Università di Cagliari

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SCIENCES AND TECHNOLOGIES FOR INNOVATION

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TITLE OF THE PHD THESIS

Immunohistochemical markers of stem/progenitor cells in the fetal human liver

Scientific Disciplinary Sector (s)
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Summary

Introduction

The liver is an essential organ that maintains vital activity through its numerous important functions, including carbohydrate metabolism, glycogen storage, byosynthesis of various biochemical components including amino acids and nucleotides, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins and hormones, and destruction of erytrhrocytes[1]. The liver has a unique capability of fully regenerating after injury. Regulating a balance between self-renewal and differentiation of hepatic stem cells, that are resources for functional mature liver cells, is required for maintenance of tissue homeostasis[2].The liver is an essential organ for maintaining systemic homeostasis. It also has a strong capacity for regeneration and has long been assumed to contain stem cells. Therefore, liver functions are properly established during development and maintained throughout life. During human fetal development, the hepatic and hematopoietic systems are intertwined. The liver forms rom an endodermal outgrowth of primitive foregut around the 3rd-4th week of gestation[3]. Hepatoblasts, the pluripotent cells of the outgrowth, give rise to both hepatic and biliary cells. They form a double-layered cylinder of cells called the ductal plate. The ductal plate cells migrate into surrounding mesenchyme to formintrahepatic bile ducts.

Those cells not in contact with the portal mesenchyme differentiate into hepatocytes[3]. During the 5th week of human gestation hematopoiesis moves from the yolk sac and /or adreno-gonadal-mesonephrenic to the liver. The liver is the principal hematopoietic organ in the human fetus from the 6th week through mid-gestation[4]. During this time, hematopoietic stem cells can be isolated from human fetal liver. In the adult liver, hematopoiesis can remerge during times of extreme stress. It is because of this intertwining of the hepatic and hematopoietic systems that a common stem cell has been proposed. Multipotent hepatic stem cells have been shown to exist within the liver in animals and humans. Both ductal plate cells, seen during fetal development, and hepatic oval cells are at least bipotential. They are able to differentiate into both hepatocytes and biliary cells.

Regarding the liver architecture, the liver is divided into lobules and each lobule consists of plates of hepatocytes lined by sinusoidal capillaries that radiate towards a central different vein. Liver lobules are hexagonal and at each of six corners there is a portal triad of vessels consisting of a portal vein, hepatic artery and bile duct. Sinusoids are composed of liver-specif capillaries with fenestrated endothelial cells, hepatic stellate cells (Io cells), liver-resident macrophages (Kupffer cell) and large granuar lymphocytes (pit cells). The liver has a dual blood supply, namel, via the portal vein and the hepatic artery. The portal veindelivers the venous

blood flowing from the intestines, pancreas and spleen. The hepatic artery supplies oxigen to the liver. The blood flows from a portal triad through a sinusoidal capillary to a central efferent vein. Hepatocytes are major parenchymal cells carrying out most of the metabolic functions and account for 60% of the total liver cell population and 80% of the volume of the organ. Hepatocytes are highly polarized epithelial cells and form cords. Their basolateral surfaces face fenestrated sinusoid endothelial cells, facilitating the transfer of materials between hepatocytes and blood flows. Tight junctions formed between hepatocytes ceate a canaliculus that surrounds each hepatocytes. Bile salts produced in hepatocytes are excreted into canaliuli that are linked to bile ducts at the portal triad. Bile ducts are formed by a specialized type of epithelial cell called a biliary epithelial cell or a cholangiocyte.

The adult liver is composed of many types of cells, including parenchymal hepatocytes that execute most of the liver's functions and non-parenchymal cells such as bile duct epithelial cells (known as cholangiocytes), Kupffer cells, hepatic stellate cells, and sinusoidal endothelial cells[1]. Previous developmental biology studies have revealed that hepatocytes and cholangiocytes, the two hepatic endothelial lineages, are derived from the foregut endoderm[5]. Thus, hepatic stem cells can differentiate into two lineages, namely hepatocyte and cholangiocytes[6]. However, these hepatic stem/progenitor cell fractions were isolated using

different markers in the fetal liver, and though they were reportedly bipotent (hepatocytes and cholangiocytes) hepatic stem/progenitor cell populations in fetal developing mouse and human livers, wheter their characteristicts were the same is unknown. It is assumed that the hepatic stem/progenitor cell population expand in early liver development and contribute as a source of mature differentiated cells during sbsequent liver development. In the adult liver, the potential to regenerate under conditions of severe parenchymal loss suggests the presence of hepatic stem/progenitor cells capable of bipotent differentiation into hepatocyte or cholangiocyte lineage. Infact, hepatic stem/progenitor cells are defined as cells that supply two types of liver epithelial cells, hepatocytes and cholangiocytes, during development, cell turnover, and regeneration. Although hepatic stem/progenitor cells in adult livers can be enriched in cells positive for cholangiocyte markers, their tissue localization and functions in cellular turnover remain obscure[7].

The existence of a liver stem cell population has only gained credence recently, following the results of animal experiments[8]. The stem cells originate from the inner cell mass during development of the blastocyst. They are pluripotent and are referred to as embryonic (ES) cells [9]. These cells give rise to somatic stem cells that differentiate further into multipotent tissue-specific stem cells [10,11]. Theese multipotent tissue-specific stem cells then give rise to progenitor cells that subsequently

proliferate and differentiate into mature somatic phenotypes that comprise organ mass. In the classical sense, a stem cell is defined as an undifferentiated cell that has continuous proliferative and has the abiity to produce progeny[12,13]. Additional characteristics include the ability to repopulate a tissue or organ after transplantation and the potential to be serially transplated[14]. Stem cells normally do not poliferate rapidly. The offspring of stem cells are referred to as progenitor cells. The early progenitor cells posses' characteristics similar to stem cells and may be multipotent. In contrast, the late progenitors appear to have progressed along the differentiation pathway and may only produce one or two cell type only. Progenitor cells are not self-renewing or capable of serial transplantation. The regulatory mechanism that underlie hepatic stem cell differentiation might be recapitulated during development and regeneration. Usually, we can explain that three criteria are applied too define a cell as a stem cell:

1) *self-renewal*: ideally for an unlimited number of cell divisions;
2) *unspecialized*: a stem celli s not has not any tissue-specific structures and, because of this, it is unable to perfom specialized functions;
3) *multipotency*: that is the ability to give rise to numerous types of differentiated cell in a process called differentiation.

Stem cells have different potentials to differentiate into multiple types. According to their potential of differentiation, stem cells are classically distinguished into:

1) *totipotent stem cells:* stem cells can differentiate into embryonic and extraembryonic cell types, produced from the fusion of an egg and sperm cell;
2) they are characterized by a potential of differentiation to all cell types of the adult organism and to self-renewal. These cells appear at the blastocyst stage (4-14 days after fertilization) and they are capable of differentiating into embryonic tissues organized in three different germ layers (ectoderm, mesoderm and endoderm);
3) *Multipotent stem cells:* they are stem cells able to differentiate into a number of cell type, but only those of a closely related family of cells;
4) *Unipotent stem cells:* these cells are present in adult tissues. They maintain self-renewal property and they can differentiate only in one cell type of the tissue to which they belong;

Within the liver, stem cells are thought to reside in a niche or microenvironment. The stem cells reside in the terminal bile duct (canal of Herring). Infact, given the pattern of proliferation of the oval cell in carcinogenesis and the histogenesis of primitive intra-hepatic bile ducts in development, it would be reasonable to assume that the stem cells should

be located in the smallest unit of the biliary tree. These units are called canal of Herring. These units line the hepatocytes and ductular cells and extend through the limiting plate to from a connection between the parenchyma and interlobular units [15,16] . The Canal of Herring have also been called cholangioles, terinal ducts or ductules [17]. Niches containing stem/progenitor cells are present in different anatomical locations along the human biliary tree and within lier acini. The most primitive stem/progenitor cellls, reside within peribiliary glands located throughout large extrahepatic and intrahepatic bile ducts. Canal of Herring (bile ductules) are found periportally and contain hepatic stem/pregenitor cells, participating in the renewal of small intrahepatic bile ducts and being precursors to hepatocytes and cholangiocytes [18]. The niche is composed of cells, extracellular matrix and soluble factors released by the niche cells that help to mantain the characteristics of the stem cells.

In adults, there are multiple niches of stem/ progenitor cells residing in different locations along the human biliary tree and niches found within the liver parenchyma. Those within the biliary tree are found in peribiliary glands and contain especially primitive stem cell populations, expressing endodermal transcription factors relevant to both liver and pancreas, pluripotency genes, and even markers indicating a genetic signature overlapping with that of intestinal stem cells[19]. Canals of Herring (bile ductules), the smaller branches of the bilisry tree, are niches

containing hepatic stem/progenitors and participating in the renewal of the small intrahepatic bile ducts and in the regeneration of liver parenchyma[20,21]. The liver stem progenitor cell response is surrounded by speciaized niche [22]. This niche furnishes several key signals driving hepatic stem cells activity. In the hepatic stem cell niche[23], hepatic stem cells are found in association with angioblasts[24]. with precursors to hepatic stellate cells and endothelial cells[25,26] and with macrophages[12,27]. These precursors release paracrine signals that are important for the maintenance of the stem progeitor cells in a quiescent state[16,28]. The hepatic stem cells, myofibroblasts and macrophagess produce a variety of signals able to drive the hepatic stem cells response[29]. Interestingly, macrophages can produce a variety of cyotkines, which have a key role in the prominent expansion of undifferentiated hepatic stem cells[30,31]. In addition to signals passing from the niche to the stem progenitor cells, there are also signals from the stem progenitor cells to the niche[32]. Hepatic stem progenitor cells can activate stellate/encothelial cells via Hedgehog (Hg), pathway resulting in release of types of matrix components (e.g. type IV collagen, laminin, sydecans and glycans) associated with normal liver regeneration[17,33,34].

Stem cells by general definition are characterized by their self-renewal properties and ablity to generate different cell lineages. Stem cells are gnerally considered to exhibit four major properties:1) capacity for self-

renewal or self-maintenance (generally slowly cycling); 2) multipotency (capable of producing progeney in at least two lineages; 3) functional, long term tissue reconstitution; 4) serial transplantability. Self-renewal is a property unique to stem cells, whereas progenitor cells that are the progeny of stem cells also proliferate and differentiate into somatic populations but do not maintain themselves. They may have single or multi-lineage potenatial, but are capable of only short-term tissue reconstitution. In differentiated tissues, adult or somatic stem cells have a more restricted capacity, generating a limited number of differentiated cells and residing in special microenvironments, defined as "niches", formed by different cells (endthelial, mesenchymal, ect.) belived to produce and release stimuli sustaining stem cell self-renewal and the generation of progeny cells.[35] Caracteristics of an ideal liver stem cell are: 1) readily available, without major ethical concerns; 2) reproducible, reliable and simple isolation technique; 3) definitive immunophenotypic signature, such as unique surface marker; 4) allowa clonal derivation; 5) ability to expand in vitro; 6) can be cryopreserved with high viability after thawing ; 7) bipotency with ability to differentiate into functional hepatocytes and cholangiocytes; 8) demonstrate functional integration in *in vivo* studies with absence of cell fusion or horizontal gene transfer ; 9) high repopulation potential in transpalnt studies; 10) demonstrable clinically relevant therapeutic effect in injury models ; 11) no risks of

malignancy over time[36]. Stem cells can be further classified into totipotent and pluripotent stem cells by virtue of how primtive they are. Progenitor cells, a term often used interchangeably with stem cells, is generally accepted to refer to descendants of stem cells[37]. Progenitor cells are still highly proliferative and can differentiate into specialized cells but they cannot self-regenerate and are less plastic compared to stem cells. Adult stem cells, particulary those derived from specialized tissues, should strictly be called progenitor cells until they fulfill the strict definition of a true stem cell.

Within niches, stem cells can generate rapidly dividing cells, usually referred to as transit amplifying cells, usually referred to as transit amplifying cells, that, in turn, lose self-renewal properties to become progenitor cells with restricted developmental potential [38,39]. Hepatic adult stem cells are believed to reside in portal areas within the Canals of Hering, envisaged as the hepatic niche for progenitor cells[40]. The existence of "stem-like" cells in the liver was first suggested over 60 years ago by Kinosita[41]. Following on from this, experiments to study liver regeneration in mice which were fed a methionine-rich diet containing bentonite to induce liver injury, first led to the description of a population of "indifferent cholangiole cells "and the hypothesis that these cells were responsible for regenerating the liver parenchyma when the hepatocytes were no onger able to fulfill this role[42]. Although stem cells

have a very important role in continuosly renewing tissues as a source for cells to replete the population that is turning over, the role of hepatic stem cells in normal liver, which has an exceedingly low level of cell turnover and a population of hepatocytes which are able to fulfill this role, is probably negligible[43].

However, stem cells may contribute to hepatocyte regeneration, or even take over this role if the liver injury is severe and associated with an impairment of hepatocyte proliferation as cirrhosis or submassive/massive necrosis, due to drug, toxins or viruses. The topic of liver stem cells is a controversial one and probably new to those pathologists who are not specialists in the hepatobiliary field. Stem cells, by Potten and Loeffler'classical definition, are "undifferentiated cells capable of proliferation, self maintenance, production of a large number of differentiated progeny, regeneration of tissue after injury and flexibility in the use of these options".[44] The stem cell concept can be a confusing one because during the lifetime of an organism hierarchies of stem cells exist with different "reproductive" repertoires. The fertilised egg is obviously totipotential, whereas, at the other end of the spectrum, cells which can maintain their own populations alone are, strictly "committed" stem cells[45,46]. In the adult organism the most actively dividing stem cells are those which subserve the continously renewing populations, e.g. epidermis, gastrointestinal mucosa and bone marrow.

