Homeobox gene expression in extravillous trophoblasts in first trimester pregnancies

Literature review and an immunohistochemical pilot study

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1. Abstract

Trophoblast dysfunction is a major factor in defect placentation, which is thought to be an important etiology of abortions. Homeobox genes are transcription factors that regulate the transcription of other genes. They encode highly conserved DNA-binding domains (homeodomains) that regulate proliferation, differentiation and migration, important for pattern formation and organogenesis during embryogenesis in the development of multicellular organisms. Homeobox genes have also been shown to control normal development of the placenta. They are thought to play an important role in trophoblast proliferation and differentiation. Chloride channels are important in many cellular processes like electrolyte transport, pH regulation, water balance, cell potensials and apoptosis. Little is known about their involvement in trophoblasts and placentation.

Our thesis consists of a literature review related to placenta, placentation, trophoblasts, homeobox genes, ion channel proteins and abortion. It also comprises a pilot study testing our hypothesis: that spontaneous/missed abortions and hydatiform mole are consequences of defect trophoblast function and signalling of homeobox genes in trophoblasts in the placental bed. We also studied the expression of an intracellular ion channel gene called CLIC3.
The pilot study was performed on a diagnostic material from first trimester abortions, partly in Oslo and partly at the Pregnancy Research Centre at the University of Melbourne.

Material: Tissue micro arrays (TMAs) from the placental bed in normal (therapeutic abortions) and abnormal (spontaneous and missed abortions and moles) first trimester pregnancies.

Methods: Immunohistochemical study of expression of the homeobox genes TGIF and HEX and the intracellular ion channel CLIC3.

Results: There was a reduction in the expression of Homeobox genes TGIF and HEX and of the ion channel protein CLIC3 in the extravillous trophoblasts in the placental bed in the clinical abortions as compared to the normal controls (therapeutic abortions). The reduced expression was most markedly for HEX in missed abortions. The findings might indicate a defect trophoblast function in clinical first trimester abortions. The staining pattern in the positive cells varied, especially in the homeobox immunostainings. Further studies are needed.

2. Literature review

2.1 Placental functions and gross anatomy

The placenta is the metabolic interchange between fetus and mother, separating the maternal and fetal circulation. It is a transient organ and an important part of the products of conception. The embryo cannot survive without it, and normal growth and development of the fetus is dependent of a normal functioning placenta with membrane. The placenta develops form the chorionic sac, in close proximity to the maternal placental bed, consisting of decidually transformed endometrial stroma with invading trophoblasts.
The most important functions of the placenta are (Langman):

- exchange of gases (oxygen, carbon dioxide and carbon monoxide) and nutrients (metabolites, nutrition, and electrolytes) between the maternal circulation and the fetal bloodstream
- hormone production (progesterone, hCG,, somatomammotropin and estrogenic hormones)
- excretion of wastes
- transmission of maternal antibodies (passive immunity)
- immunological barrier
- detoxification of drugs

An examination of the placenta is routine procedure following delivery. At term, the placenta normally is discoid with a diameter of 15 to 25 cm. The thickness is around 3 cm, and the weight 400 – 600g. The chorionic plate and the umbilical cord could be seen under the thin amnion at the fetal side. The maternal side is dominated by cotyledons. No focal lesions, discolorations or hemorrhagic areas should be seen.

