206X antibody, Santa Cruz Biotechnology, dilution 1:300).

After washing, the blots were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, dilution 1:10000). α Tubulin was used for data normalization (α Tubulin (E-19)-R antibody, Santa Cruz Biotechnology, dilution 1:1000). Bands were visualized by LiteAblot reaction (EuroClone) and quantified (OD/mm^2) by Quantity One 4.2.1 image analysis software (Bio-Rad).

2.5 Statistical analysis

Clinical data are expressed as mean ± standard deviation (SD). Densitometric analysis of immunoblots are reported as mean and standard error of the mean (SEM). Clinical characteristics were compared by the Student t test for unpaired samples. The Kruskal-Wallis test was used to

compare LC3-II, CRF and HIF-1α protein levels in placental samples. P values <0.05 were considered significant. Statistical test were performed using Instat 3, GraphPad software.

3. Results

3.1 Clinical characteristics

Maternal age, BMI, gestational age at delivery, newborn and placental weight, placental surface, F/P ratio, umbilical arterial blood parameters and time to vaginal delivery were similar in the three group of women undergoing labor induction, thus the data were pooled together and are presented in table 1, compared to those obtained in women in spontaneous labor. As expected time to vaginal delivery was significantly shorter (p=0.03) in women with spontaneous labor.

3.2 Autophagy localization and expression

By immunohistochemistry we detected cytoplasmic staining of LC3 in the amniotic epithelium, in the villous vessels, in villous syncytio- and cyto-trophoblasts, in decidual stromal cells and in extravillous trophoblasts (Figure 1A, 1B). The spatial distribution of staining were the same in cases with spontaneous and induced labor.

Figure 1C shows the expression of LC3-II according to the mode of the onset of labor: no significant difference was found among groups.

3.3 Correlation between autophagy and CRF and HIF-1α

The pattern of immunostaining for CRF and HIF-1α was the same that for LC3 (Figure 1D-G), but not the intensity: HIF-1α staining was weaker than LC3 and CRF staining.

Figure 1H-I shows the expression of CRF and HIF-1α according to the mode of labor onset. Similarly to LC3-II, HIF-1α expression was not significantly different between any of the groups. On the contrary, as expected, a significantly higher expression of CRF was detected in placentas

177 from spontaneous onset of labor; however, we found no significant relationship between the level of
178 CRF and LC3-II (Figure 1M).

179 *3.4 Correlation between clinical characteristics and autophagy expression*

180 We found no relationship between LC3-II expression and any of the clinical parameters (time to
181 vaginal delivery; maternal and fetal characteristics) with the exception of pre-pregnancy BMI: as
182 BMI increased, placental autophagy expression decreased ($p<0.005$, Figure 1N).

183

184 **Discussion**

185 Autophagy is a Greek term coined by Christian DeDuve to indicate "self-eating" [15]. During the
186 autophagic process, macromolecules (such as sugars, proteins, lipids) and organelle (such as
187 mitochondria) are degraded by lysosome to warrant a cellular adaptive response during
188 compromised conditions [16]. In our previous work, in uneventful term pregnancies, we
189 demonstrated a higher autophagy expression in placentas obtained from cesarean section than from
190 vaginal delivery [2]. This suggested that the mode of delivery *per sè*, or any other factor linked to it,
191 such as fasting before cesarean section, could affect the expression of the markers of autophagy. In
192 other words, the presence of a healthy mother and newborn at the end of an uneventful pregnancy is
193 not sufficient to consider the level of autophagy in placenta as a "basal" level and the mode of
194 delivery should be taken into account when comparing placentas from normal and abnormal
195 pregnancies. For this reason we were interested in investigating the influence, if any, of the mode of
196 onset of labor on autophagy expression.

197 According to expectation [17], placental CRF expression in induced labor was lower than in
198 spontaneous onset; however no significant relationship was found with LC3-II expression.

199 Pharmacological induction of labor aims to achieve vaginal delivery through the processes of
200 cervical ripening and onset of uterine contractions. Synthetic prostaglandin E2 mimics the natural
201 process of cervical softening through collagen breakdown and movement of an inflammatory

infiltrate into the cervix [18]. Synthetic oxytocin is chemically identical to the endogenous form and stimulates uterine contractions when administered continuously by intravenous infusion [19], while the endogenous oxytocin is released in pulsatile manner.

