Changes in transcription pattern lead to a marked decrease in COX, CS and SQR activity after the developmental point of the 22nd gestational week

Kolarova H[1], Krizova J[1], Hulkova M[1], Hansikova H[1], Hulkova H[2], Smid V[3], Zeman J[1], Honzik T[1] and Tesarova M[1]

[1]Department of Pediatrics and Adolescent Medicine, [2]Institute of Inherited Metabolic Disorders and [3]Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

Corresponding author:

Ing. Markéta Tesařová, Ph.D., Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Ke Karlovu 2, Prague 2, 120 00, Czech Republic

Phone: +420-2-24967748

Fax: +420-2-24967099

e-mail: marketa.tesarova@lf1.cuni.cz

Short title: COX, CS and SQR changes follow the 22nd gestational week

Summary

Background: Tissue differentiation and proliferation throughout foetal development interconnect with changes in the Oxidative Phosphorylation System (OXPHOS) on the cellular level. Reevaluation of the expression data revealed a significant increase in *COX4* and *MTATP6* liver transcription levels after the 22nd gestational week (GW) which inspired us to characterize its functional impact.

Methods: Specific activities of cytochrome *c* oxidase (COX), citrate synthase (CS), succinate-coenzyme Q reductase (SQR) and mtDNA determined by spectrophotometry and RT-PCR were studied in a set of 25 liver and 18 skeletal muscle samples at 13th to 29th GW. Additionally, liver haematopoiesis (LH) was surveyed by light microscopy.

Results: The mtDNA content was positively correlated with the gestational age only in the liver. The activities of COX, CS and SQR in both liver and muscle isolated mitochondria significantly decreased after the 22nd GW in comparison with earlier GW. A continuous decline of LH, not correlating with the documented OXPHOS-specific activities, was observed from the 14th to the 24th GW indicating their exclusive reflection of liver tissue processes.

Conclusion: Two apparently contradictory processes of increasing mtDNA transcription and decreasing OXPHOS-specific activities seem to be indispensable for rapid postnatal adaptation to high energy demands. The inadequate capacity of mitochondrial energy production may be an important factor in the mortality of children born before the critical developmental point of the 22nd GW.

Keywords: human foetal development, mitochondrial biogenesis, liver, skeletal muscle, mtDNA

Abbreviations: GW, gestational week; LH, liver hematopoiesis; OXPHOS, oxidative phosphorylation system; mtDNA, mitochondrial DNA; COX, cytochrome *c* oxidase; CS, citrate synthase; SQR, succinate-coenzyme Q reductase.

Introduction

Mitochondrial biogenesis is a target of long-term interest in various physiological or pathological situations, such as endurance training or heart failure (Wallace 1992), with specific attention to its pharmacological modulation (Whitaker *et al.* 2016). However, the functioning of mitochondrial biogenesis during foetal development still represents a highly unexplored field with only a few relevant reports available to date. A better understanding of the molecular mechanisms involved in mitochondrial biogenesis during this extremely dynamic period may help us not only to improve the diagnosis of mitochondrial disorders but also to provide better care to very premature neonates (Wenchich *et al.* 2002, Honzik *et al.* 2008).

Studies on rat models in the early 80s postulated that the activities of respiratory enzymes increase in the course of the human development, and significant differences in activity rate occur between foetal and adult life in both tissue homogenates (Warshaw 1969, Mackler *et al.* 1970, Jakovcic *et al.* 1971, Sordahl *et al.* 1972) and in isolated mitochondria (Jakovcic *et al.* 1971, Pollak *et al.* 1975). We previously showed that the activities of several oxidative phosphorylation system (OXPHOS) enzymes in isolated mitochondria were significantly lower in immature neonates and suggested that the low functional activity of the mitochondria is responsible for their high mortality rate (Wenchich *et al.* 2002, Honzik *et al.* 2008). However, contrary to the previous observations, no positive correlation between OXPHOS activities and gestational age was found (Honzik *et al.* 2008, Pejznochova *et al.* 2010). Moreover, the extensive report on OXPHOS activities in homogenate samples originating from five different human tissues did not show any correlation with the age of gestation (Minai *et al.* 2008).

To explain the regulation of mitochondrial function on a genomic level, we performed a study of the expression pattern of several proteins involved in mitochondrial DNA (mtDNA)

transcription and regulation both in human foetal liver and skeletal muscle (Pejznochova *et al.* 2010). The increasing mitochondrial biogenesis as a preparation to postnatal life was demonstrated based on significant changes in mtDNA, mRNA and protein molecular levels throughout the studied period. These results were in accordance with the hypothesis that prenatal preparation leans mainly on a transcriptional level spearheading for more flexible post-transcriptional regulation early after birth (Cuezva 1997). Our data, however, were not considered in an organ-specific manner, and it was further indicated that developing tissues, especially liver and skeletal muscle, differed in the control of mitochondrial biogenesis depending on their specific energy demands and the phase of development (Pejznochova *et al.* 2010).

We further examined our expression data (Pejznochova *et al.* 2010) using the quadratic regression model and we revealed a significant increase in *COX4* and *MT-ATP6* transcription levels in the liver after the 22nd gestational week (GW) (Fig. 1.). This finding prompted us to further examine enzyme activities in two distinctly developing foetal tissues, liver and skeletal muscle. In the present study, the specific activities of three mitochondrial enzymes, cytochrome *c* oxidase (COX), citrate synthase (CS), succinate-coenzyme Q reductase (SQR), and mtDNA amount were studied in human foetal tissues between the 14th and 29th GW. Data on COX, CS and mtDNA during the human foetal development were already published in our previous report (Pejznochova *et al.* 2010). In the present work, a different statistical evaluation of enzyme activities is used. To determine whether ongoing liver haematopoiesis (LH) influences our data, we surveyed a consecutive series of foetal livers. Moreover, to determine whether marked changes of transcription capacity will be reflected in mitochondrial function, we decided to divide our results into two specific groups with a 22nd GW limit. To our knowledge, no study has described both OXPHOS and LH mass throughout this particular period.