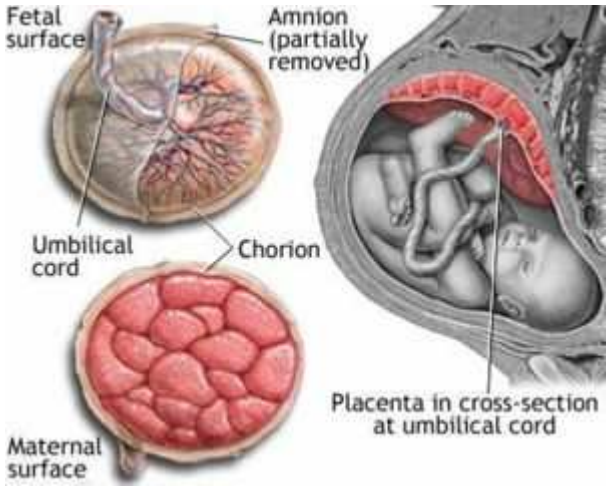


Fig 1. Normal placenta at term
www.nlm.nih.gov

Macroscopic and microscopic examination of placenta is especially important if there have been maternal or fetal complications during the gestational period, delivery or perinatal period. The examining of the placenta can provide important information related to:

- Fetal growth restriction
- Neonatal illness and infant death

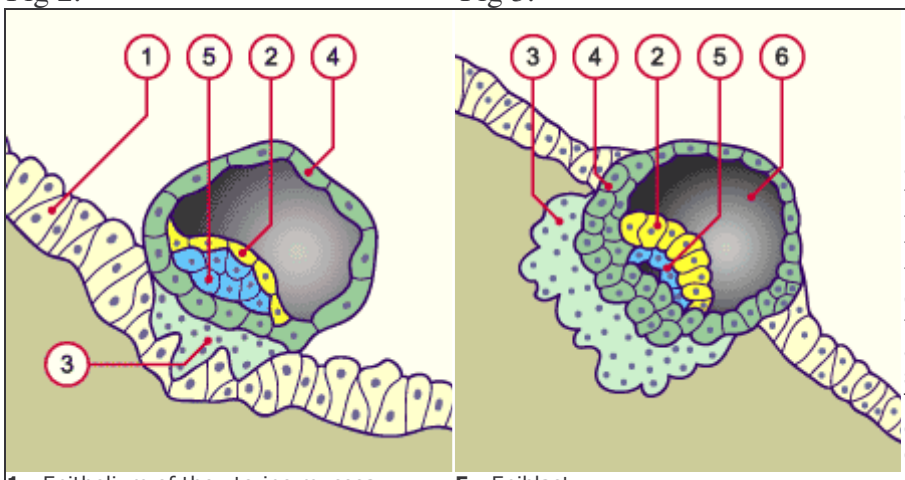
- Information valuable for the follow up in subsequent pregnancies
- Placental function and dysfunction

In addition it can provide new knowledge and insight in gestational and perinatal clinical problems.

2.2 Placental development

Normal embryonic and placental development requires implantation of the fertilized ovum into the uterine mucosa (endometrium). The blastocyst (with starting differentiation into embryonal and placental structures) attaches to the mucosa. Placentation, i.e. the growth and formation of the placenta and placental bed, is a highly coordinated process.

Fig 2. Fig 3.



1 Epithelium of the uterine mucosa
2 Hypoblast
3 Syncytiotrophoblast
4 Cytotrophoblast

5 Epiblast
6 Blastocyst cavity

Fig. 2
Free blastocyst (following the dissolution of the pellucid zone) in adplantation phase on the uterine wall (6th to 7th day). The trophoblast cells of the embryonic pole differentiate themselves, multiply, and form the invasive syncytiotrophoblast. The abembryonic pole consists of cytotrophoblast cells.

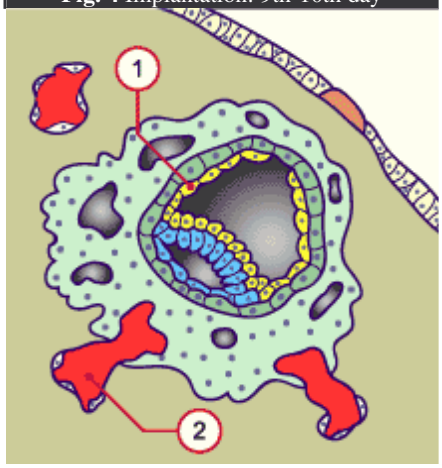
Fig. 3
Didermic embryonic disk (hypoblast and epiblast) after 8 days. The ST continues its invasive, lytic activity into the maternal tissue.