We hypothesized a possible interference on the placental oxygenation by "artificial" contraction, resulting in a modification of the autophagy expression. In our population, cases with induction of labor had achieved the neonatal birth after a longer time to vaginal delivery. It is known that during labor, blood flow to the intervillous space is intermittent due to the interruption of the diastolic flow of the spiral arteries at the pick of the uterine contraction [20]. In placentas obtained after labor, many markers of injury from hypoxia-reoxygenation have been detected, suggesting the presence of an oxidative stress [21,22,23] and autophagy can be induced by oxidative stress [24]. Our *a priori* hypothesis was that induction of labor can increase autophagy in placenta, actually we did not observed significant differences either in LC3 localization or LC3-II expression between cases with spontaneous labor and cases induced with synthetic oxytocin and/or prostaglandin; no significant relationship was also found between LC3-II and the time to vaginal delivery. Moreover, we observed similar pO2 and pCO2 level in umbilical artery between groups, consequently no differences in HIF-1α expression were found according to the mode of onset of labor. Hypoxia stabilizes HIF-1 and HIF-1 is a major regulator of the cellular response to hypoxia [10]; moreover hypoxia can induce autophagy [25,26]. Therefore we speculated that similar levels of oxidative stress and hypoxia can be present in spontaneous and pharmacologically induced labor, although this hypothesis needs to be confirmed.

An interesting results of our study was to find a tight inverse relationship between LC3-II expression and the maternal pre-pregnancy body mass index: as BMI increased, placental autophagy decreased, irrespective of the mode of labor onset. A possible explanation of this findings might reside in the nutritional maternal habits before and throughout pregnancy rather than in rapid metabolic changes during labor, since we found no difference in umbilical arterial glucose concentrations in the groups of women.

In conclusion, this is the first study assessing the influence of the mode of the onset of labor on placental expression of LC3. Our results suggest that autophagy expression is unaffected by the pharmacological induction of labor.

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Table 1. Clinical characteristics and autophagy

	Spontaneous labor *(n=7)*	**Induced labor** *(n=21)*
Maternal age *(years)*	29.7±5.8	31.9±5.3
Gestational age *(weeks)*	39.0±0.8	39.9±1.2
Maternal pre-pregnancy BMI *(kg/m^2)*	22.6±3.7	22.5±2.9
Neonatal birth-weight *(grams)*	3271.4±499.3	3412.0±354.6
Placental weight *(grams)*	434.6±90.1	441.3±78.7
F/P ratio	7.6±1.1	7.9±1.3
Placental surface *(mm^2)*	199.2±55.9	219.1±58.8
Time to parturition *(minutes)*	276.7±499.3	660.2±388.5 *
pH	7.25±0.09	7.25±0.03
BE (*mmol/l)*	-5.4±2.8	-4.3±2.5
Lac (*mmol/l)*	6.1±2.7	5.2±1.8
pO2 (*mmHg)*	25.4±5.5	20.5±6.8
pCO2 (*mmHg)*	50.4±11.8	51.1±6.4
Glu (*mmol/l)*	4.9±0.9	5.1±1.2
LC3-II /α-Tubulin *(ODu x mm^2)*	1.4±0.3	1.1±0.1

BMI= body mass index, F/P ratio= ratio between fetal weight at birth and placental weight, BE= base excess, Lac= lactate, Glu=glucose, ODu= optical density unit.