Materials and methods

Ethics

The work described has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) after approval was granted by the Committee of Medical Ethics at the General University Hospital in Prague. Informed consent was obtained for experimentation with human samples.

Material

A set of 25 and 18 samples of human foetal liver and skeletal muscle, respectively, were collected subsequently to the termination of pregnancy for genetic indications unrelated to OXPHOS deficiency (Table 1). Informed consent was obtained from women prior to tissue samples collection. Small blocks of liver and skeletal muscle tissue were taken at autopsy not later than 60 min post-mortem and immediately frozen and stored at -80°C until used. The foetal gestational age varied from the 13th to 29th GW and there are 1−5 samples from each analysed GW in this period. The same set of liver and muscle samples was already used for our previous analyses (Pejznochova *et al.* 2010). However, in the latter paper, there was enough material for further analyses and the set was larger, including 26 liver and 25 muscle samples. In this paper, another 5 liver samples from GW 16th to 19th were included for additional analysis of haematopoietic activity (Table 1).

Isolation of mitochondria

Mitochondria from the liver and muscle were isolated from 5% tissue homogenates (w/v) prepared from frozen tissues according to the standard differential centrifugation procedure (Rickwood 1987). STE buffer was used for isolating liver tissue contained 0.25 M sucrose, 10

mM Tris-HCl, 2 mM EDTA, 2 μg/ml aprotinin, and 0.4 μM PMSF, pH 7.4; KTEA buffer for muscles contained 150 mM KCl, 50 mM Tris-HCl, 2 mM EDTA, and 2 μg/ml aprotinin, pH 7.5 (Makinen and Lee 1968). Freshly isolated mitochondria were used for activity evaluation and the rest of samples were kept at -80 °C for other analyses.

Respiratory enzyme activities

Activities of respiratory enzymes COX, SQR, and CS were measured spectrophotometrically in isolated mitochondria as described elsewhere (Rustin *et al.* 1994). Protein concentration was determined by the method of Lowry (Lowry *et al.* 1951).

MtDNA content

The quantification of mtDNA was executed by the method previously described in detail by Pejznochova *et al.* (2008).

Haematopoietic mass

Small blocks of liver tissue were fixed in 10% paraformaldehyde and embedded in paraffin. Serial ultrathin sections (6 µm) were cut and fixed doubly stained with haematoxylin and eosin.

Six representative sections from each foetal liver sample were chosen for light microscopy. The whole area of the section was systematically examined, and every third frame was photographed using stratified sampling method (Hamilton 1995). Five images were taken for each out of 6 sections, which led to a complete yield of 30 images for one foetal liver sample. All photographs were taken under constant conditions at 40× magnification of the objective.

The mass of LH was expressed as a proportion of haematopoietic islands to photographed field of view. The cell surface was quantified as the mean optical density of analysed areas as determined by the densitometric program cellSens Dimension 1.6 (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Vessels and fixation artefacts were excluded from the measurement. Considering mild variations of a preparation thickness and intensity of staining, the program adjustment of density evaluation for each preparation needed to be made.

Statistical methods

Correlation of selected observed variables was examined due to the lack of normality of the data, using a non-parametric Spearman correlation coefficient and a corresponding test of its statistical significance. Differences between groups of subjects were tested by a non-parametric two-sample Wilcoxon test. P-values less than 0.05 were considered as statistically significant. Analyses were conducted using R statistical package, version 3.1.1.

Results

Specific activities of the three mitochondrial enzymes COX, CS, and SQR and the quantity of mtDNA were studied in foetal tissues between 13th and 29th GW. In the present study, compared to previous results (Pejznochova *et al.* 2010), the quantity of mtDNA was positively correlated with the age of gestation only in the liver (p=0.011) and not in muscle, a fact we ascribe to the diminution of a sample set and the use of a different statistical evaluation (Fig. 2). To compare the activities of these enzymes in distinctly developing tissues, both liver and skeletal muscle samples were used for the analysis. We recognize that these two tissues represent a heterogeneous population of cell types, and our data need to be considered in this concept.

COX, CS and SQR specific activities as a function of gestational age

To determine whether there are age-dependent differences during the human development, the specific activities of COX, CS and SQR were determined in isolated mitochondria. Specific activities of COX were observed in the range of 5.22 - 580.89 $nmol.min^{-1}.mg^{-1}$ in the foetal liver, and in the foetal skeletal muscle, the range was $20.5 - 196.2$ $nmol.min^{-1}.mg^{-1}$. Activities of SQR ranged in interval $14.5 - 273.2$ $nmol.min^{-1}.mg^{-1}$ and $5.9 - 67.4$ $nmol.min^{-1}.mg^{-1}$ and for CS, it was $9.37 - 256$ $nmol.min^{-1}.mg^{-1}$ and $15.7 - 189.7$ $nmol.min^{-1}.mg^{-1}$ in liver and muscle, respectively. Although no significant correlation with the age of gestation was observed for all studied enzymes in both prenatal tissues, the profile of enzymatic activities shown in Fig. 3 indicates a similar pattern of decreasing specific activity in both tissues. The higher levels were consistently observed in the liver.

Group subdivision at the 22nd GW

According to the pattern of increasing gene expression after the 22nd GW (Fig. 1), this period was chosen as a critical point for a group subdivision. This way, we could evaluate specific activities and their ratios as a dependent variable of changes in the transcription rate. After dividing the data into two subgroups with the 22nd GW age limit, significant differences were revealed both in liver and muscle. The specific activities of COX, CS and SQR showed a significant decrease following the GW 22 in the liver (Fig. 4 A, C, E) (p=0.003, p=0.003 and p=0.005, respectively). Activities of both COX and CS in muscle also showed a significant decrease (p=0.003, p=0.034, respectively), but of a lesser magnitude, except for SQR, which did not significantly differ before and after the 22nd GW (Fig. 4 B, D, F).