Figs 2 and 3 show embedding of the blastocyst in to the uterin mucosa.
http://www.embryology.ch/anglais/gnidation/etape02.html#apposition

Implantation starts approximately six days after fertilisation with attachment of the outer trophoblast cells of the blastocyst to the uterine lining. The attached trophoblasts differentiate into syncytiotrophoblasts, which invade the uterine endometrium. The trophoblasts thus enable the developing conceptus with embryo to invade deep into the endometrial stroma of the uterus and establish a connection with the maternal circulation.

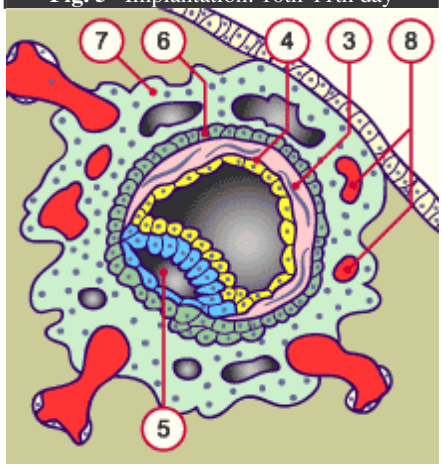
Trophoblasts cells establish the uteroplacental circuit at approximately day twelve of pregnancy. The placenta is mostly derived from trophoblast (fetal) cells.

Fig. 4 Implantation: 9th-10th day



1 Hypoblast growing ventrally
2 Eroded maternal capillaries

Fig. 5 - Implantation: 10th-11th day



3 Extraembryonic reticulum
4 Heuser´s membrane
5 Amniotic cavity
6 Cytotrophoblast
7 Syncytiotrophoblast
8 Lacunae, filled with blood

Legend

Fig.4, Fig.5
The destructive lytic activity of the ST reaches the capillaries of the endometrium. The maternal blood flows into the lacunae. The ST envelops the maternal capillaries, expands its lacunae network, and forms an arterial inflow and a venous outflow system.

Figs 4 and 5: Destruction of endometrial capillaries and development of lacunae filled with maternal blood. http://www.embryology.ch/anglais/gnidation/etape02.html#apposition

Placenta contains two parts: the major one is the fetal part, made from chorion frondosum In addition there is a small, but important maternal part comprising decidua basalis (transformed endometrium) with extravillous trophoblasts and remodelled spiral arteries with trophoblasts in the fibrinoid replacement wall and partly trophoblast lining (the placental bed).

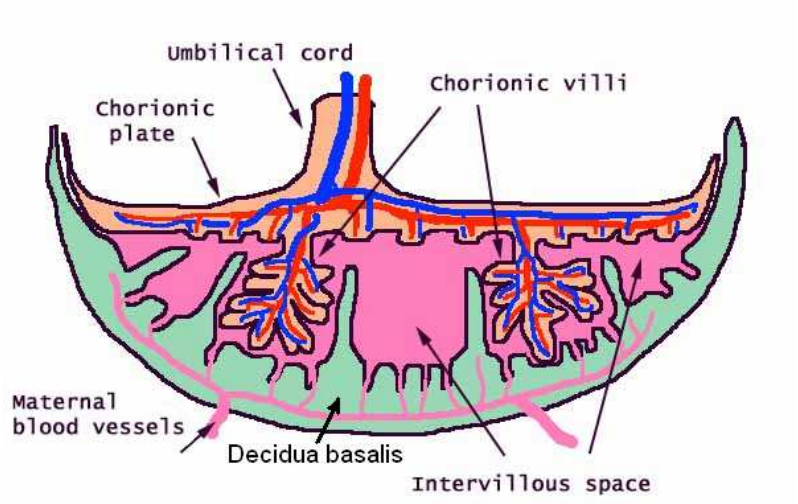


Fig 6. Schematic drawing of the major components of the placenta.
www.siumed.edu/~dking2/erg/resource/placenta.jpg