On the other hand, conditionally renewing populations such as endocrine organs have stem cells which are only sporadically active.

The situation regarding stem/progenitor cells, however, is further complicated in adult liver. The nature and role of tissue stem cells in adult organ/tissues can be considered for simplicity, in the context of two distinct (closely related) processes; namely homeostatic maintenance tissue repair/regeneration under pathological conditions, upon various types of injury[47]. Functionally, stem cells are the multipotential, self-renewing cells that sita t the top of the lineage hierarchy and proliferate to make differentiated cell types of a given tissue in vivo. It is important to restrict this definition to single cells that, once developed, self-renew for the life time of the organism in order to distinguish stem cells from the many types of more transient progenitor cells (with limited self-renewal life –spans) that are present, especially in complex organism[48]. In vivo in adult organism, stem cells can divide repeatedly to replenish a tissue or may be more quiescent[49]. Rather than considering stem cells as undifferentiated cells, it may be more productive to think of the mas appropiately differentiated fro their specific tissue niches[33], with peraphs the ability to display more potential phenotypes in alternate niches. Stem cells can divide symmetrically during development to expand theor numbers and asymmetrically to self-renew and give rise to a more differentiated progeny[50].

The human hepatic stem cells are 9 μm in diameter, express cytokeratin 7,8,14,18 and 19, CD133/1, telomerase, CD44H, claudin 3 and albumin (weakly). They are negative for alpha-fetoprotein (AFP), intercellular hemopoietic cells (CD45), and mesenchymal cells (vascular endothelial growth factor recptor and desmin)[51].

The cytokeratins (CK) are the intermediate filaments of cytoskeleton characteristic for epithelial cells and they are a family of many different filament-forming proteins (polypeptides) with specific physicochemical properties and are normal components of epithelial cell cytoskeleton[52,53]. Cytokeratins are expressed in various type of epithelia and in different combinatons[53,54]. The CK8/CK18 have been found in simple epithelia[55], while CK5/CK14 in stratified[56]. In addition, these epithelial cells can also produce secondary cytokeratins, such as CK7/CK19 in simple epithelial or CK15 and CK6/16 in stratified epithelia[57,58].

Different cytokeratins have been identified and catalogued[59].Normal adult human liver parenchymal cells express only CK8 and 18; intrahepatic bile duct cells express in addition the CK7 and 19[60] and 20[61]. CK7 and 8 have been hypothesized to represent a population of common stem cells for hepatic and hematopoietic tissues in the human fetal liver[62]. CK7 and 8 cells are conspicuosly different from hematopoietic stem and progenitor cells being larger in size and having a more complex cytoplasm. CK7 and 8 are intermediate filament proteins htat interact with keratin 19 and

18, rispectively. CK8/18 can be found in hepatocytes biliary cells, stellate cells and adult hepatic progenitor cells. CK7/19 are located on biliary and adult hepatic progenitor cells[63].

In its earliest developmental stages, the human embryonic liver is composed of epithelial liver cell precursor (hepatoblasts) that express CK8, 18, and 19[64,65], and in addition CK14 from 10 to 14 weeks of gestation[66]. Around the eighth week of gestation, the primitive hepatoblasts adjacent to the mesenchyme around the largest hilar portal vein branches become more strongly immunoreactive for their CK8,18, and 19. In the meantime, the hepatoblasts not involved in ductal palte fromation gradually lose CK19, and by 14 weeks of gestation the future parecnhymal cells are immunoreactive only for CK8 and 18, the cytokeratin pair normally expressed in adult liver parenchymal cells[67]. By 20 weeks of gestation, weak immunoreactivity for CK7 appears in the cells of the developing ducts, again appearing first in the older ducts near the hilium[68]. The immunoreactivity for CK7 gradually increases and extends into more peripheral ducts, to reach the level of immunorectivity observed in ducts of the adult liver at approximately 1 month after birth[69]. In human at 8-14 weeks gestation, liver progenitor cells may be both CK14 and CK19-positive, and commitment to the biliary lineage migth to be signalled by increased CK19 expression, loss of CK14and transient expression of vimentin[49].

The main function of cytokeratins is to give mechanical strength to the epithelial cells. Cytokeratins are not evenly distributed throughout the cytoplasm. CK19 is most abundant at the apical end below microvilli. Defect in CK19 expression affects the polarity of the cell[70]. Cytokeratin filament are aso importaant in intercellular context. They are attached to the desmosomes as well as hemi-desmosomes. Thus, they help in cell-cell adhesion and also in the attachment of the epithalial cells to the underlning connective tissue. Besides this structural function, cytokeratins also play a role in trasport of some membrane protein[71,72,73]. It's important to know liver development is a sequential array of distinct biological events. Each step of differentiation is regulated by intrinsically programmed mechanisms as well as by extracellular signals. During embryological development, around 8 gestatonal week (GW), bipotential hepatoblasts differentiate into both hepatocytes and ductal plate cells. The ductal plate cells are remodeled into intrahepatic bile duct and can generate periportal hepatocytes and oval cells. Around 20 GW, these embryological progenitor cells express a broad range of cytokeratyns CK8, CK18, CK19 and (transiently) CK14. Ductal plate cells continue to express CK8, CK18 and CK19 and at 20 weeks of gestation begin to express CK7. This immunophenotype is retained by mature bile ducts at birth. Fully differentiated hepatocytes express CK8 and CK18, but not CK7 or CK19[74]. In vitro expanded human fetal liver progenitor cells

express CK18, CK8 and some CK19. A cell type termed the oval cell has been dscribed as a putative hepatic stem cell in animal (especially rat) models. These cells appear in the portal and periportal regions of animal livers within a few days of liver injury and may express biliary markers, such as CK7 and CK19.

The changes in cytokeratin expression during liver development as detected by immunohistochemistry are schematically represented in Fig.1[4].

Fig.1[4]

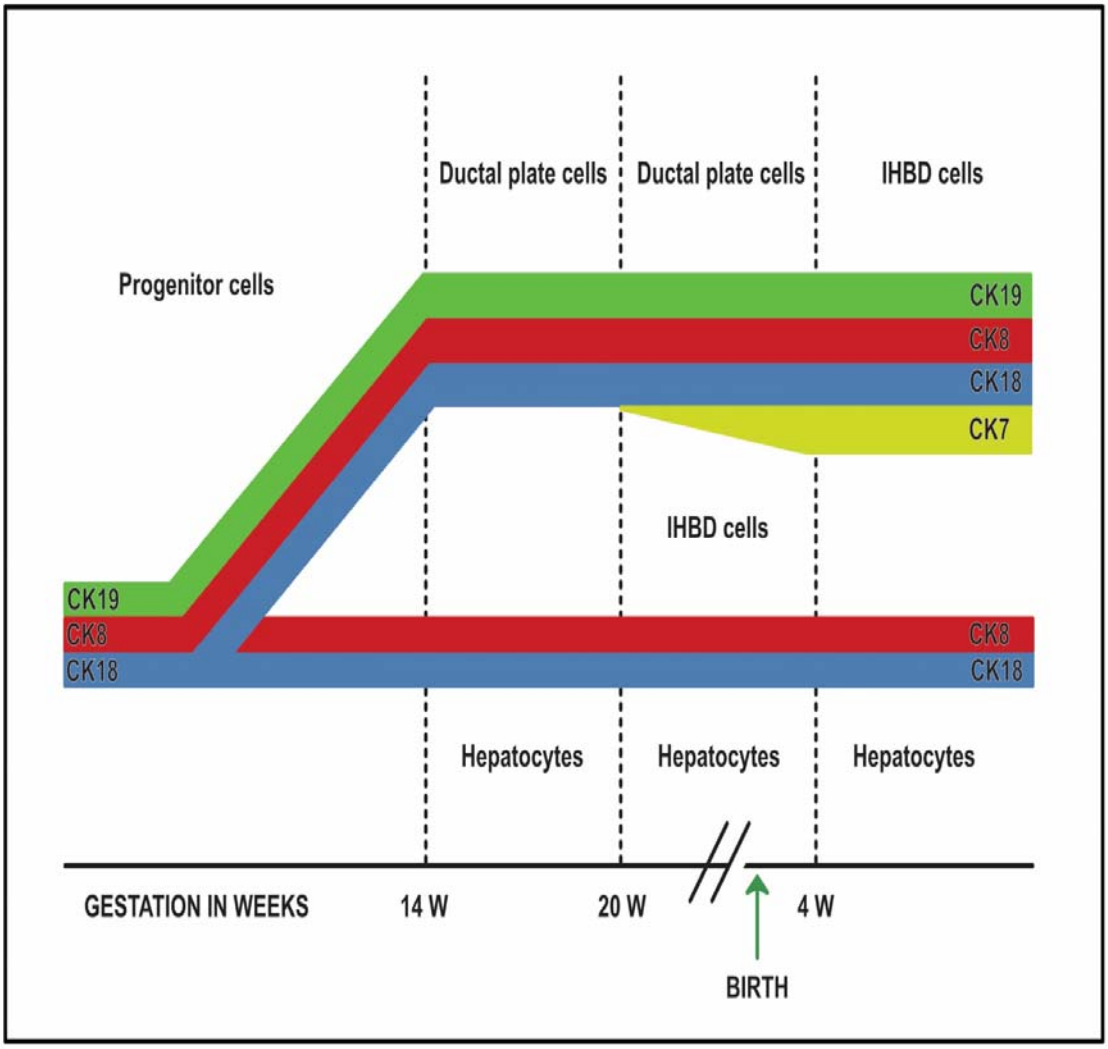


Fig.1[4] Cytokeratin expression, particulary Ck7 and Ck19, during gestation and after birth.

Aim of the study

The aim of this study was to identify the stem/progenitor cell markers, by immunohistochemistry, in order to highlight the immunoreactivity of CK7, CK14 and CK19 in human liver progenitor cells at different gestational ages.

Evaluating both stem/progenitor and matur cell markers lead to identification of multiple stages of differentiation of liver progenitors during gestation, in order to better understand the development of fetal human liver.

Materials and methods

Liver samples were obtained from 14 fetal liver from 7 to 38 weeks of gestation, received from Department of Pathological Anatomy. All procedures performed were approved by the Ethics Human Studies Committee of University Medical Centre of Cagliari (according to the instructions of the Declaration of Helsinki). All the fetuses included in this study had no congenital malformation.

Samples were fixed in 10% buffered formalin, routinely precessed, and paraffin-embedded. A total of 4 serial 3 μm-thick sections were obtained from each paraffin block; after dewaxing and rehydrating, one of these was stained with hematossilin-eosin, while the others were pre-treated for immunoistochemical analysis, with 20 minutes heat-induced epitope retrival in buffer pH 9.00 (*Envision^{TM}FLEX Target Retrieval Solution High pH; Dako Denmark A/S, Glostrup, Denmark; Code K8004).* Slides were then incubated for 20 minutes at room temperature with: anti-CK19 (*Dako Denmark A/S;* Code M0888; monoclonal mouse antihuman clone RCK108 at 1:50 diluition); anti-CK7 (*Dako Denmark A/S;* Code M7018 monoclonal mouse antihuman clone 0V-TL 12/30 at 1:150 dilution); and anti-CK14 (*Cell Mark;* Code CMC887; mouse antibody clone LL002 at 1:100 dilution); Staining procedures were performed by Dako REAL EnVision Detection System Peroxidase (*Dako Denmark A/S, Glostrup,*

Denmark, Code: k5003) following dealer's instructions. Data were obtained by evalutation of positivity (+) and negativity (-) for CK19, CK7 and CK14 immunoreactivity in each fetal liver sample.

Results

Progenitor cell markers:

Cytokeratin 7 (CK7)

From 7 to 12 weeks of gestation, the positivity for ck7 is particularly evident in the cytoplasm of progenitor cells of hepatocytes, in development, with a perinuclear granular appearance. From 15w to 19w, no immunoreactivity is found in progenitor cells. At about 27w up to 38w, we have a cytoplasmic, moderate and more homogeneous positivity than before.

The bile ducts in the first 7 weeks of gestation are absent[4]. Then towards the 9w, transitional cells begin to appear, around the branches of the portal vein. From 15w onwards, we have a strong positivity of ck7 in ductal cells, remaining until late gestational age. The positivity of ck7 in the bile ducts is cytoplasmic and perinuclear.