Determination of haematopoietic mass

Haematopoiesis represents an important process that takes place in the developing foetal liver. LH was determined in liver samples of human foetuses at the 14th to 24th GW (Fig. 5). Altogether, 12 samples of the human foetal liver were examined by light microscopy. Twelve ultrathin tissue sections were examined under light microscopy. The amount of haematopoietic mass was expressed as a proportion of haematopoietic cells to the liver tissue.

A significant negative correlation was observed between the LH mass and gestational age (q=-0.602, p=0.042). Stem cells were observed only in the extravascular location; however, the mature cells migrated into the sinusoids and were not included in our evaluation. Haematopoiesis was fully established around the third month, and the haematopoietic islands occupied considerable spaces between the parenchymal cells, altering the architecture of hepatic cords (Fig. 5 A). Hepatic blood cell formation declined continually throughout the development from the maximum percentage of 15 % at the outset of the studied period and it was apparent that by the end of the second trimester, haematopoiesis diminished and became only focal (Fig. 5 B, C). Unusually high level of LH was found in three patients, which may be due to their primary diagnosis of oligohydramnios (Fig. 5 C). Moreover, there was no correlation between OXPHOS specific activities and LH mass (data not shown).

Discussion

Given its extreme time-specific variability, the period of foetal development represents an exciting but rather challenging field. Tissue differentiation and proliferation are interconnected with the changes of metabolism, including OXPHOS on the cellular level. The main objectives of this study were to further characterize several markers of mitochondrial biogenesis throughout the human foetal development with its time and tissue-specific changes.

COX is a terminal enzyme of the mitochondrial electron transfer chain determining the functional status of mitochondria. It represents a multicomponent structure with rather complicated biogenesis not only due to its dual genetic origin but also because of the existence of tissue and age-specific isoforms of its subunits (Bonne *et al.* 1993, Rohdich and Kadenbach 1993). The SQR or Complex II is the only complex encoded only by nuclear DNA. Both the tricarboxylic acid cycle and respiratory chain share its enzymatic function; therefore, it is a suitable factor informing us about the overall metabolic function of the cell, including the fatty acid β-oxidation. CS, a mitochondrial matrix enzyme, is one of the three regulatory steps of the tricarboxylic acid cycle and, similarly as SQR, it is frequently used as important normalization factor for OXPHOS enzymatic activities. However, CS seems to be inappropriate for this application when two different organs are being compared, as there exists important inter-tissue variability between its specific activity (Fernandez-Vizzara *et al.* 2011). According to our observations, CS and SQR also seem to be inappropriate normalization parameters or markers of mitochondrial biogenesis within the foetal tissues throughout their development (Fig. 6).

Human mtDNA are self-replicating circular molecules present in mitochondria in variable numbers of copies. MtDNA content is an important marker of mitochondrial biogenesis (Malik *et al.* 2011). It was previously shown that the quantity of mtDNA increases throughout the development (Heerdt and Augenlicht 1990). Early studies on the rat liver (10 – 21 days of gestation) or bovine heart models (first and third trimester) determined that also COX and SQR activities increased throughout foetal development in tissue homogenate (Jakovcic 1971, Mackler *et al.* 1971, Marin-Garcia 1994) or in isolated mitochondria (Jakovcic 1971, Pollak 1975). Moreover, Marin-Garcia *et al.* later proved that COX activity in the human heart homogenates significantly grows during foetal life (6th to 17th GW) (Marin-Garcia *et al.* 2000). We found no correlation of COX, CS and SQR enzymatic activities with

the age of gestation in both human liver and muscle isolated mitochondria within the second trimester of gestation. In both tissues, we observed high variability of enzymatic activities that can be explained by small number of samples in each GW and/or by heterogeneity of clinical conditions that led to pregnancy termination (Table 1). Nevertheless, none of clinical conditions was prevalent in any GW.

Interestingly, following the pattern of increasing gene expression, we could observe similar time-specific changes in both studied tissues after the 22nd GW. Specific activities of COX, CS and SQR were significantly lower in the group following the critical point of the 22nd week of development, although overall, the specific activities were lower in the muscle tissue (Fig. 3). At birth, the full switch from glycolysis to oxidative phosphorylation relies on the mitochondrial biogenesis (Valcarce *et al.* 1994). However, GW 22 to 23 seem to be a limit of viability (Ogawa *et al.* 2013). A profound change in the transcription pattern was observed for several COX subunits (Fig. 1 A, Mrhalkova *et al.* 2010) after the 22nd GW, and a similar observation was made for several ATP synthase subunits (Fig. 1 B, Mrhalkova *et al.* 2010). The expression level of mitochondrially encoded gene *MT-ATP6* was distinctively increasing after the 22nd GW in both the liver and muscle and a parallel increase of nuclearly-encoded *ATP5G2* was shown in liver, although compared to mitochondrially-encoded genes, levels of both *ATP5G2* and *ATP5O* were four-fold lower (Mrhalkova *et al.* 2010). High levels of mitochondrial mRNAs seem to be a mechanism to compensate the relatively less efficient translation compared to a high nuclear elongation rate, which might be caused by significantly greater ribosomal spacing (Thames *et al.* 2000). Lightowlers *et al.* (1990) suggested that it is either inefficient translation or the rapid turnover of COX8L protein that is responsible for the isoform switch within bovine heart tissue. Similarly, COX6AL destabilization led to increased mRNA degradation within skeletal muscle development. However, other COX subunits that do not undergo isoform switching, such as COX5A, were observed to be relatively stable up

to >15-20 h (Thames *et al.* 2000). The accumulation of transcripts must, therefore, be accomplished rather by increased transcriptional rate than the increased half-life of mRNAs (Izquierdo *et al.* 1995b, Ostronoff *et al.* 1996).