The chorionic villi are the main functional units of the placenta. Villi have grown out of the chorionic plate and are peripherally anchored to the maternal decidua with an outer cytotrophoblast shell. In the maturing placenta there are a growing number of villi, like branches on a tree. The villi are subdivided into stem-, primary-, secondary- and tertiary-

villi according to size, amount of stroma, and, most importantly, the fetal capillaries that merge towards the outer layer of syncytiotrophoblastic cells. In the mature, tertiary villi, the syncytium and endothelial cells are the only layers that separate the maternal and fetal circulation. This enables an effective substance exchange. The villi are surrounded by the intervillous space, filled with maternal blood from the uteroplacental arteries (transformed endometrial spiral arteries).

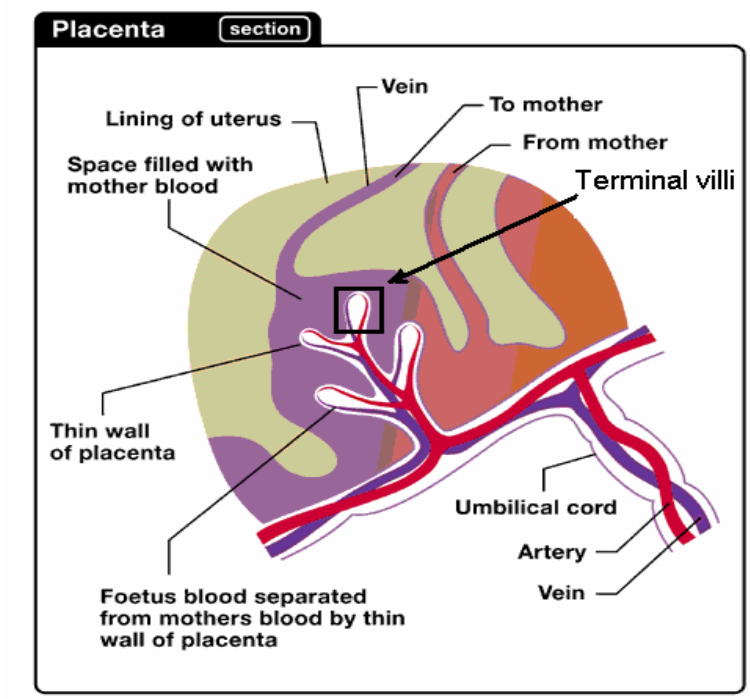


Fig 7. Schematic drawing of the major components of the placenta involved in substance exchange. www.natracare.com/.../illustrations/placenta.gif

2.3 Role of villous and extravillous trophoblasts in normal and abnormal placental development:

Following normal implantation and start of placentation, trophoblast cells undergo extensive proliferation and differentiation. Trophoblast differentiation occurs by two major pathways, villous and extravillous. In our pilot study, we focussed on the extravillous trophoblasts.

The villous pathway starts with villous stem cell cytotrophoblasts that differentiate into villous cytotrophoblasts. Villous cytotrophoblasts will then differentiate into villous syncytiotrophoblasts. The syncytiotrophoblasts are the primary site of placental transport, protective and endocrine functions.

The extravillous pathway starts with villous stem cell cytotrophoblasts that differentiate into cytotrophoblast cells. By day thirteen to fourteen of pregnancy, cytotrophoblast cells penetrate the layer of syncytiotrophoblast surrounding the fetus to form columns of extravillous cytotrophoblast cells. Extravillous trophoblasts cells then invade the decidua basalis and migrate so that they penetrate the wall of maternal spiral arteries (media and endothelium), hence the arterial walls are replaced by trophoblasts and fibrinoid material and become sinusoidal sacs. This transformation is vital to insure enough blood supply to the fetus.