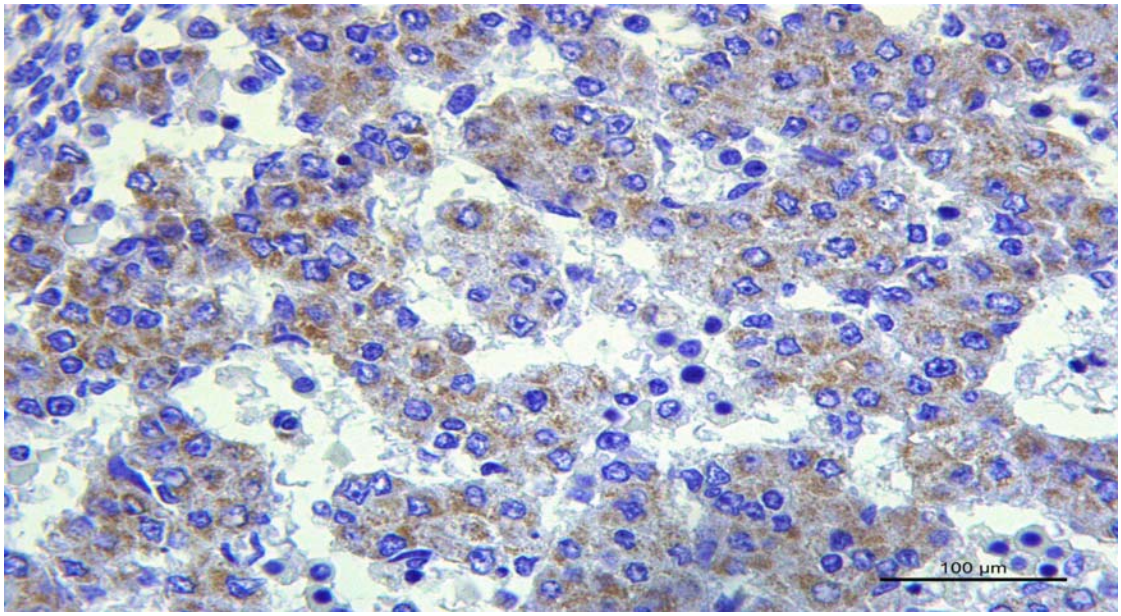


Fig.2 Gestational week 7. Immunoreactivity for CK7-citoplasmatic and perinuclear - of hepatocytes. No presence of bile ducts. 40x

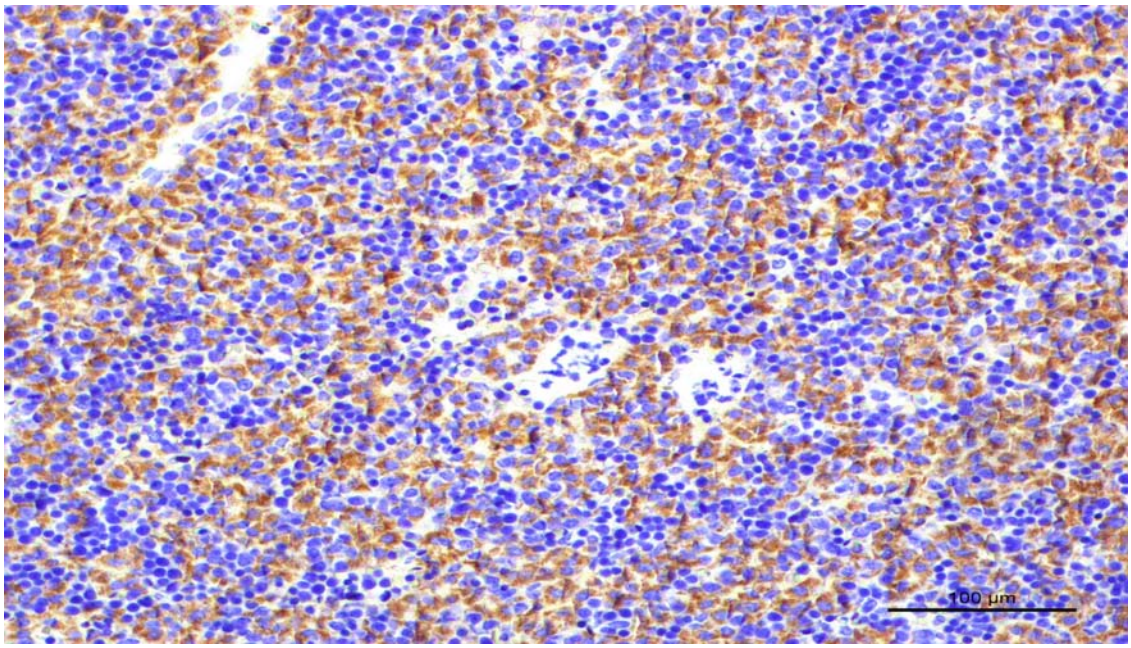


Fig.3 Gestational week 7. Immunoreactivity for CK7-citoplasmatic and perinuclear- of hepatocytes. No presence of bile ducts. 40x.

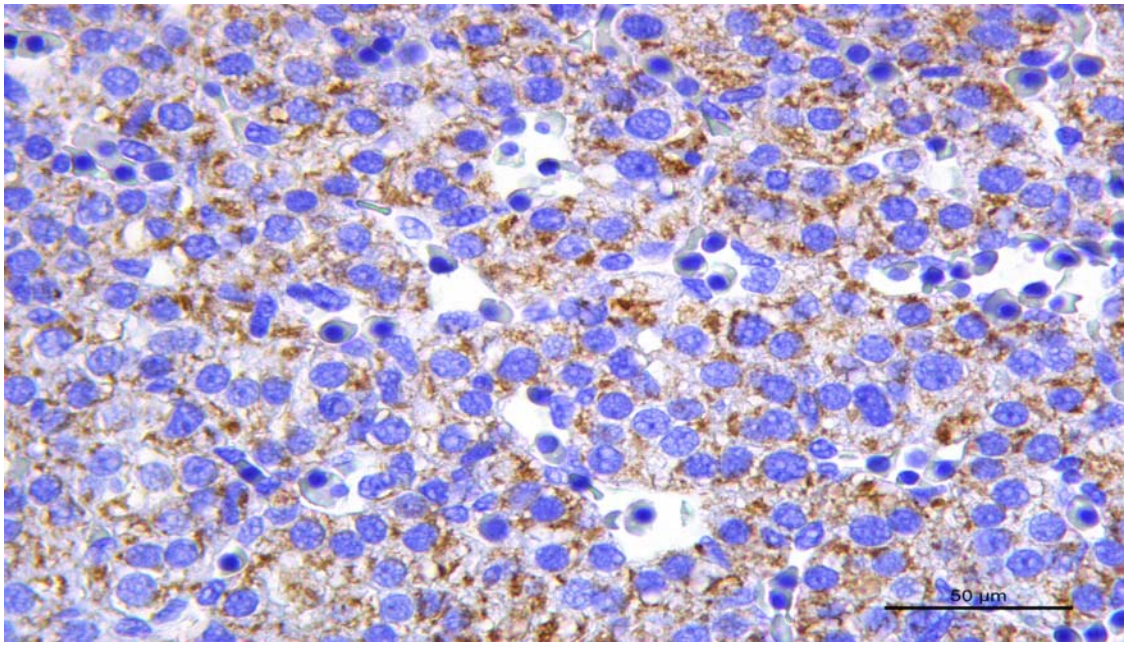


Fig.4 Gestational week 9. Immunoreactivity for CK7 of hepatocytes. No presence of bile ducts. 40x.

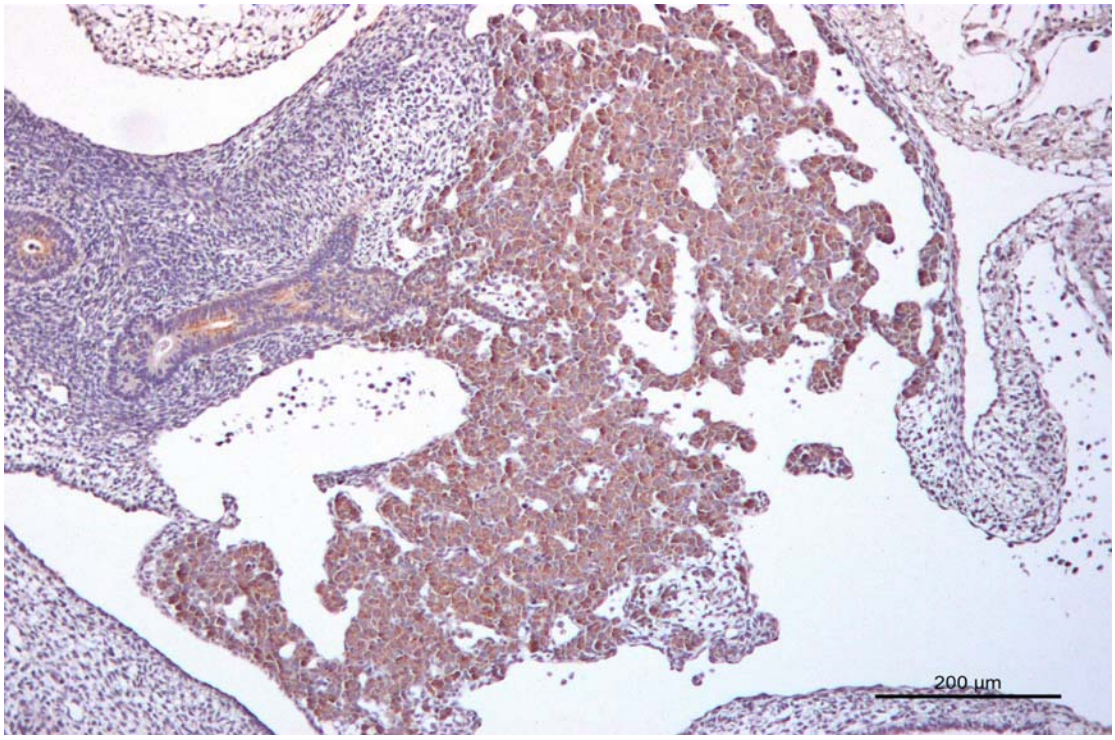


Fig.5 Gestational week 11. Immunoreactivity for CK7. 10x

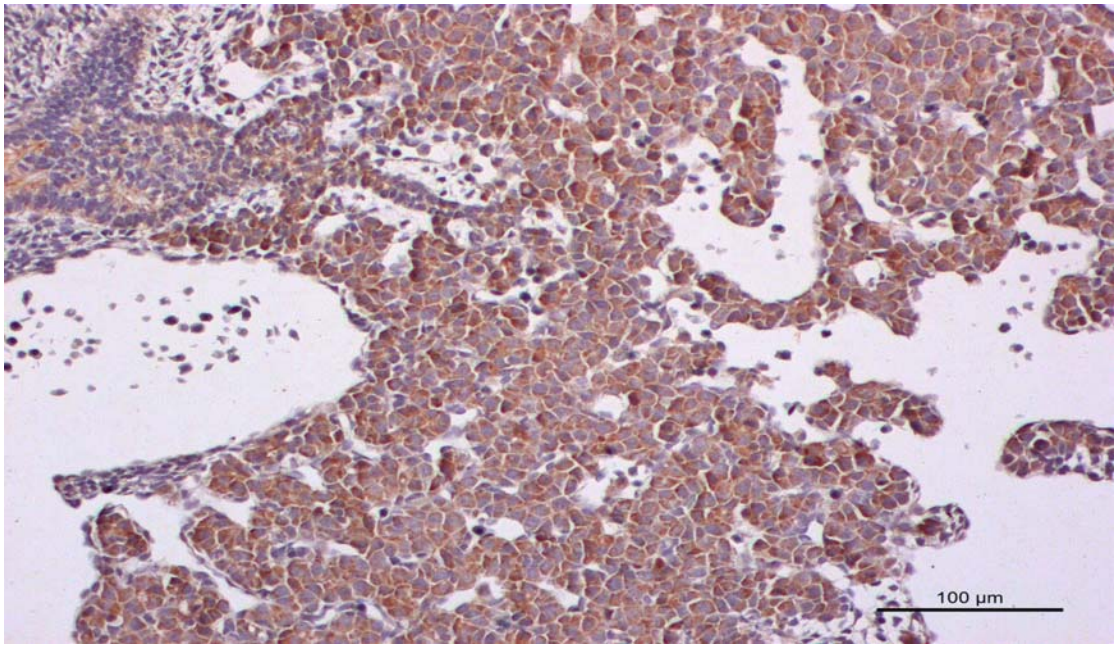


Fig. 6 Gestational week 11. Immunoreactivity for CK7 of hepatocytes. No presence of bile ducts. 20x.