It is hypothesized that protein expression in the liver is exerted mainly after birth (Cuezva *et al.* 1997). Similarly, no major difference in protein levels or enzyme-specific activities of all respiratory complexes were noted in the study examining 5 foetal tissues (Minai *et al.* 2008). We could have clearly observed in the liver tissue a marked decrease in COX activity after the 22nd GW. However, at the same time, its transcript levels started increasing. From these inverse processes and considering the stability of COX subunit protein levels throughout the studied period, we can speculate that around the 22nd GW, possibly regulatory changes in COX (OXPHOS) biogenesis are proceeding especially on the posttranslational level.

Based on our results, we suggest that sufficient levels of mtDNA and mitochondrially encoded transcripts that are accumulated throughout gestation but mainly after the 22nd GW are crucial for the adequate onset of respiration after birth. Translational activity reflected in enzyme specific activity would rather stay behind the scene to establish more effective energy production early after birth.

In the work of Pejznochova *et al.*, it was suggested that the foetal liver and muscle differ in the control of mitochondrial biogenesis (Pejnochova *et al.* 2010). The correlation of mtDNA and COX specific activity informed us about the effectiveness of the mtDNA template utilization. We showed that there was a significant difference between liver and muscle when the enzyme specific activity and mtDNA are correlated (Fig. 6 A). A significant negative correlation between mtDNA and specific activity of COX is only present in the liver. One can presume that the gene dosage in the liver may be one of the factors affecting gene expression; however, it does not determine the final COX activity.

This is also supported by the fact that while mtDNA levels are higher in the muscle, the enzyme specific activities are significantly lower in the muscle than in the liver (Fig. 2, Fig. 3 A, B, C). These results are in line with the work of Fernandez-Vizzara *et al.*, who suggested that while in adult skeletal muscle, the gene dosage determines COX activity; in other tissues, including the adult liver, other levels of mtDNA expression regulation must have a more relevant contribution to the regulation of the final OXPHOS activity (Fernandez-Vizzara *et al.* 2011). Nevertheless, it only speaks in favour of the fact that similar mechanisms regulate mitochondrial gene expression in foetal and in adult organisms.

It is evident from our results that all of the studied enzymes follow the same trend. Similarly, the overall activity of CS and SQR did not change throughout the studied period in either liver or muscle, but it was significantly lower in the liver after the 22nd GW. The mitochondrial oxidation of fatty acids plays a critical role in energy metabolism after birth (Oey *et al.* 2005). However, it was only recently proven that β-oxidation is active also during foetal life, as it was long thought that the major energy supply comes from glucose. In the last trimester of intra-uterine life, fat accumulation becomes more important in the human foetus due to its important function in energy storage and temperature maintenance. Some of the deposited fatty acids will accrue from foetal lipogenesis, but the great bulk of foetal lipid is derived from the maternal circulation. This is further supported by observations in the placenta where the activity of long-chain 3-hydroxyacyl-CoA dehydrogenase and short-chain 3-hydroxyacyl-CoA dehydrogenase were inversely correlated with gestational age (Oey *et al.* 2005). In this way, SQR as a more multifunctional enzyme may follow the same trend as other enzymes of the respiratory chain, leaving space for more effective β-oxidation early after birth. We suggest that metabolic pathways work inter-connectively in the organism and that the enzymes of tricarboxylic acid cycle need to develop hand in hand with OXPHOS.

Although differences exist in the regulation of liver and muscle mitochondrial biogenesis, we presume that both tissues need to be prepared for increased energy demands after birth. The study of three cattle muscles suggested that hoofed animals are more mature relative to other mammals, as they get up and start to walk within hours after birth and the development of fast phasic muscle composed of type II glycolytic fibres is more important at birth (Gagniére *et al.* 1999). In a physiologic human neonate, hypertonia is a compensatory mechanism for the change of external conditions and the effect of gravity. Since postural muscles that keep the adequate muscle tonus consist mainly of type I oxidative fibres, rich in mitochondria, a sufficient OXPHOS capacity early after birth becomes crucial. This is further supported by the fact that in preterm infants with immature OXPHOS (Honzik *et al.* 2008), hypotonia is a frequent feature found at birth.

The studied parameters undergo important changes throughout the second trimester, especially in the liver. However, other specialized processes, such as LH, are limited to the foetal period, and their own mitochondrial functional status may be responsible for our observations. Throughout embryonic development, haematopoiesis represents a highly dynamic process, taking place in several different localizations. The blood formation in the human liver begins in embryos of approximately 12 mm in length, i.e., around the 6th GW (Minot *et al.* 1912) and it becomes fully established around the third month of intrauterine life (Zamboni 1965, Brugnara and Platt 2003). In our study, we observed a moderate, continuous decline of LH mass from the 14th to 24th GW, with a maximum of 15 % at the beginning of the studied period (Fig. 5). These results would indicate that participation of haematopoiesis in the overall liver metabolism is rather limited. This is further supported by the fact that haematopoietic cells in foetal liver rely mainly on glycolysis and have both low mitochondrial content and amounts of respiratory chain complexes (Imanirad *et al.* 2013, Simsek *et al.* 2010). Additionally, there were no correlations between OXPHOS-specific activities and LH

mass (data not shown). We concluded that the documented developmental changes of respiratory chain enzyme activities are not affected by concomitant haematopoiesis and, above all, reflect processes in liver tissue.