Endovascular trophoblasts invade arteries and also form plugs which are in continuity with the cytotrophoblast shell. The plugs act as valves and sieves to prevent pressure damage to

the gestation sac in the first trimester. Giant cells are found in a deeper part of the decidua and myometrium. These cells have lost invasive ability and are more passive, but they seem to play an important part for placentation (Loke).
Giant cells seem to regulate blood flow to the implantation site, and production of angiogenic factor by these cells seems to be critical for maternal vascular development in the pregnant uterus (Hemberger).

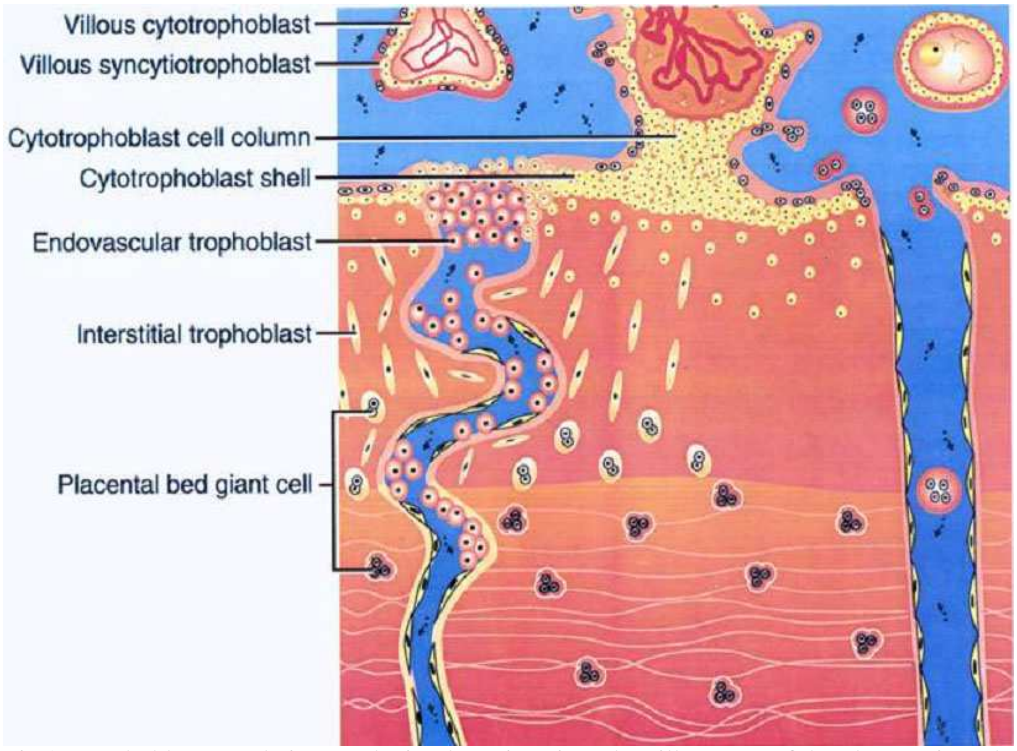


Fig 8. Trophoblast populations at the implantation site. The villous part of the placenta is at the top surrounded by intervillous space. Decidua basalis with the myometrium at the bottom with a spiral artery on the left and with a vein on the right. The picture shows a remodelled spiral artery were the endothelium and media of the decidual portion are replaced by trophoblasts and fibrinoid material. This is due to infiltration by endovascular and interstitial trophoblast. Placental bed giant cells are seen in the myometrium (Loke).

Limitation of trophoblast invasion is likely the main feature of defective placentation and the failure to develop a normal uteroplacental blood supply. Normal fetal growth and development must ultimately depend on how trophoblast invasion is controlled (Loke).

2.4 Gene control and cell differentiation.

The exact number of genes encoded by the human genome is still unknown. National Human Genome Research Institute (NHGRI) and the Department of Energy (DOE) estimated the number of human protein coding genes to 20,000-25,000 in 2004.