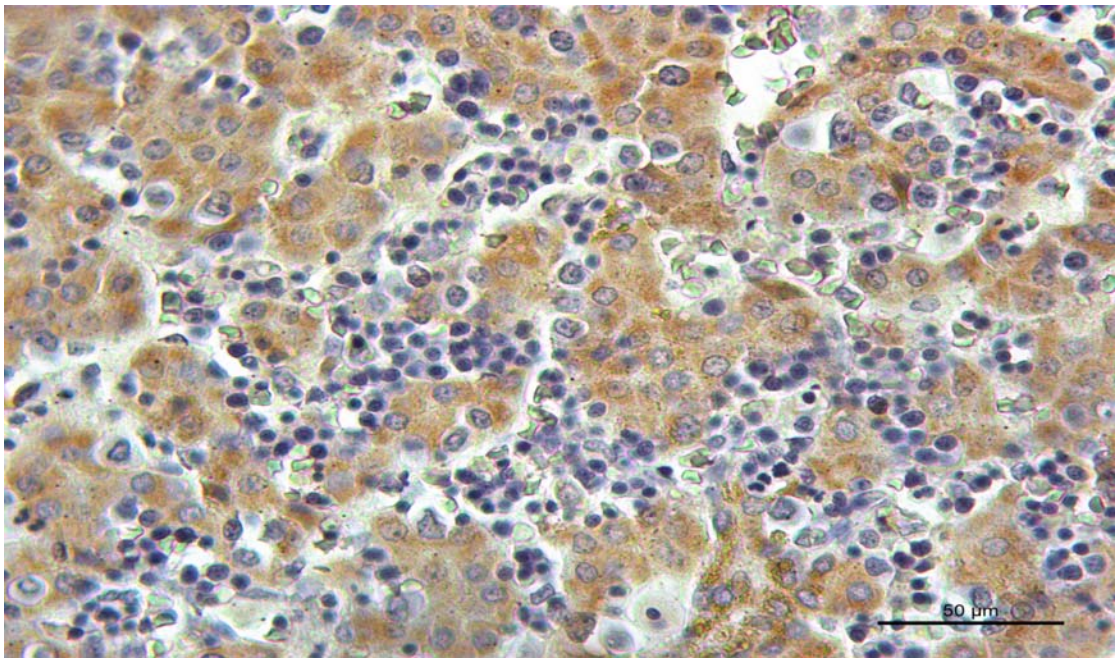


Fig. 7 Gestational week 12. Immunoreactivity for CK7 of hepatocytes. No presence of bile ducts. 40x

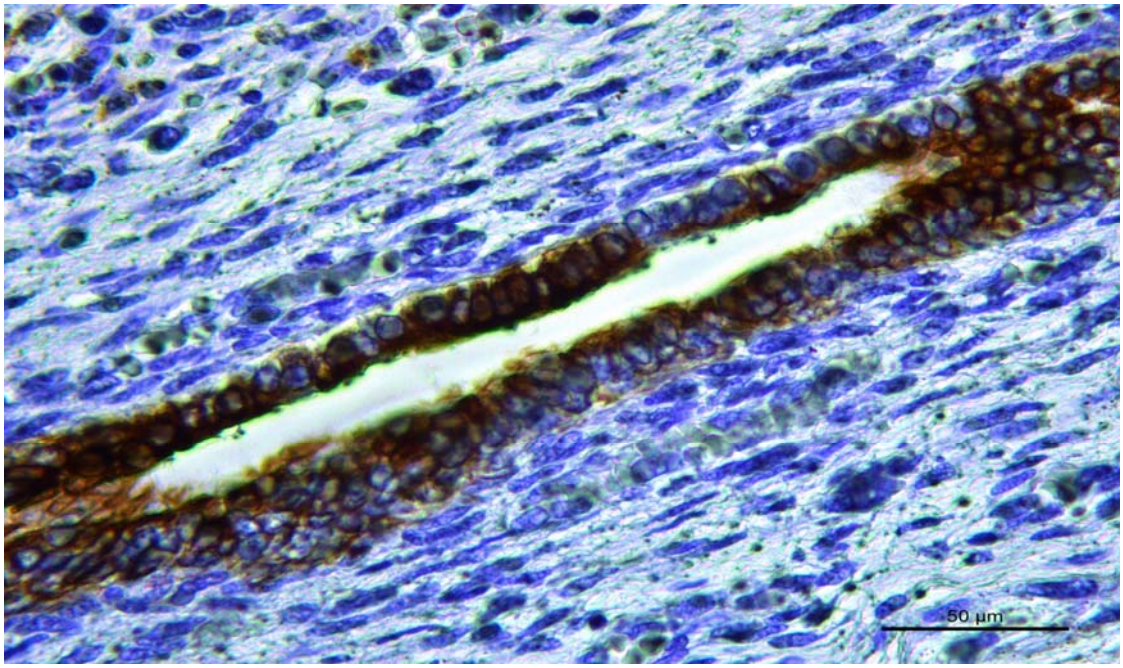


Fig. 8 Gestational week 15. Evident immunoreactivity for CK7 on bile duct cells. Negativity for hepatocytes. 40x.

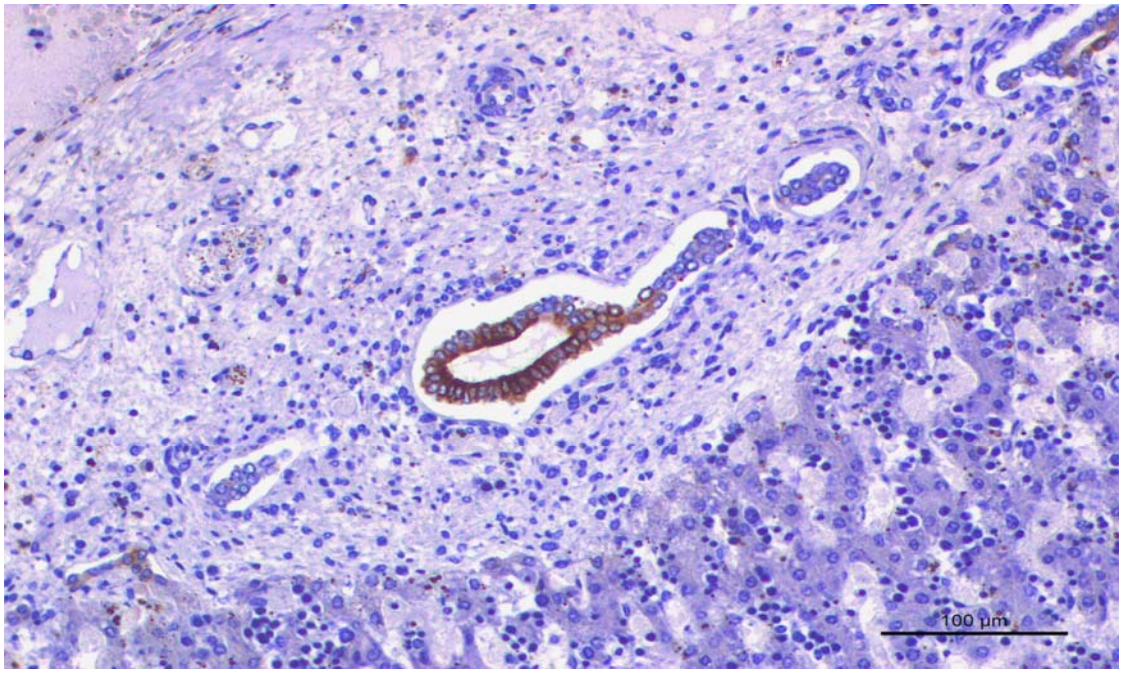


Fig.9 Gestational week 18. Immunoreactivity for CK7 on bile duct cells. No immunoreactivity in progenitor cells of hepatocytes.

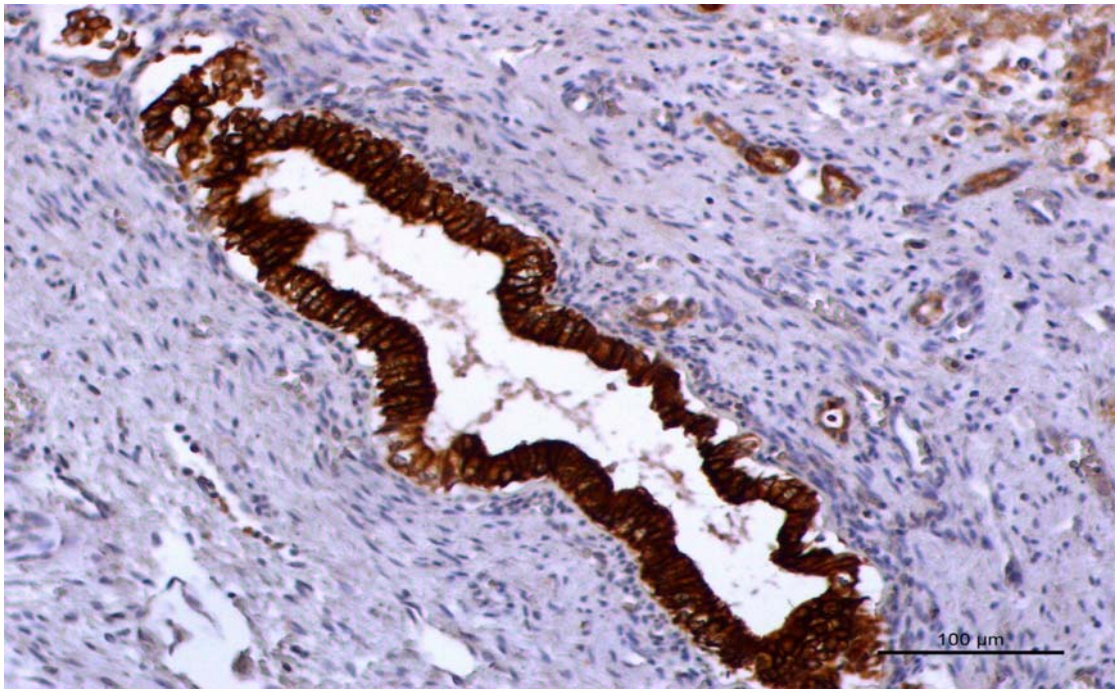


Fig. 10 Gestational week 27-28. Imunoreactivity for CK7 on bile duct cells.

Cytokeratin 14 (CK14)

From 7w to 12w, a cytoplasmic positivity, mainly perinuclear, is present in the cytoplasm of progenitor cells.

From 15w to 19w, no immunoreactivity was found in hepatoblasts. From 27w up to 38w, there is a positive recovery.

On the other hand, there is no positive effect during the development of the ductal system.

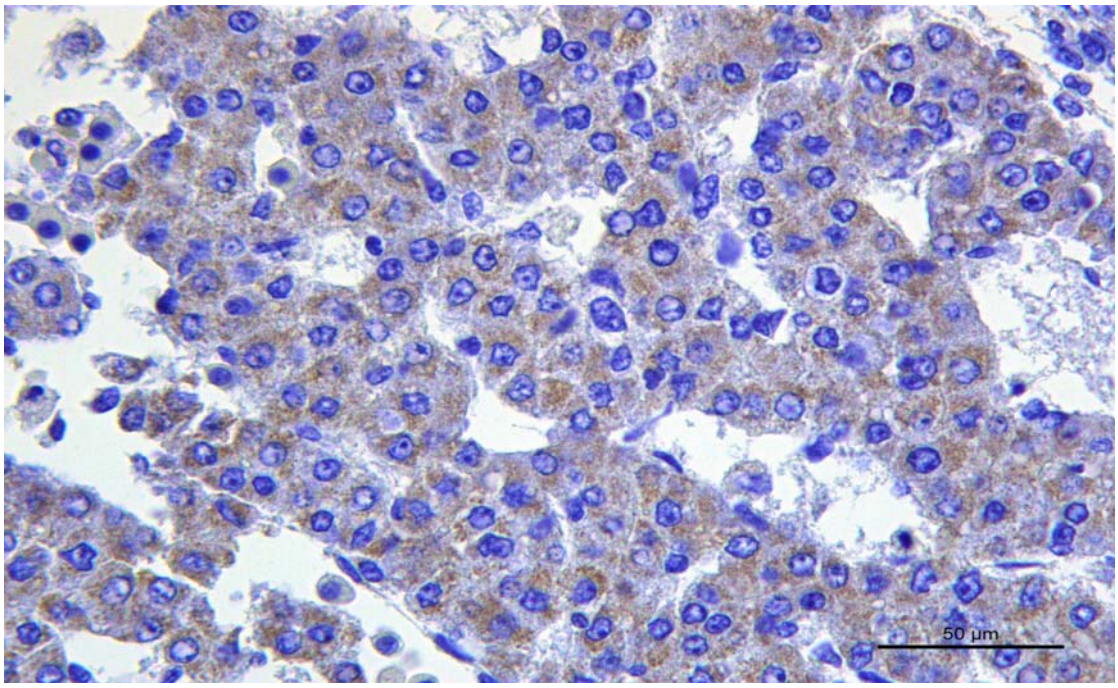


Fig. 11 Gestational week 7. Immunoreactivity for CK14 of hepatocytes. No presence of bile ducts.40x.

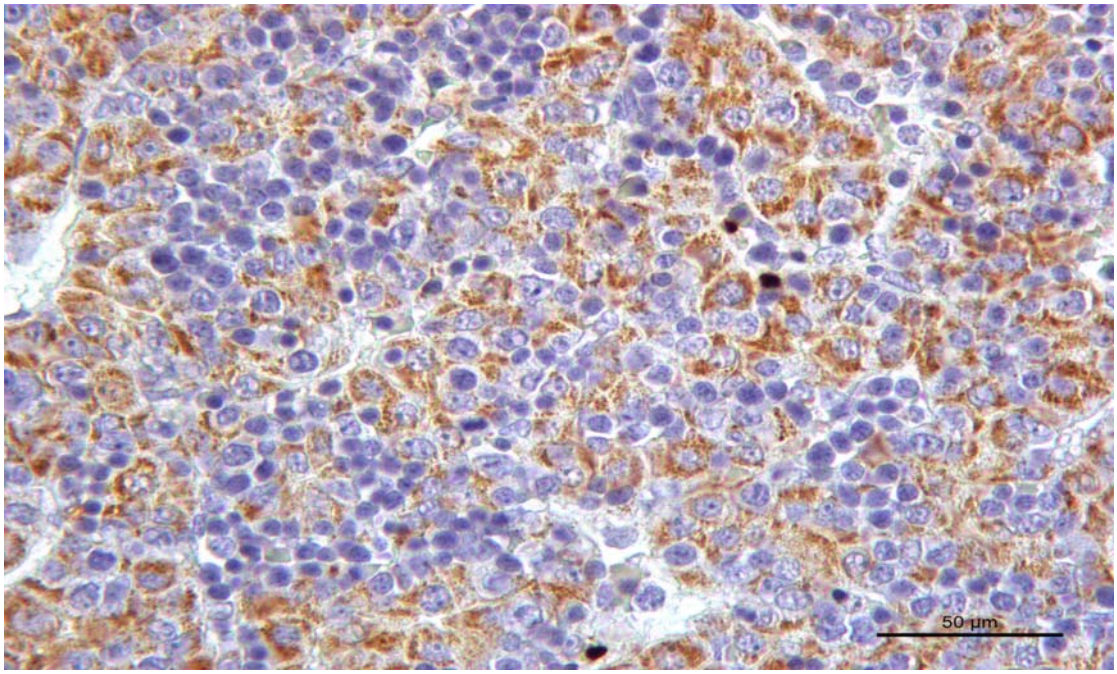


Fig. 12 Gestational week 9. Immunoreactivity for CK14 of hepatocytes.No presence of bile ducts. 40x.