In conclusion, the period of foetal development represents an exciting but rather challenging field. With great developments in perinatal intensive care, the limit of viability was shifted back to GW 22 to 23 (Ogawa *et al.* 2013). A better understanding of the molecular mechanisms involved in mitochondrial biogenesis during this extremely dynamic period may help us in improving diagnostics and in providing better care of very premature neonates (Wenchich *et al.* 2002, Honzik *et al.* 2008). Within the course of the second trimester, profound tissue-specific changes of mitochondrial energy metabolism are underway. In our study, we observed that specific activities of COX, CS and SQR were significantly lower following the 22nd week of development; however, mtDNA levels continuously increased. These processes seem to be indispensable for rapid postnatal adaption and increasing energy demands. We conclude that the inability of OXPHOS to easily adapt to extrauterine life may be an important factor in the mortality of children born before this critical developmental point.

Conflict of interest: none

Legends to figures (in the order given in the attachment)

Table 1.

Fig. 1 Expression levels of *COX4* and *MT-ATP6* mRNA (a.u.; quadratic regression model). In the liver, there was a significant increase in *COX4* and *MT-ATP6* transcription levels after the 22nd week of gestation (p=0.001 and p=0.002, respectively). Adapted from Mrhalkova *et al.* 2010.

Fig. 2 Time-specific differences in mtDNA content per cell (a.u.) in the liver and skeletal muscle (Spearman's correlation). The quantity of mtDNA was positively correlated with the age of gestation only in the liver and not in the muscle (p=0.011).

black points - liver; blank points – skeletal muscle

Fig. 3

The relationship between the age of gestation and the enzyme-specific activities (nmol.min^{-1}.mg^{-1}) in mitochondrial fraction (Spearman's correlation). In liver, there was no significant correlation between gestational age and all three cytochrome *c* oxidases (COX) (p=0.342) (A), citrate synthase (CS) (p=0.161) (B) and succinate-coenzyme Q reductase (SQR) (p=0.124) (C). Insignificant results were also present in the muscle for all COX (p=0.082) (A), CS (p=0.216) (B) and SQR (p= 0.766) (C).

black points - liver; blank points – skeletal muscle

Fig. 4

Tissue-specific differences in COX, SQR and CS enzyme activities (nmol.min^{-1}.mg^{-1}) in isolated liver and muscle mitochondria divided into two groups with a critical point at the 22nd

gestational week (GW). In the foetal liver tissue, the specific activities of all studied enzymes, cytochrome c oxidase (COX), citrate synthase (CS) and succinate-coenzyme Q reductase (SQR), presented significant differences between the two categories (Wilcoxon test) (A, C, E). COX was the most age-dependent enzyme with a significant decrease within the groups in the liver (p=0.003) (A). Moreover, both CS and SQR demonstrated a similar significant decrease in the liver (p=0.003 and p=0.005, for CS and SQR, respectively) (C, E). In the muscle, the second group showed significantly lower levels after the 22nd GW for COX (p=0.003) (B) and CS (p=0.034) (D).

Fig. 5 The haematopoietic mass (%) in 12 liver samples in the course of foetal development from the 14th to 24th gestational week (GW) (Spearman's correlation). Haematopoiesis was fully established around the third month and the haematopoietic islands occupied considerable spaces between the parenchymal cells, altering the architecture of hepatic cords (A). Hepatic blood cell formation declined continually throughout the development, and by the end of the second trimester, it became only focal, with sparse haematopoietic islands (B, C). The correlation between haematopoiesis and gestational age presented with a significant negative decrease (q=-0.602, p=0.042) (C).

Fig. 6 The correlation between COX-, CS- and SQR-specific activity ($nmol.min^{-1}.mg^{-1}$) and mtDNA amount (a.u.) in both liver and muscle tissue. There was a significant difference between the studied tissues only for COX-specific activity (p=0.000) (A). A significant negative correlation between CS-specific activity, and the mtDNA content is present in both the liver (p=0.008) and muscle (p=0.049) (B). On the contrary, between COX- and SQR-specific activity and mtDNA amount it was present only in the liver (p=0.000, p=0.010) and not in the muscle tissue (A, C).

black points - liver; blank points – skeletal muscle

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WHITAKER RM, CORUM D, BEESON CC, SCHNELLMANN RG. Mitochondrial Biogenesis as a Pharmacological Target: A New Approach to Acute and Chronic Diseases. *Annu Rev Pharmacol Toxicol* **56**: 229-49, 2016.

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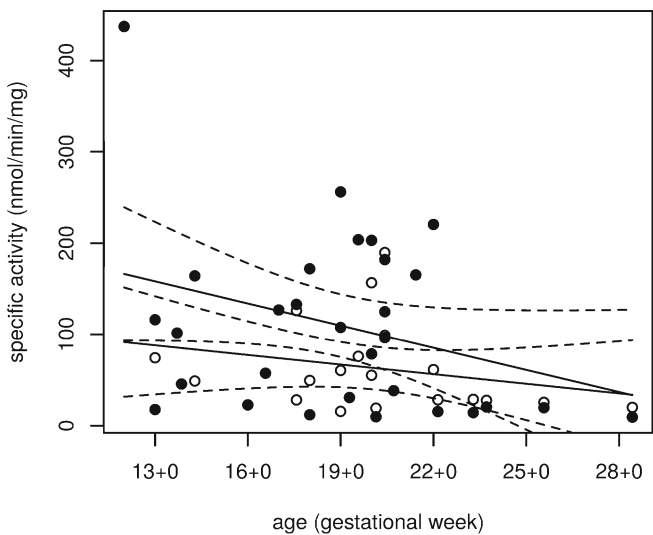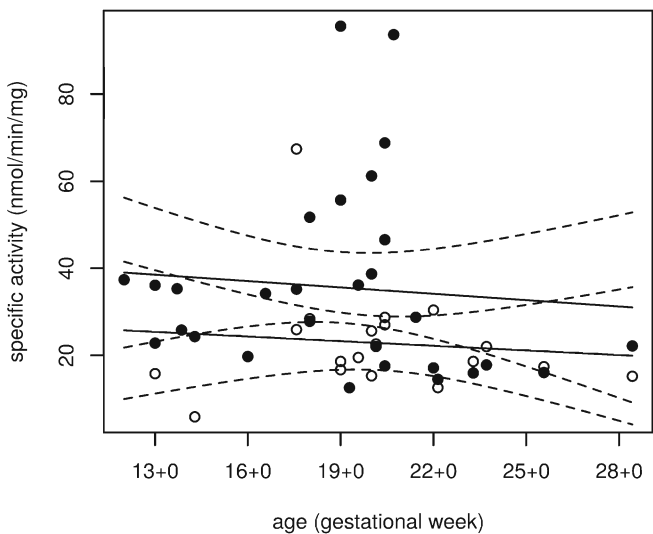Table 1. List of indications for pregnancy termination in individual foetuses.