Human cells in different tissues express only a fraction of their genes. The conversion of the genetic information in DNA through RNA into proteins is called gene expression. Tissues are made differentiation of the cells, where genes are switched on and off by gene regulatory proteins which bind to a particular DNA sequence and initiates transcription. Transcription refers to the transfer of genetic code information by which a base sequence of messenger RNA s synthesised on a template of complementary DNA. The transcription of specific genes is switched on and off in cells by gene regulatory proteins. These bind to short stretches of DNA called regulatory DNA sequences, and initiates transcription with

RNA polymerases.
Different gene regulatory proteins in different cells ensure the expression of only those genes appropriate to that cell. A single gene regulatory protein, if expressed in an appropriate precursor cell, can trigger formation of a specialized cell type or even an entire organ (Alberts).

2.5 Homeobox genes

Homeobox genes are transcription factor genes which encode highly conserved DNA-binding domains (homeodomains) that regulates proliferation, differentiation and migration. This is important for pattern formation and organogenesis during embryogenesis. Homeobox (180 basepairs) is a part of a homeobox gene and encodes the homeodomain (60 amino acid). The homeodomain is a DNA-binding domain that is a part of proteins that are usually transcription factors. These transcription factors regulate the transcription of other genes and hence very frequently play important roles in development of multicellular organisms.

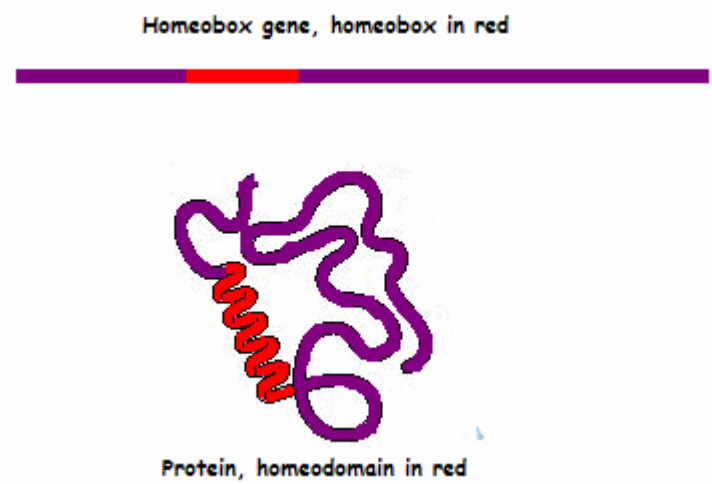
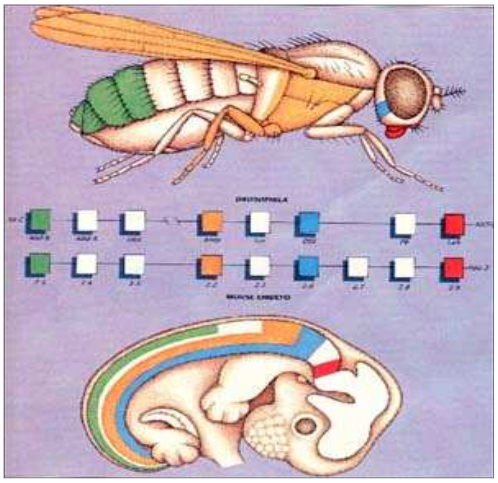


Fig 9. Schematic drawing of a homeobox gene and its expressed protein with the homeodomain.

A subgroup of homeobox genes is called Hox genes. In humans (and other vertebrates) these genes are found in gene clusters (four in mammals) on the chromosomes. These are called Hox clusters. Only genes in the HOX cluster should be named Hox genes.

2.6 Homeobox genes control normal development in the embryo

Hox genes are important in the regulation of cell proliferation, differentiation and migration. They play an important part in organogenesis and tissue formation during embryogenesis



Genes have a common evolutionary origin, shown by similarity in homeobox sequences. They are nearly identical in all species.
The genes specify location alongt hemain body axis in almostall animals.
Example: fly and a human embryo had a common ancestor half a billion years ago, and the Homeobox sequence has hardly changed during that long period. (Zhilman)
The genes determine where limbs and other body segments will grow in a developing fetus.