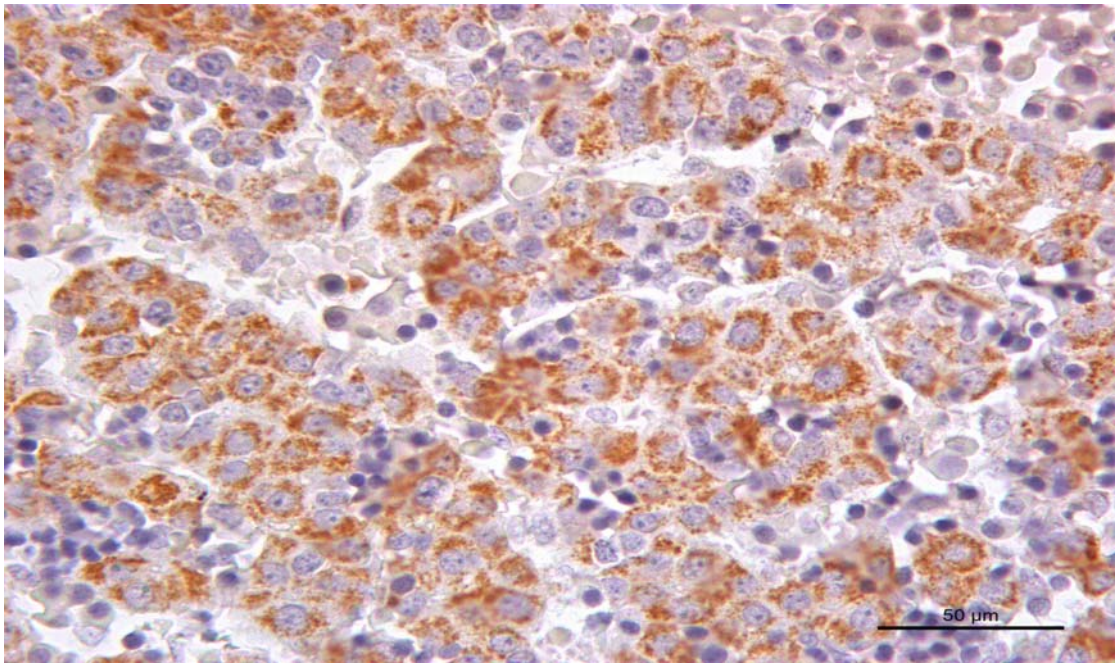


Fig. 13 Gestational week 10. Immunoreactivity for CK14 of hepatocytes. No presence of bile ducts.40x.

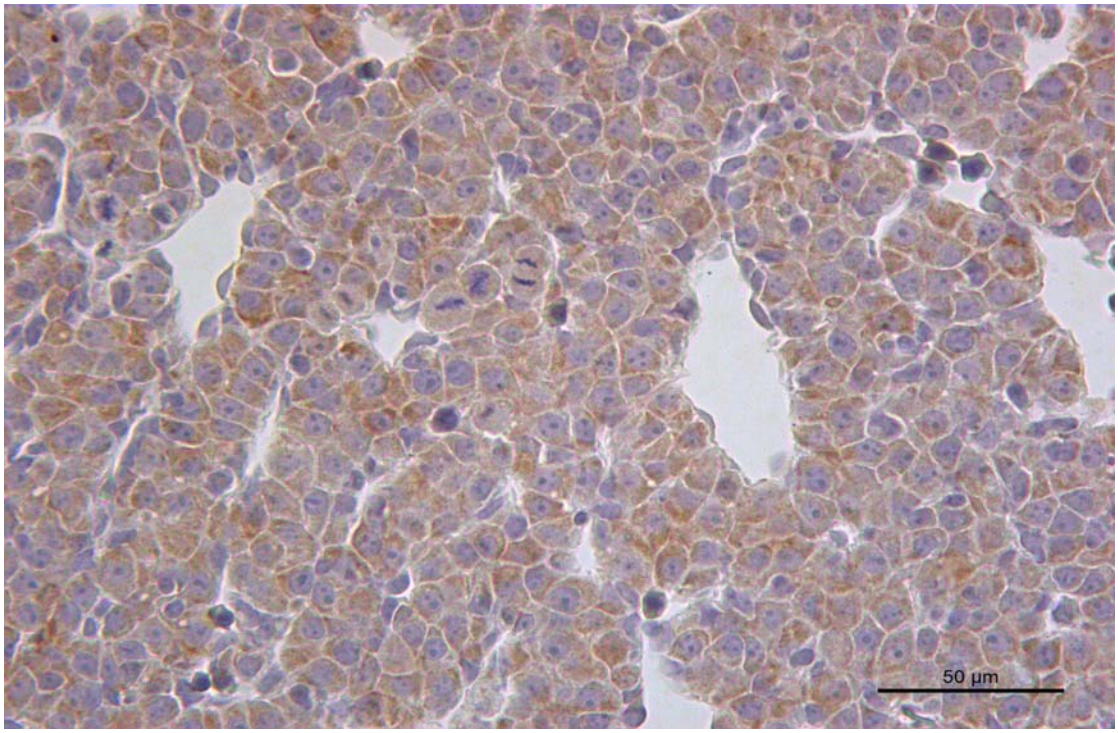


Fig. 14 Gestational week 11. Immunoreactivity for CK14 of hepatocytes. No presence of bile ducts. 40x.

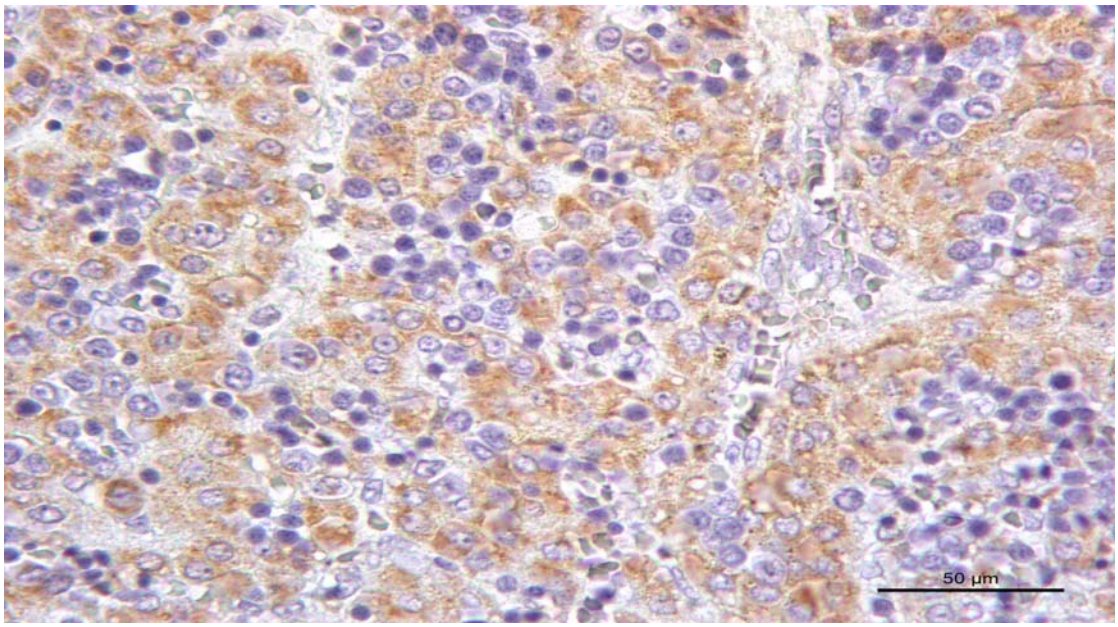


Fig. 15 Gestational week 12. Immunoreactivity for CK 14 of hepatocytes. No presence of bile ducts. 40x.

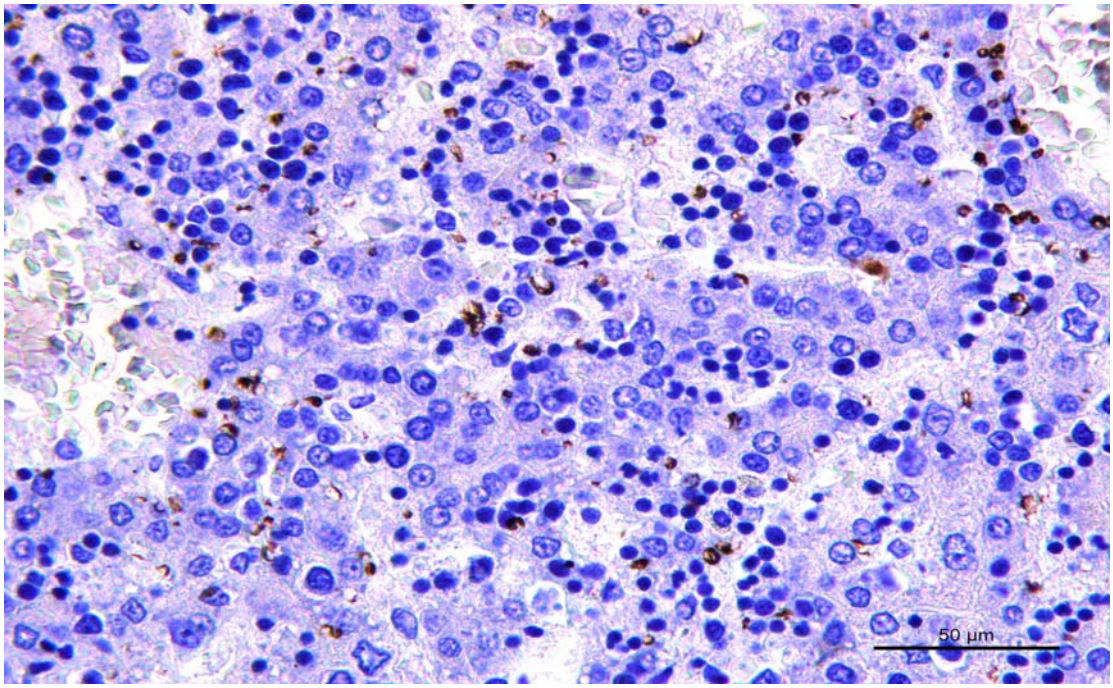


Fig.16 Gestational week 16. Immunoreactivity for CK14. Negativity of hepatocytes and bile ducts.40x.

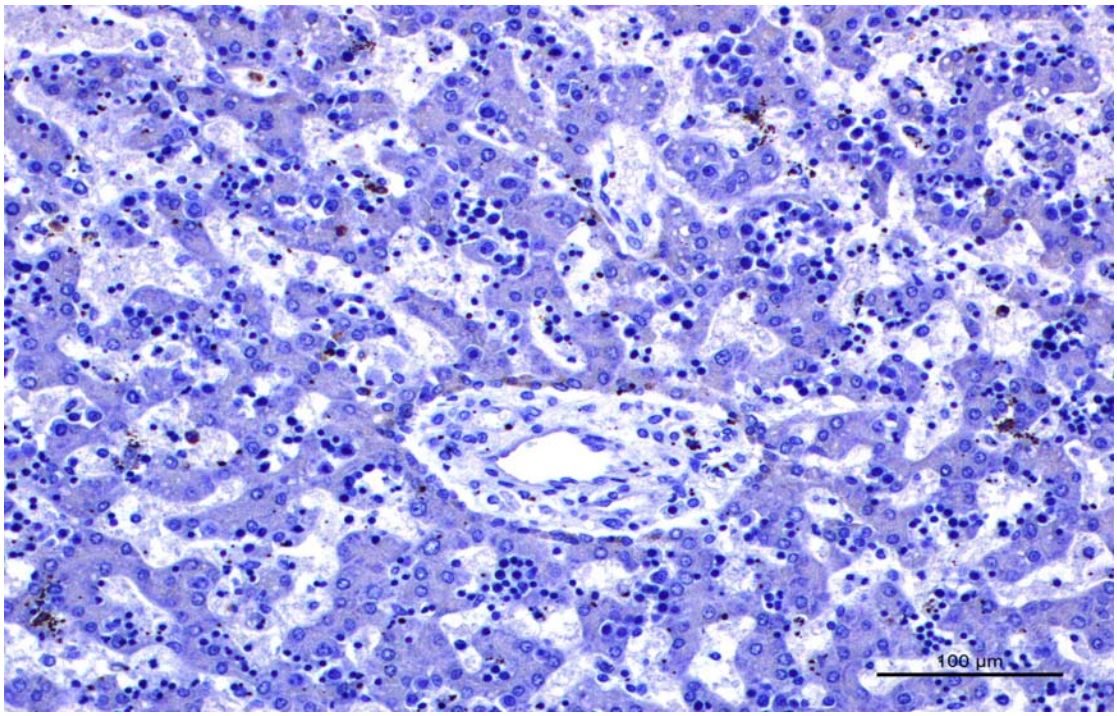


Fig.17 Gestational week 19. Immunoreactivity for CK14. Negativity for hepatocytes and bile ducts. 20x.