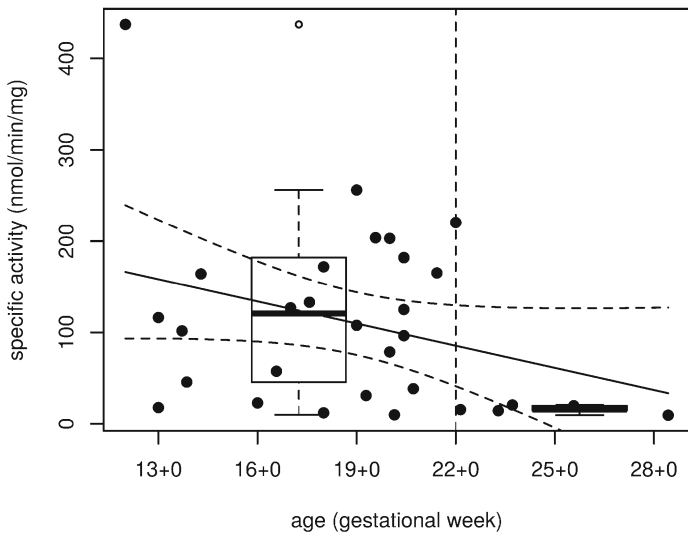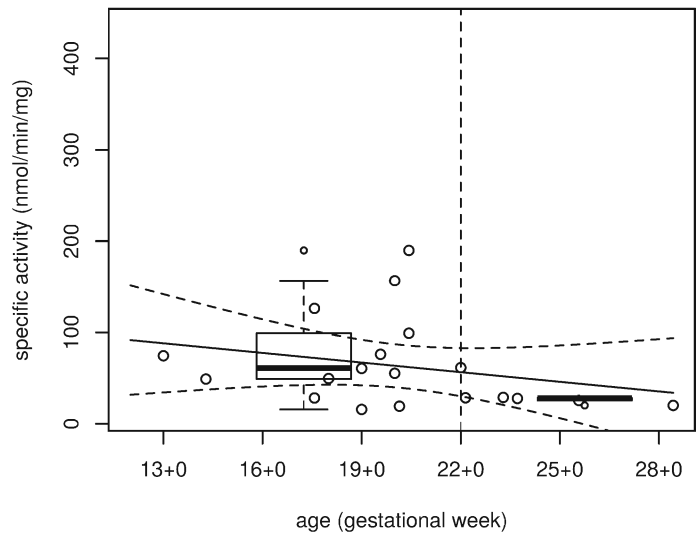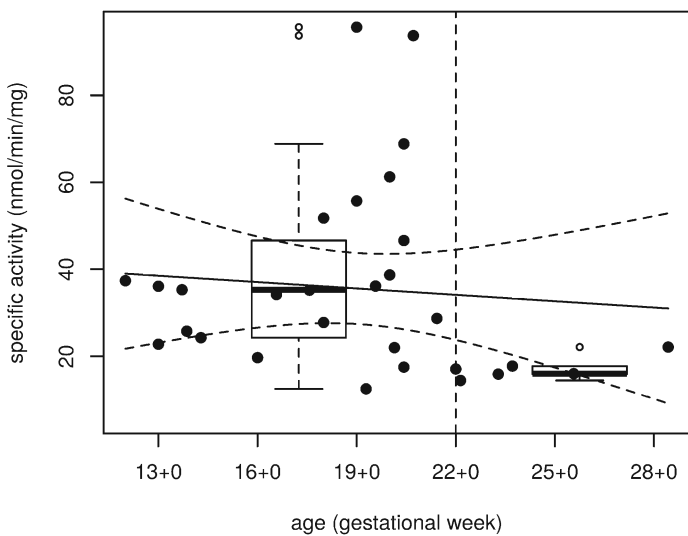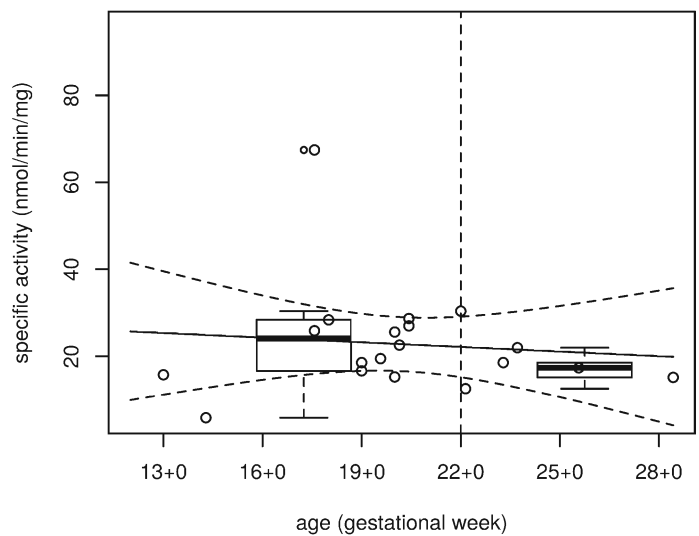| No of sample	Liver	Muscle	Gestational age (weeks+days)	Indication for pregnancy termination
1	+	+	13+0	Hygroma colli cysticum
2	+*		13+5	Trisomy 21
3	+		13+6	Trisomy 21
4	+*	+	14+2	Trisomy 21
5	*		15+5	Trisomy 18
6	+		16+0	Intrauterine infection
7	*		16+4	Trisomy 18
8	+		16+4	Trisomy 18
9	+	+	17+4	Meningocele
10	*		17+6	Diaphragmatic hernia
11	+	+	18+0	Trisomy 21
12	+		18+0	Gastroschisis
13	*		18+2	Fluidothorax
14	+*	+	19+0	Spontaneous abortion, oligohydramnion
15	*		19+0	Congenital malformation
16	+*	+	19+4	Trisomy 21
17	+	+	19+4	Trisomy 21
18	+*	+	20+5	Renal agenesis, oligohydramnion
19	+	+	20+0	Unknown
20	+	+	20+1	Sacrococcygeal teratoma
21	+	+	20+3	Intrauterine infection
22	+		20+5	Omphalocele
23	+	+	21+3	Limb deformity
24	+		21+3	Meningocele
25	+*	+	22+1	IUGR, oligohydramnion
26	+	+	22+2	Renal agenesis, oligohydramnion
27	+	+	23+2	Trisomy 21
28	+*	+	23+5	Urinary tract malformation
29	+	+	25+4	Sacrococcygeal teratoma
30	+	+	28+3	Meningocele