*p= 0.03 vs spontaneous labor

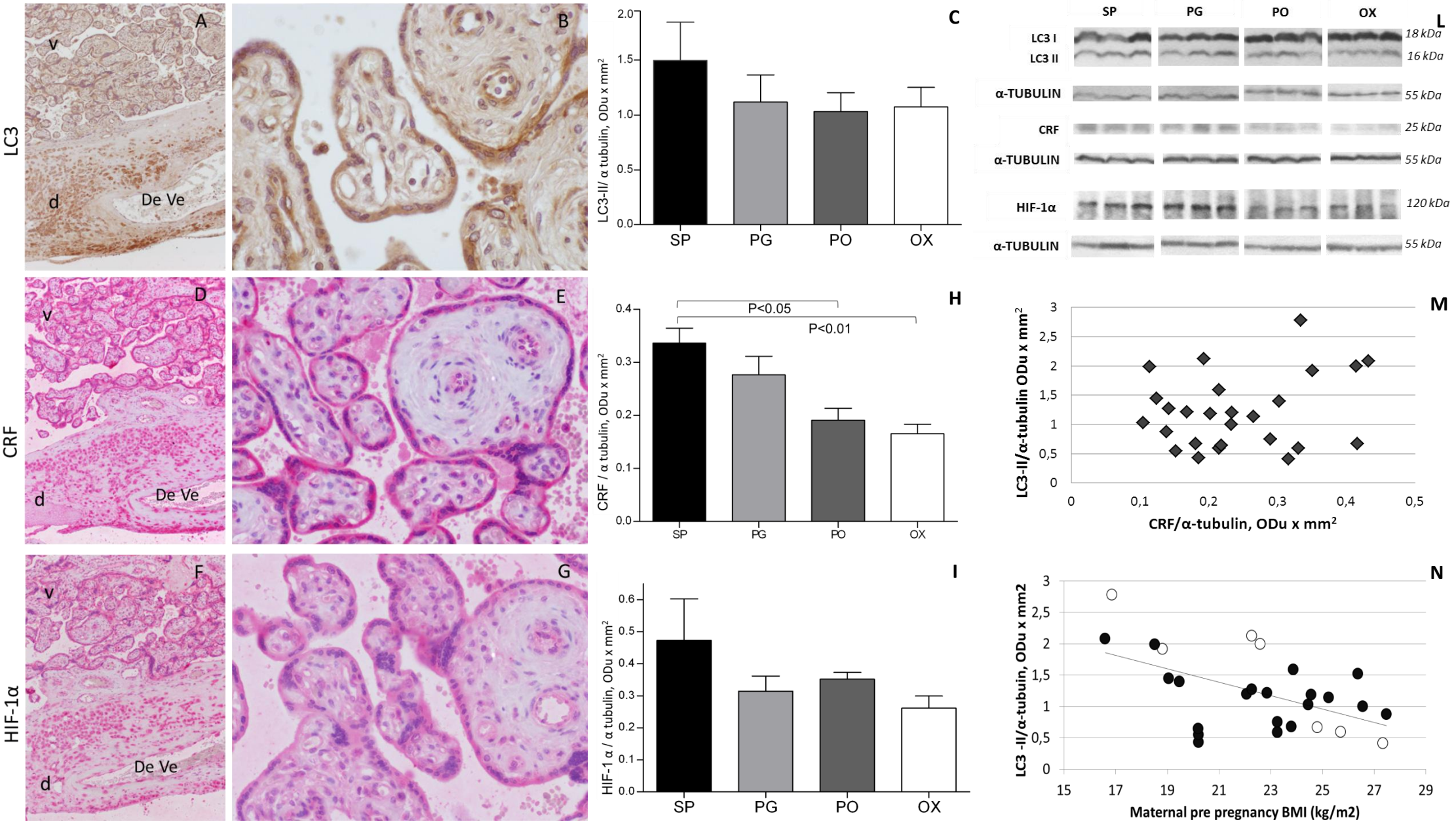
Figure legends

Figure 1. Autophagy in placenta from term uneventful pregnancy and its correlation with clinical, biochemical and hormonal characteristics of our population.

The immunohistochemical staining shows that LC3, CRF and HIF-1α have an overlapping localization in villous and extravillous trophoblast. d= decidua, v= villi, DeVe= decidual vessel (A, B: Immunohistochemical LC3 expression, original magnification 10x and 40x respectively; D, E: Imunohistochemical CRF expression; original magnification 10x and 40x respectively; F, G: Immunohistochemical HIF-1α staining; original magnification 10x and 40x respectively).

The analysis of data from Optical Density values of Western blotting bands shows no differences of LC3-II (C,L) and HIF-1 α (I,L) expression between groups. On the contrary CRF is higher in spontaneous labor (H,L). LC3-II, HIF-1 α and CRF are normalized onto α-tubulin (L).
SP= spontaneous labor, PG= induction with prostaglandin, PO= induction with prostaglandin and oxytocin, OX= induction with oxytocin.

We found no correlation between LC3-II and CRF expression (M).
A significant correlation was found between LC3-II expression and pre-pregnancy maternal body mass index (N). open circles: spontaneous onset of labor, closed circles: pharmacological induction of labor. LC3-II= 3.6-0.11BMI. R^2 0.29. p=0.003.


LC3
CRF
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A
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LC3-II/ α tubulin, ODu x mm²
SP
PG
PO
OX
LC3 I
LC3 II
18 kDa
16 kDa
α-TUBULIN
55 kDa
CRF
25 kDa
HIF-1α
120 kDa
P<0.05
P<0.01
CRF / α tubulin, ODu x mm²
LC3-II/α-tubulin ODu x mm²
CRF/α-tubulin, ODu x mm²
HIF-1 α / α tubulin, ODu x mm²
LC3 -II/α-tubuin, ODu x mm2
Maternal pre pregnancy BMI (kg/m2)