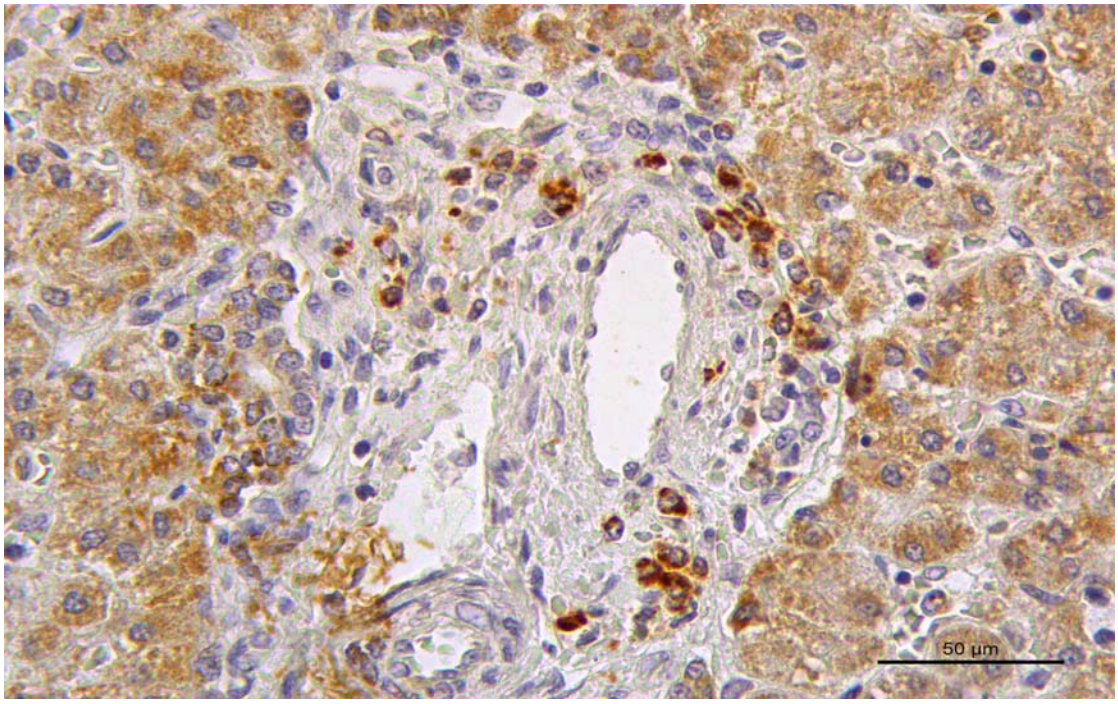


Fig. 18 Gestational week 27-28. Immunoreactivity for CK14 of hepatocytes. No presence of bile ducts.40x.

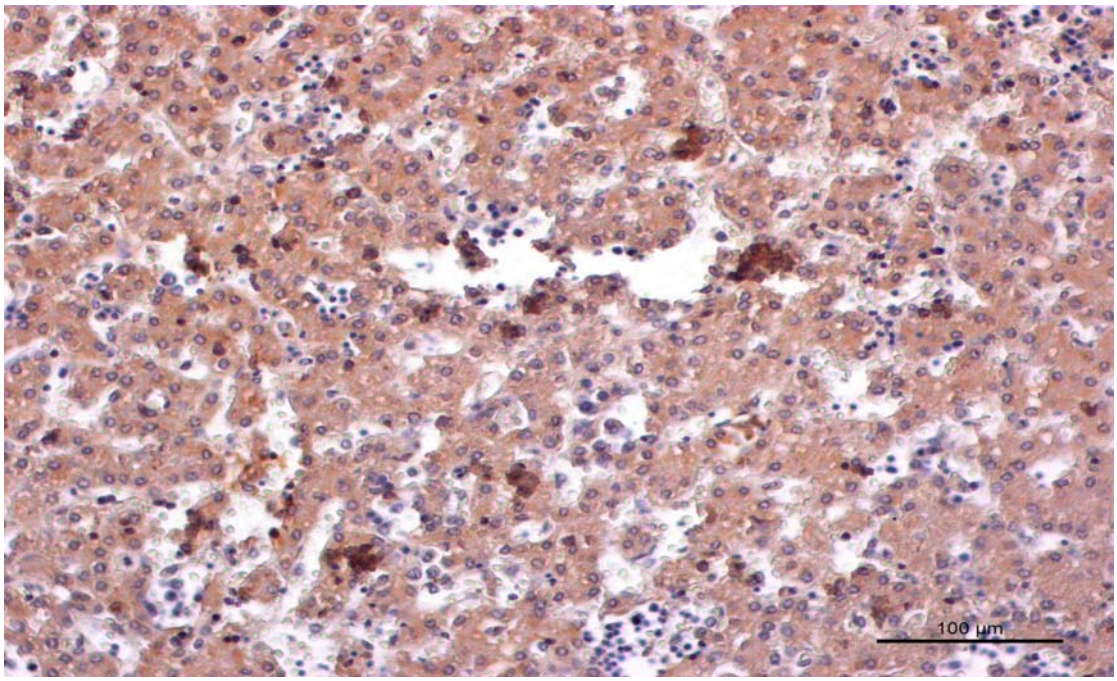


Fig. 19 Gestational week 38. Immunoreactivity for CK14 of hepatocytes. No presence of bile ducts. 20x.

Cytokeratin 19 (CK19)

From 7 to 12 weeks of gestation, we have an intense cytoplasmic positivity homogeneously spread at the level of progenitor cells. At 15 w it is more light and focal and then negativize and start again from 27 w. Like ck 7, also the ck 19 is intensely expressed in the bile ducts from 15w and then maintained until the 38th gestational age studied.

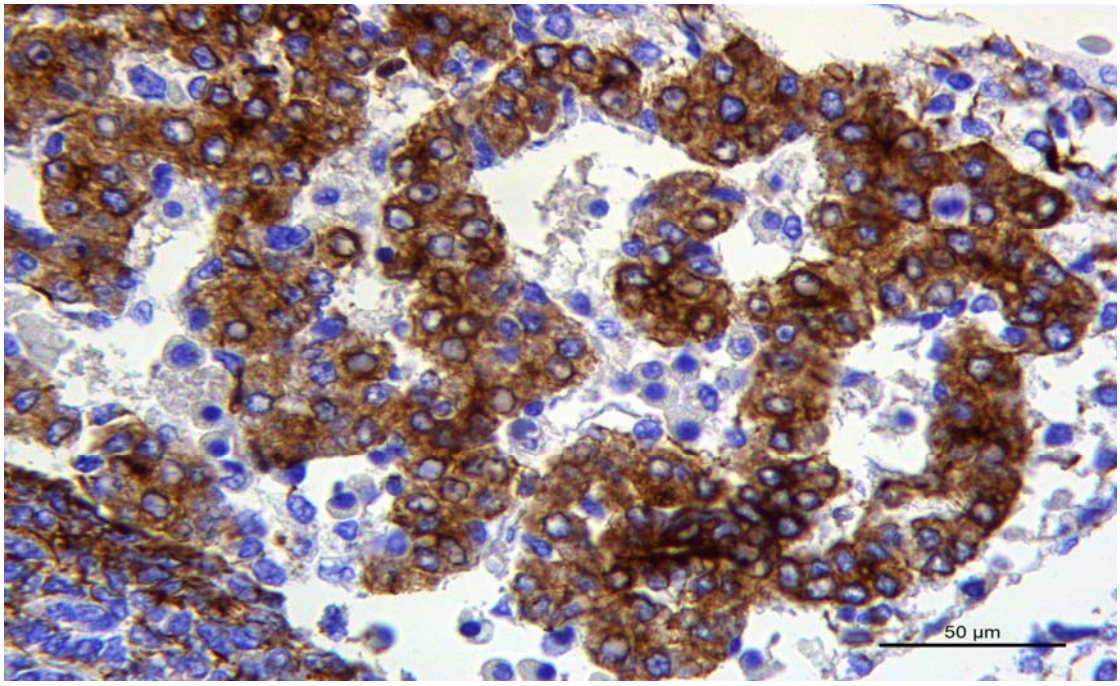


Fig. 20 Gestational week 7. Strong immunoreactivity for CK 19 of hepatocytes. No presence of bile ducts.40x.

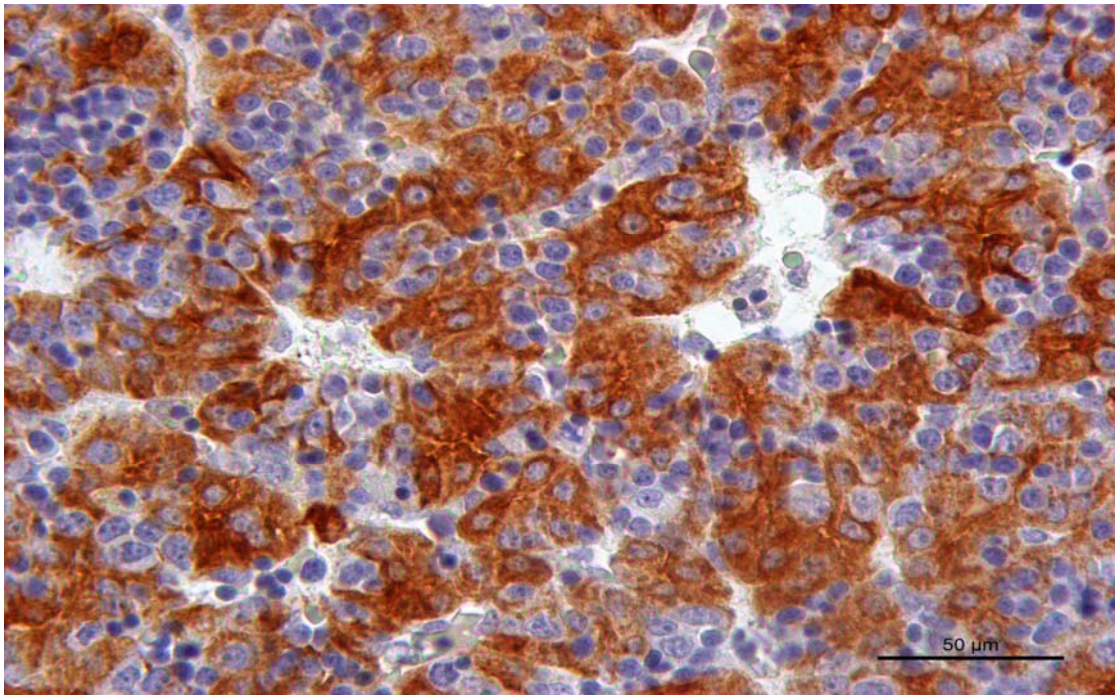


Fig. 21 Gestational week 9. Immunoreactivity for CK 19 of hepatocytes. No presence of bile duct. 40x.

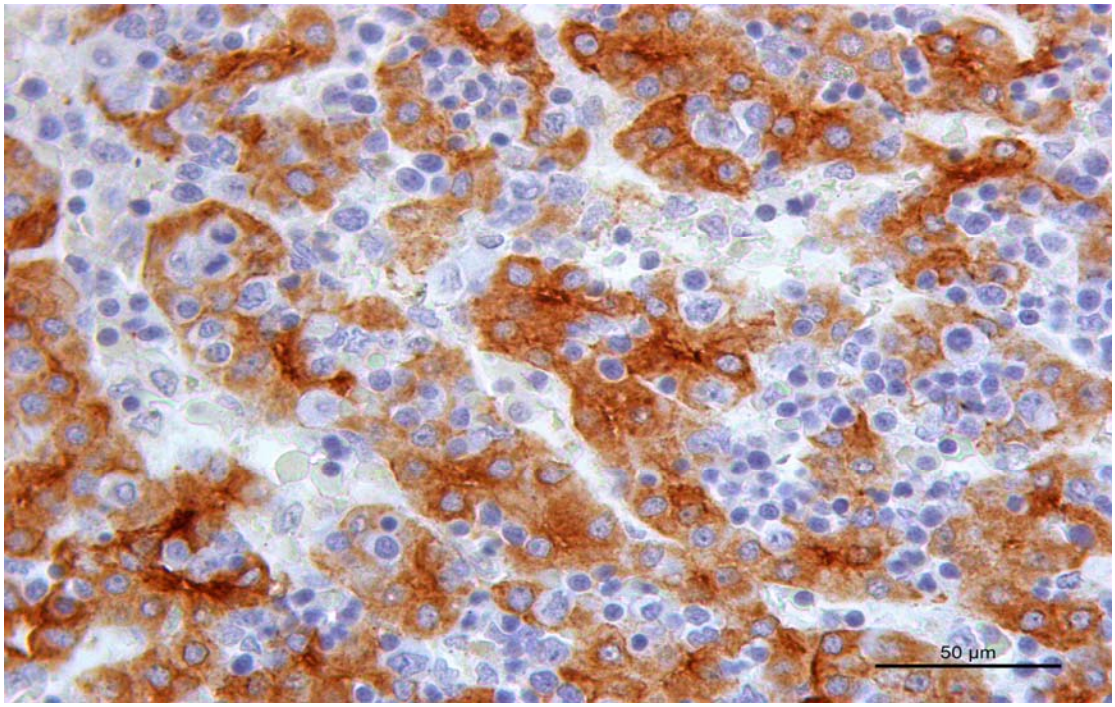


Fig. 22. Gestational week 10. Immunoreactivity for CK 19 of hepatocytes.No presence of bile ducts. 40x.