IUGR: Intrauterine Growth Restriction, *samples used for additional analyses of liver hematopoiesis

A COX4

COX4 mRNA level (a.u.)

age (gestational week)

B MT-ATP6

MT-ATP6 mRNA level (a.u.)

age (gestational week)
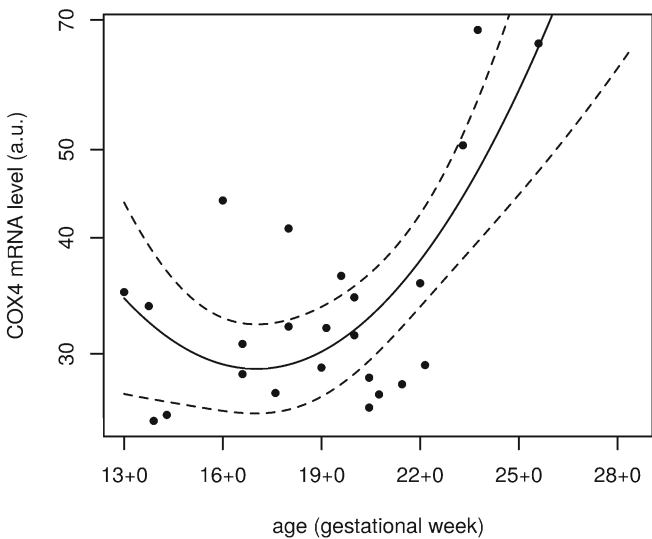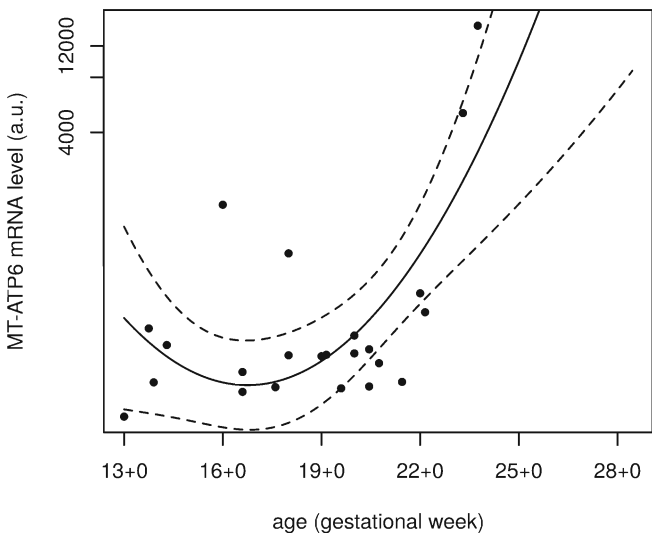
mtDNA
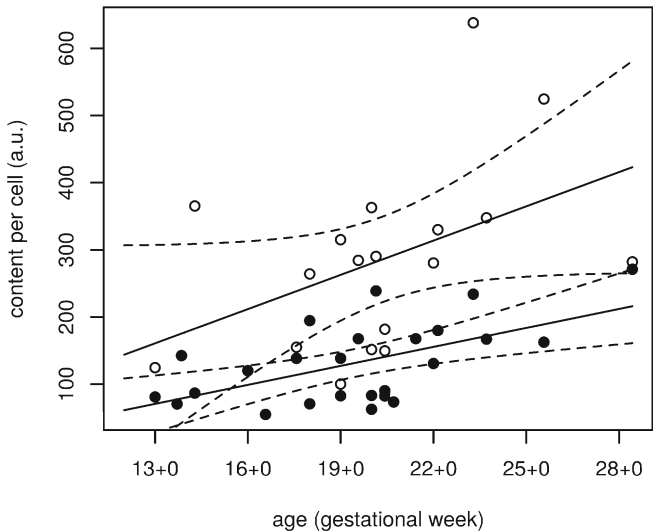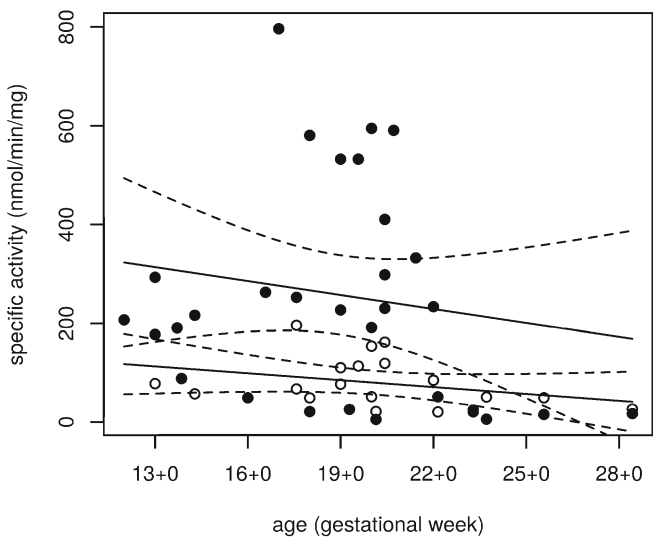
A COX