Fig 10. Homeobox genes and the vertebrate body plan (Robertis, Scentific American Jul 90)

The specificity of a single homeodomain is normally not enough to recognize the target genes. Thus it is believed that homeodomain proteins act together as complexes in the promoter region of their target genes. These complexes have much higher target specificity than a single homeodomain.

Mutations in any one of these genes can lead to the growth of extra, typically non-functional body parts in invertebrates. A known example is an antennapedia complex in *Drosophila,* which results in a leg growing from the head in place of an antenna; a result of a defect in one single gene. Duplication of homeobox genes can produce new body segments, and such changes are likely to have been important in the evolution of segmented animals. Mutation in vertebrate Hox genes usually results in miscarriage (Lodish).

2.7 Homeobox genes in trophoblasts and placenta

The role of homeobox genes in the placenta is not fully understood. The research focus in this field has increased in recent years. The PRC (Pregnancy Research Centre) in Melbourne have performed studies to gain knowledge about the function of these transcription factor genes, which are though to be important for trophoblast function and placental development.

Studies at the PRC in Melbourne resulted in five homeobox genes being cloned from the human placenta: HLX1, DLX4, DLX3, MSX2 and MOX2. In human trophoblast cell culture, HLX1 can be inactivated by siRNA technique. siRNA studies show that when HLX1 is inactivated, there is reduced trophoblast proliferation (Kalinois, unpublished data).
Further studies concluded that there is an association between alterations in homeobox gene expression level of HLX1/DLX4 and pathological development of the human placenta. By reverse transcriptase PCR, real time PCR, and western blots on placenta samples from normal and IUGR (Intra Uterine Growth Restriction) pregnancies, a consistent drop in HLX1 levels was seen in IUGR samples compared to normal, and a rise in DLX4 expression (Murthi).

Other studies have focused on different homeobox gene expression patterns in the placenta of multicellular organisms. Trophectoderm (progenitor tissue in mice for trophoblasts) is only specified in mouse when the homeobox-gene Oct4 becomes down-regulated. The formation of the trophectoderm is not simply a default pathway initiated by the down regulation of Oct4, but probably requires specific transcription factors. Examples of these homeodomain protein are Cdx2 and the T-box gene Eomes (Roberts 2004). The role of these and other homeobox genes in terms of trophoblast and placental function are still unknown.

2.8 Specific homeobox genes

The following homeobox genes are of specific interest for our pilot study.

TGIF (Transforming Growth Factor-Beta-Induced Factor) belongs to a family of evolutionary conserved, atypical homeodomain proteins that act as transcriptional repressors and corepressors in retinoid and transforming growth factor signalling pathways (Shen and Walsh, 2005)

HEX (Hematopoietically expressed homeobox) is a homeobox gene isolated from hematopoietic tissue. It is expressed in a range of hematopoietic progenitor cells and cell lines, and is an early marker of endothelial cell precursors (Kubo). The gene contains 1.9 kbp which encodes a 35-37 kDa protein. It is considered important in the initial stages of vasculogenesis and angiogenesis (Thomas). It is acting as a negative regulator for angiogenesis by modulating the expression genes in angiogenesis-related endothelial cells in vitro" (Nakagawa). HEX is expressed in extravillous trophoblast cells in first trimester placenta (Kalionis, unpublished data).

2.9 Intracellular ion channels and CLIC3

Channel proteins are transmembrane pores that allow passive and active movement of small water soluble molecules into or out of the cell or intracellular organeles. Chloride Channels are critical for important cellular functions like:

- Cell volume regulation.
- Electrolyte transport.
- Ph regulation.
- Control of cell-potential.
- Apoptosis.