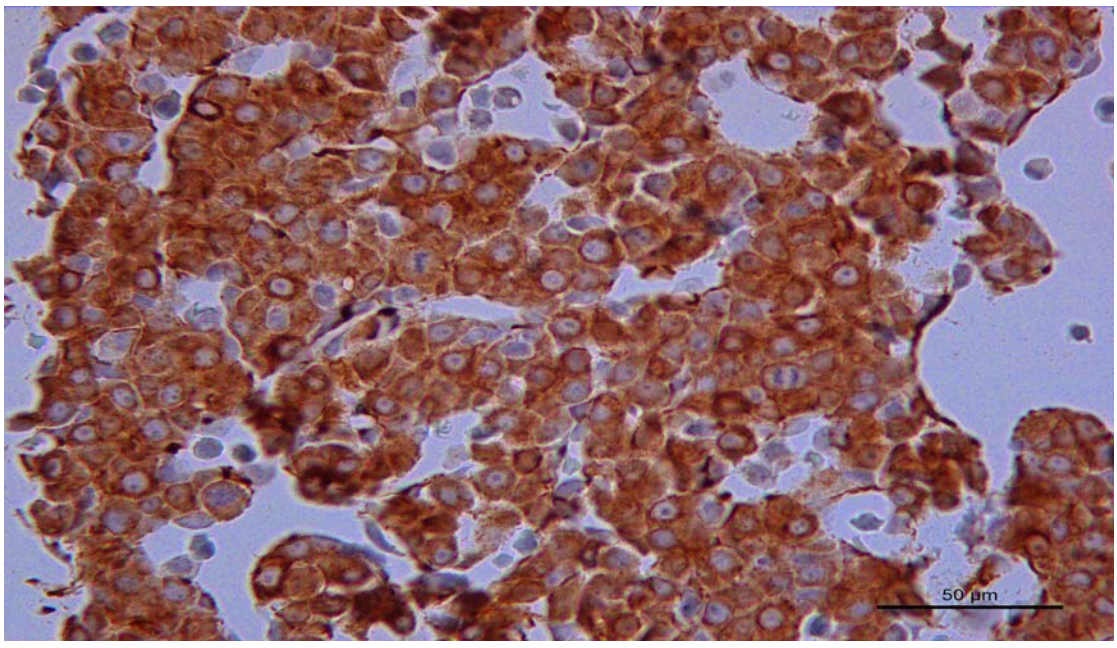


Fig. 23 Gestational week 11. Immunoreactivity for CK 19 of hepatocytes.No presence of bile ducts. 40x.

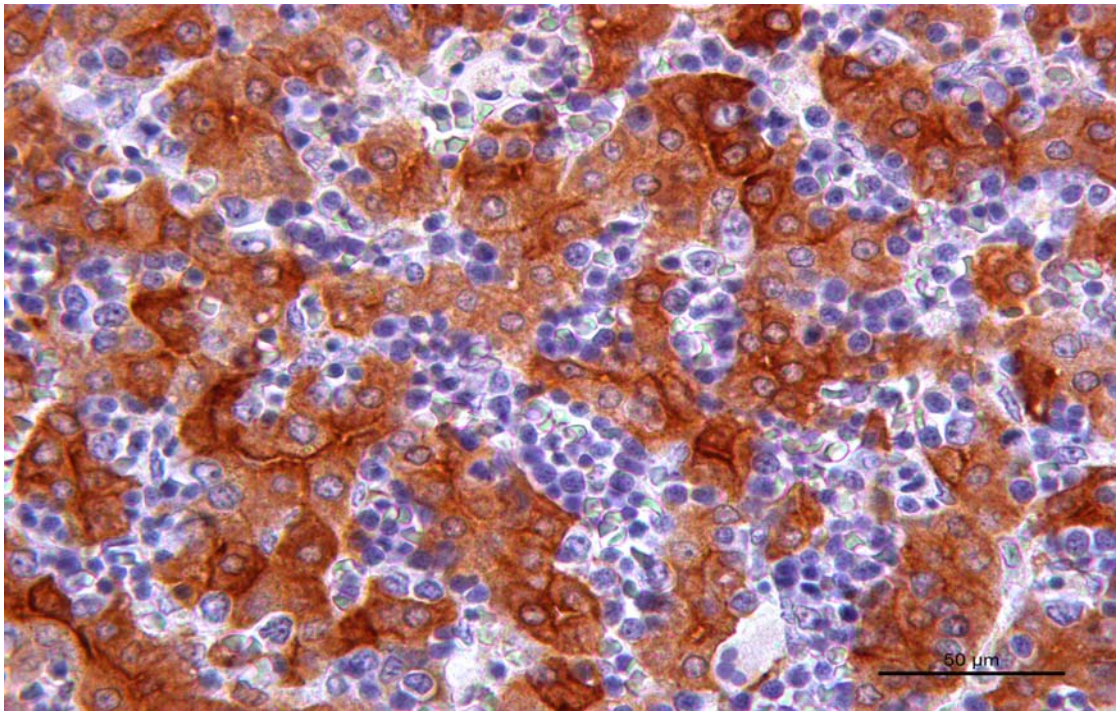


Fig. 24 Gestational week 12. Immunoreactivity for CK 19 of hepatocytes. No presence of bile ducts. 40x.

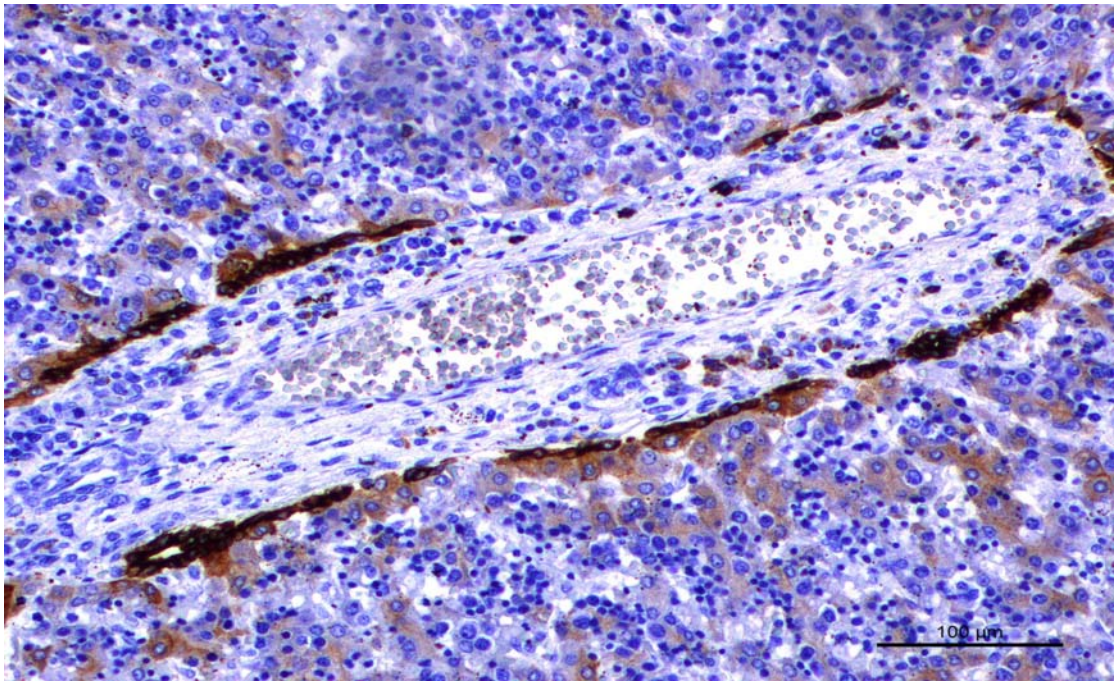


Fig. 25 Gestational week 15. Immunoreactivity for CK 19 on bile duct cells. Weak positivity on hepatocytes.40x.

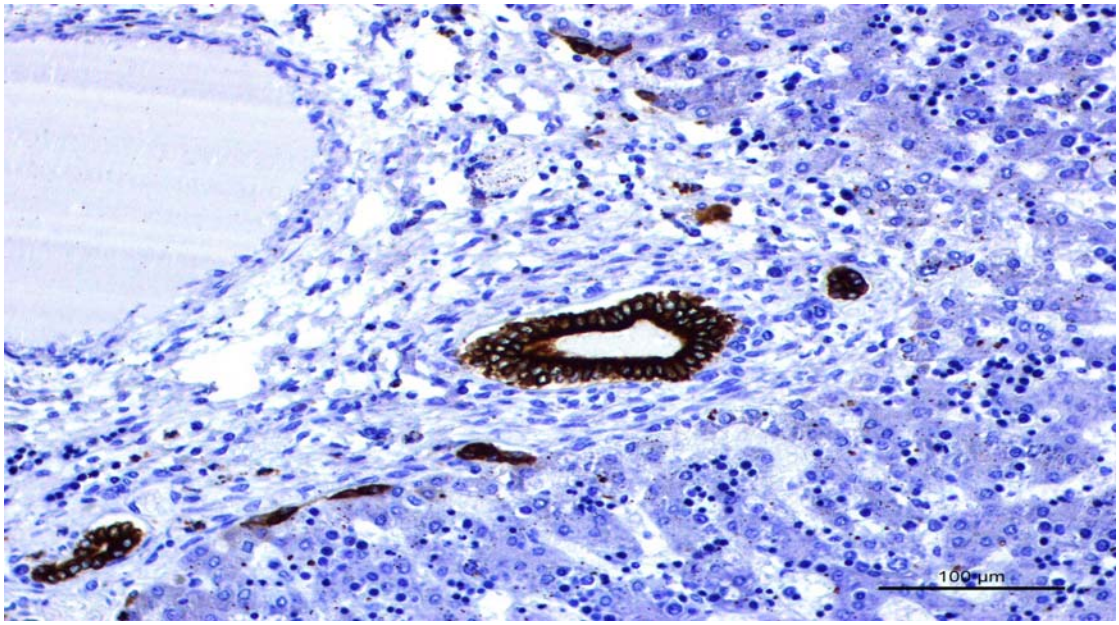


Fig. 26 Gestational week 18. Immunoreactivity for CK 19 on bile duct cells. Negativity on hepatocytes. 20x.

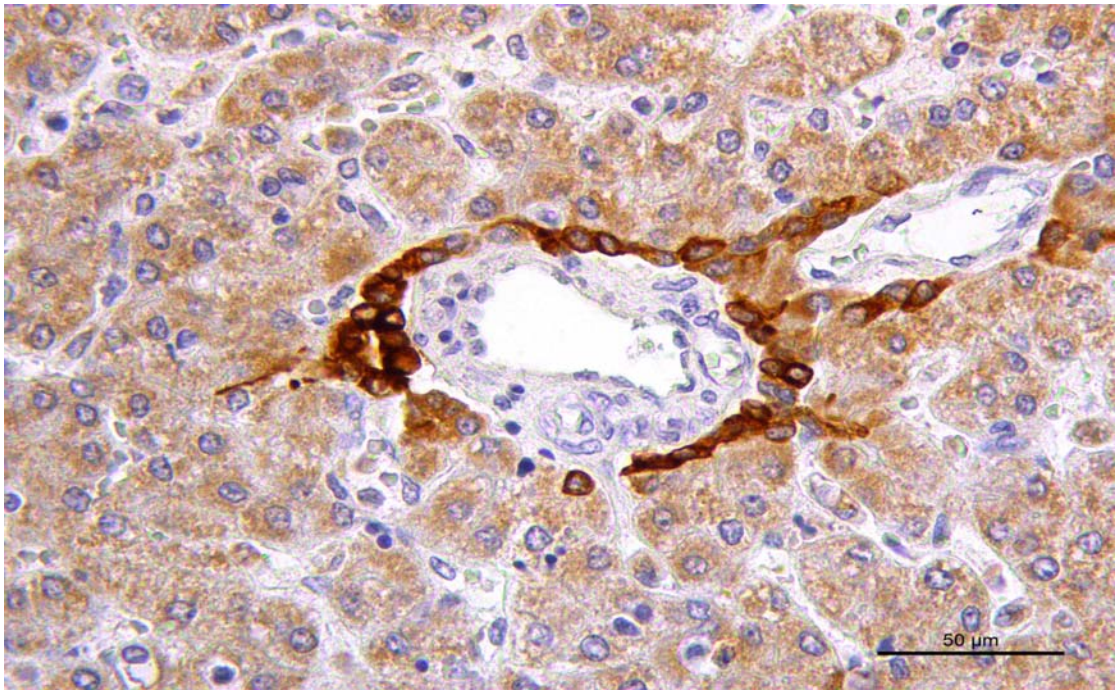
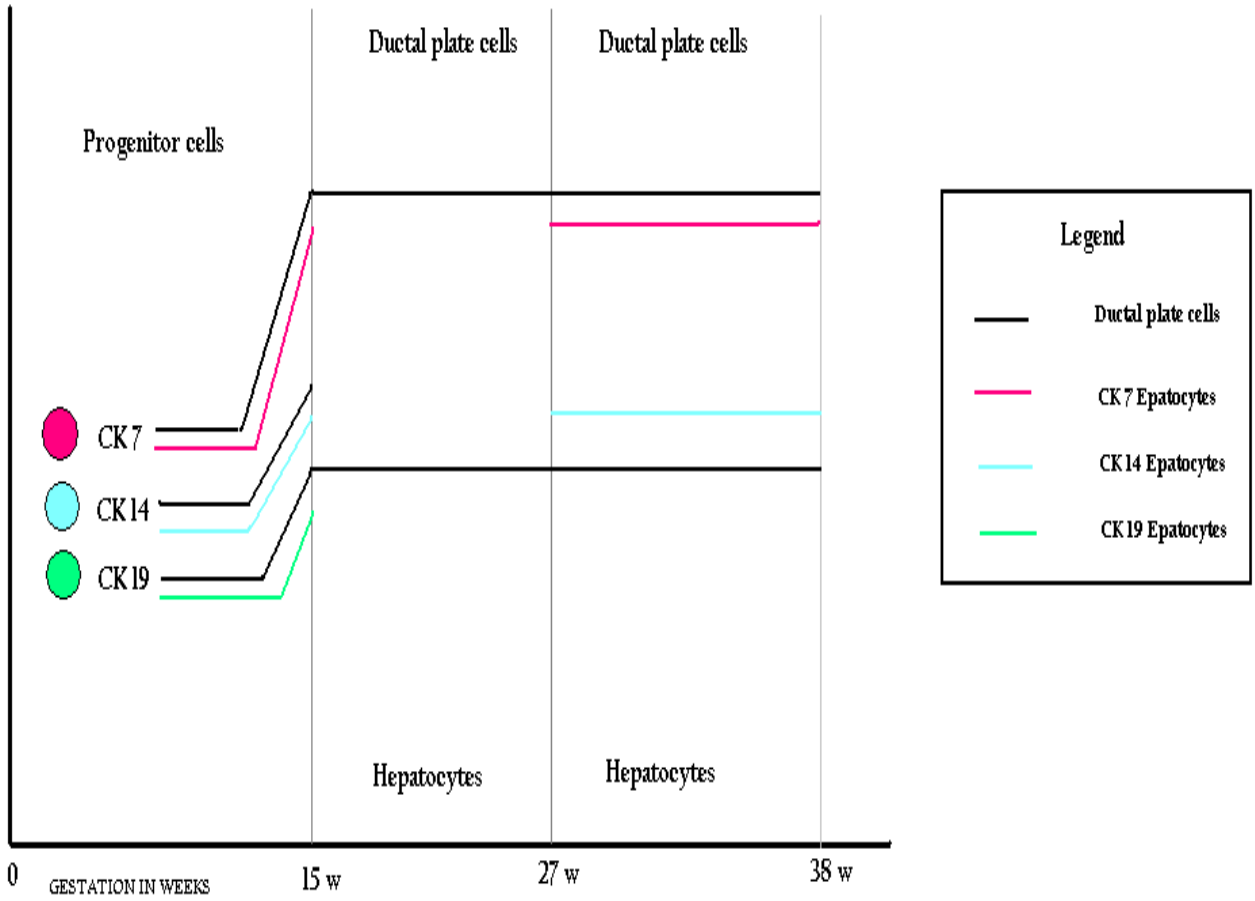