B CS

C SQR

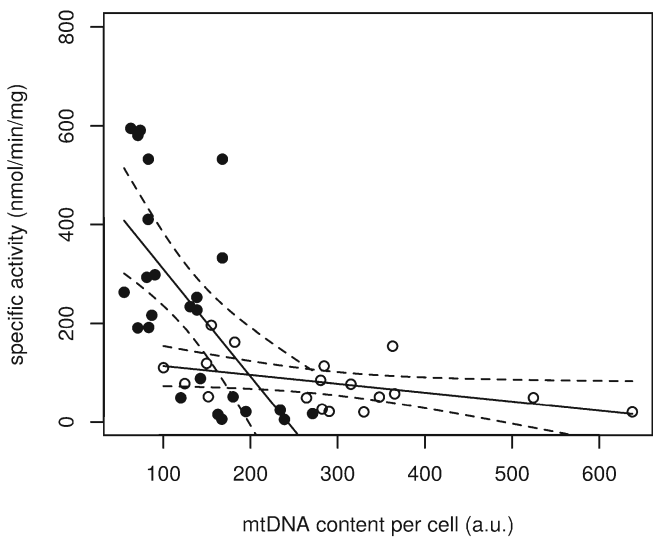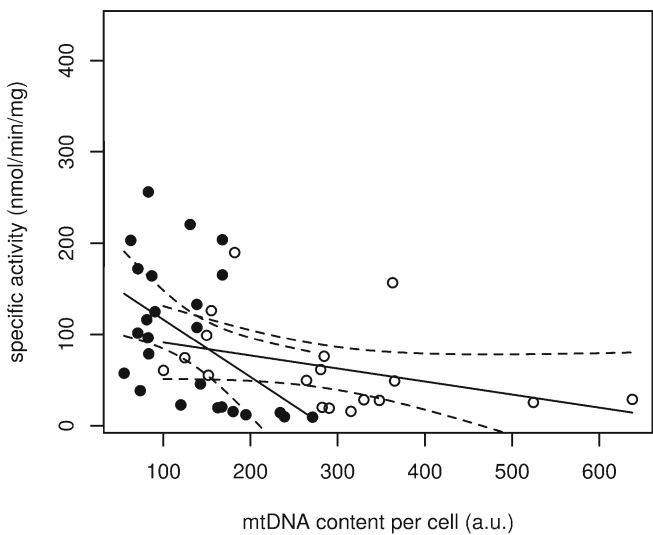**A** COX liver
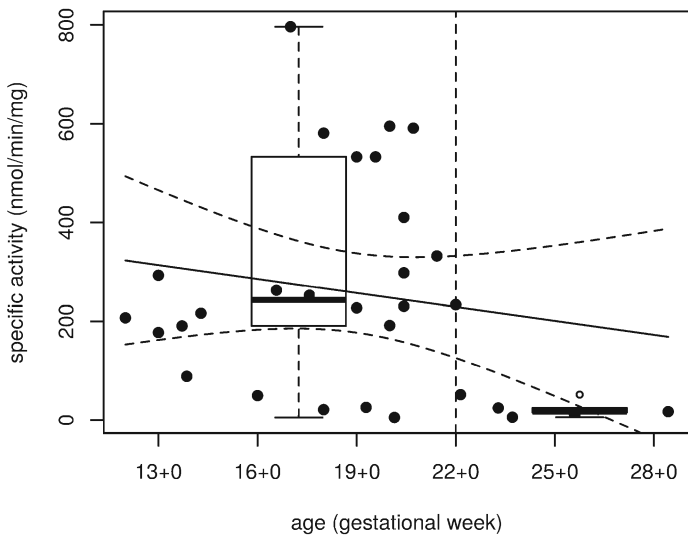
B COX muscle
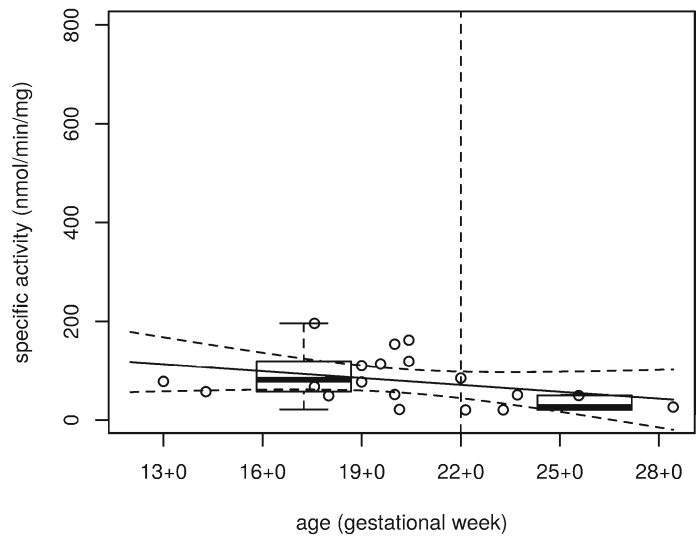
C CS liver

D CS muscle

E SQR liver

F SQR muscle

A

B

C

A COX

B CS

C SQR