Fig. 27 Gestational week 27-28. Immunoreactivity for CK 19 on bile duct cells and hepatocytes. 40x.

In this grafich (Fig. 28), we have a schematic rapresentation of the expression of cytokeratins (CK7, CK14 and CK19) in the fetal human liver at different gestational age, based our results:

Fig.28



Discussion

The documentation and the characterisation of human stem/progenitor cells of the liver are an interesting subject of the current scientific literature[75]. The identification of hepatic stem/progenitor cells was first claimed in experimental animal models, while the chief support for their existence arises from several studies on cirrhosis, liver disease, and carcinogenesis. Liver stem/progenitor cells are typically characterised by the self-renewal ability that means being able to differentiate into diverse lineage after injury or damage[76]. The regenerative potential of the adult human liver after damage is based on the aptitude of hepatic stem/progenitor cells to differentiate both into hepatocytic or cholangiocyte lineage[77,78]. Besides liver stem/progenitor cells may also differentiate into extrahepatic cell types, like intestinal, pancreatic[79,80] and insulin-producing cells or may give rise to liver cancer[81]. The presenc of hepatic stem/progenitor cells were discovered in human liver[82], in human liver disease[83] and cirrhosis[84]. Hepatic stem/progenitor cells were identified in hepatoblastoma[85], as well. The identification of hepatic stem/progenitor cells was first claimed in experimental animal models[86,87], where prolonged and severe hepatic injury with acetylaminofluorene in rats fed with choline devoid diet was restored by

pre-existing parenchymal cells derived from undifferentiated cholangiole cells[79,88]. The liver sections from these rats models prove the presence of proliferating small periportal scant cytoplasm oval cells, that spread across the liver[79,89]. Further studies then discovered other poisons able to induce oval cells in combination or alone [79]. The markers expressed or upregulated by ratio oval cells are cytokeratins 8/18, cytoketins 19, cytokerains 7, cytokeratins 14, CD90, OV6, alfa-fetoprotein[90,91], CD-34 and c.kit[79].

Liver stem/progenitor cells hypothesis embody a heterogeneous population through a range of morphological and immunohistochemical features extending from bile duct cells to hepatocytes. According to with the niche hypothesis, the stem/progenitor cells reside within a not specific compartment including the surrounding matrix, cells, and signals required for proliferation and differentiation control[92]. The Wnt, Notch, and Hedgehog pathways regulate the stem/progenitor cell quiescence and proliferation. Particulary Notch and Wnt signals manage cell fate by regulating the switch between cells division or differentiation[93]. The niche consists of the interaction with cell types, cellular and extracellular microenvironment, signaling and adhesion molecules contribute to self-renewal, regulates stem/progenitor cell maintenance[93,94]. The hepatocyte niche is composed of numerous cell types: hepatocytes, cholangiocytes, hepatic stellate cells, Kupffer cells, Pit cells, myofriboblasts, endothelial

cells, and immune cells. All cells interact and cross-talk with hepatic stem/progenitor cells[90].

Adult human hepatocytes contain cytokeratyn 8 and 18[95]. Differentiation of liver stem/progenitor cell towards hepatocyte evolves from the hepatoblast (cytokeratin 19 positive), through the commited hepatocytic intermediate cell with hepatobiliary phenotype (both cytokeratin 19 and cytokeratin 7 positive), to intermediate cell with hepatocytic phenotype (cytokeratin 19 negative and cytokeratin 7 positive) and finally to mature hepatocytes (cytokeratin 7 negative)[96]. From 9 weeks of gesttional age, the portal vein branches are surrounded by a layer of cells referred to as the ductal plat, showing a strong positivity for CAM 5.2, KL-1, cytokeratin 8/18, cytokeratin 19 and negative for cytokeratyn 7 until 20 weeks of gestation[95]. Small- undifferentiated oval cells (hepatoblasts) with scant cytoplasm are easily identified in the developing portal tracts. At the periphery of developing portal tracts in the ductal plate, these cells are easily identifiable by the expression of cytokeratin 7. Moreover, these cells are characterized by the expression of stem markers as SOX9 and c-kit[75].

In the 18 day heman embryo (2.5 mm stage), the liver bud arises as thickening of the endoblastic epithelium in the ventral wall of the foregut, near the origin of the yolk sac[96,97]. By 22 days of gestation (19-somite embryo, 3-4 mm), the hepatic diverticulum can be seen to protude into

the mesenchyme of the septum trasversum[98,99]. Available data indicates that the liver primordium is made up of a uniform population of at least bipotential progenitor cells that will give rise to both liver parenchymal cells and biliary epithelial cells[97,98,100,101,102]. In human, the liver stem progenitor cells are immunoreactive for cytokeratins 8, 18 and 19[103,104,105,106]. In normal adult rat liver, parenchymal cells express only cytpkeratin 8 and 18; intrahepatic bile duct cells express in addition cytokeratin 7 and 19[107] and 12[108]. In its earliest developmental stages, the human embryonic liver is composed of epithelial liver cell precursors (hepatoblasts) that express cytokeratins 8, 18 and 19[109,110] and in addition cytokeratin 14 from 10 to 14 weeks of gestation[111]. Around the eighth week of gestation, the primitive hepatoblasts adjacent to the mesenchyme around the largest hilar portal vein branches become more strongly immunorective for their cytokeratins 8, 18 and 19[112]. This layer of cells, surrounding the portal vein branches like a cylindrical sleeve, is termed ductal plate[113]. During the following weeks, ductal plates also appear around the smaller portal vein branches at a distance from the hilium. In the meantime, the hepatoblasts not involved in ductal plate formation gradually lose immunoreactivity for cytokeratin 19 , and by 14 weeks of gestastion the future parenchymal cells are immunoreactive only for cytokeratin 8 and 18, the pair of cytokeratin normally expressed in adult liver parenchymal cells. By the 20th weeks of gestation, weak

immunoreactivity for cytokeratin 7 appears in the cells of the developing ducts, again appearing first in the older ducts near the hilium[114]. The immunoreactivity for cytokeratin 7 gradually increases and extend into more peripheral ducts, to reach the level of immunoreactivity observed in ducts of the adult livera t approximately 1 moth after birth[114]. Anyway, from 10 to 14 weeks of gestation, liver stem progenitor cells express in addition cytokeratin 14[115]. The majority of liver stem progenitor cells will differentiate into liver parenchymal cells. During this process, they gradually lose cytokeratin 14 and 19[105,113]. Cyotkeratin 19 is no longer detectable in human hepatoblasts after 20 weeks of gestation[105,106].

Instead, regardinf fetal development, cytokeratin filaments are scare but are uniformly distributed inside the cytoplasm[116]. Later in development, contacts between hepatocytes become more numerous and bile canaliculi become well developed. The density of cytokeratyn filaments increases and appears to be very high near the bile canaliculi. Cytokeratin filaments show a symmetrical distribution in relation to the nuclear region. The highest density of filaments is found near the cytoplasmatic membrane[117]. Cytokeratin 7 and cytokeratin 19 are strongly expressed by interlobular bile ducts, intraportal and intralobular bile ductules and the biliary epithelial cells that partly line the canal of Herring. It has been suggested that the individual cytokeratin 7+ and cytokeratin 19+ cells that partly line the canal of Herring represent hepatic progenitor cells. Biliary

epithelial cells also express cytokeratyn 8 and cytokeratyn 18. In contrast, normal hepatocytes express cytokeratin 8 and cytokeratin 18 but not cytokeratin 7 or cytokeratin 19.

At this point, it's important say something about the intrahepatic bile duct. During the first 7 weeks of embryonic life, there are no intrahepatic bile duct[97,118]. The intrahepatic biliary tree develops in close association with the portal vein branches[119]. Between 5 and 9 weeks of gestation, formation of intrahepatic bile ducts starts around large portal vein branches close to the liver hilium[114]. Morphologucally, intrahepatic bile duct develepment is characterized by the formation of the "ductal plate" i.e. duplicated layers of epithelium surrounding the portal vein branches[97,101]. By 12 weeks of gestation, not only hiliar portal vein branches but also more peripheral ones are surrounded by ductal plates[114]. The first generation of ducts (left and rigth hepatic ducts) are present from 12 weeks on[120]. By 35 weeks of gestation, most portal tract contain an individualized bile duct. However, even at 40 weeks, some of the smallest ramifications of the portal vein are still surrounded by a discontinuous ductal plate[97] indicating that the development of the intrahepatic bilary tree is not terminated at birth. Throughth development, cells of the ductal plates and of the bile ducts show a strong immunoreactivity for cytokeratin 8, 18 and 19. At around 20 weeks of gestation, individual bile ducts in large portal tracts show a weak apical

positivity for cytokeratin 7, thereby acquiring the full set of cytokeratins characteristic of normal biliary epithelial cells, i.e. cytokeratin 7, 8, 18 and 19[113,121]. Over the following weeks of gestation, bile ducts and ductal pate cells display an increasing immunoreactivity for cytokeratin 7; however, "adult" staining intensity is reached only at 1 month after birth[113].

Based on our results and the literature[4], from 7 to 12 weeks of gestation, the positivity for ck7 is particularly evident in the cytoplasm of progenitor cells of hepatocytes, in development, with a perinuclear granular appearance. From 15w to 19w, no immunoreactivity is found in progenitor cells, as in letterature[74]. At about 27w up to 38w, we have a cytoplasmic, moderate and more homogeneous positivity than before.

The bile ducts in the first 7 weeks of gestation are absent[4]. Then towards the 9w, transitional cells begin to appear, around the branches of the portal vein. From 15w onwards, we have a strong positivity of CK7 in ductal cells, remaining until late gestational age, this differs from the literature[95], according to which at 20w there is a weak positivity of ck7 on the bile duct cells. The positivity of ck7 in the bile ducts is cytoplasmic and perinuclear.

Based on our results, from 7w to 12w, a cytoplasmic positivity for CK14, mainly perinuclear, is present in the cytoplasm of progenitor cells.

Instead in literature[95,109,110], the positivity for CK14 in the cytoplasm of progenitor cells start from 10 gestational week until 14w.

From 15w to 19w, no immunoreactivity was found in hepatoblasts. From 27w up to 38w, there is a positive recover, that it is not seen in literature[95,109,110].

On the other hand, there is no positive effect during the development of the ductal system.

In our results, from 7 to 12 weeks of gestation, we have an intense cytoplasmic positivity homogeneously spread at the level of progenitor cells for CK19. In literature as for CK14[95,109,110], there is a strong positivity for liver progenitor cells from 10w until 14w. Based on our results, at 15w it is more light and focal and then negativize and start again from 27 w.

Like CK7, also the CK19 is intensely expressed in the bile ducts from 15w and then maintained until the 38th gestational age studied. In literature[95], the portal vein branches are negative for CK19 from 9w until 20w.

Conclusion

In conclusion, this study gives new immunohistochemical data reinforcing the hypothesis on existence of cytokeratin expression in the fetal human liver during development. Future study are needed in order to evaluate mRNAexpression of these markers and to evaluate other markers of stem/progenitor cells in other gestational ages, in oder to better clarify their role in cell proliferation, migraton and differentiation of the human liver during development, post-natal and adult life.

The study of stem cells in the human fetal liver represent a fascinating toping of research, in that they are interconnected with hematopoietic stem cells. In fact, the fetal liver behaves also as hematopoitic tissue.

The thesis “immunohistochemical markers of stem progenitor cells in the fetal human liver” well focus this aspect and analyzed several different markers of stemness, starting of field research that suerly will represent the basis for future molecular approaches.

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