CLIC family (Chloride Intracellular Channel) has eight members: p64, parchorin and CLIC 1-6 (Littler). The exact function of CLIC proteins is not known. Chloride channel regulators interacting with cellular signalling pathways (Qian). CLIC 3 was cloned by Qian et al in 1999, and shows homology with some other members of CLIC family. Compared to other organs (brain, muscle, lung, liver, pancreas, kidney, skeletal), the CLIC 3 is highly expresses in the human placenta (Qian). An unpublished study from PRC (Gude, unpublished observations), CLIC 3 was located in the nucleus in cultured cells, suggesting that the protein is transported into the nucleus by other proteins. The structure and function of the CLIC3 is uncertain, it could also be a channel regulator. CLIC3 may play a role in pregnancy as CLIC3 was found in higher concentrations in placenta perfusate from women with PE, when compared to normal placenta perfusate.

2.10 Abortion

Abortion or miscarriage is defined as the expulsion of a fetus before it reaches viability. Most abortions occur naturally between the sixth and 10^{th} weeks of pregnancy. An estimate of about 25% of all pregnancies end in an abortion, many early, clinically presenting as a delayed menstruation. 10 to 15% of clinically diagnosed pregnancies end in an abortion.

In approximately 50% of spontaneous abortions there are major chromosomal defects in the embryo. Other factors include infections, anatomic features of the uterus, luteal phase insufficiency and immunological factors. It is thought that defective implantation or trophoblast dysfunction also play and important role in abortions.

Abortions can be divided into the following categories
1) Spontaneous abortion (**SA)** -the loss of a pregnancy before 20 weeks, the point at which a fetus might be able to survive outside the womb. The woman's body expels all or some of the fetus, the placenta and the fluid surrounding the baby.

2) Missed abortion (MA) — Abortion in which the fetus died prior to 20 weeks gestation, but neither the fetus nor the placenta has been expelled from the uterus. The products of conception thus remain in the womb, but is no longer developing.

3) Hydatiform mole (MH- mola hydatididosa → here BM (Blære Mola)) is a disease of trophoblast proliferation. The hyperplastic trophoblast tissue proliferate on the surface of placental villi.
Hydatiform mole (Robbins) are principally divided into two types: Complete or partial.

- **Complete** – are diploid, and occurs when an empty ovum is fertilized by 2 spermatozoa (or a diploid sperm), (purely paternal, process called androgenesis). No fetus is developed, only placental, and it carries a risk for choriocarcinoma. (90% are 46,XX and 10% are 46,XY)
- **Partial –** are triploid, and results from a fertilization of a haploid ovum and a duplication of paternal haploid chromosomes. Can also occur if a normal haploid ovum is fertilized by two spermatozoa. Partial moles have no malignant potential and there is often a fetus. (69XXX, 69XXY, and some tetraploid cases)

4) Therapeutic abortion (TA) - In many countries, as in Norway, induced (therapeutic) abortion is legal. The majority are performed for social or psychiatric reasons.

3. Relevant techniques

The specimens used in our pilot study were all archive material, fixed in neutral buffered formalin and paraffin embedded.

Fixation agents are needed to preserve tissues in an original way. This is done by chemical coagulation of the tissue. Most commonly used are aldehydes, like formaldehyde and glutaraldehyde. They make cross-bindings between the molecules. Alcoholes like ethanol, acethon and metanole work through denaturation. Some fixatives are combinations of these.
In order to ease the technical and analytic parts of the study, tissue micro arrays were produced from the original archive paraffin blocks, the procedure detailed below. For routine staining we used HE, i.e. hematoxylin and eosin, visualizing DNA (the nucleus)

and the cytoplasm in the cells. Protein identification in the cells was done by immunohistochemistry.

3.1 Tissue micro arrays (TMAs)

Tissue micro arrays consist of sample paraffin blocks where small areas of relevant tissue for a study is removed from a series of original (often archive) tissue blocks and put into a new (recipient) paraffin block in a coordinate fashion (an array).

The area of interest is identified microscopically in the HE section from the routine, archive material and marked. With a hollow needle, a tissue cores from this area is removed from the paraffin block. The removed tissue cores are inserted into the recipient paraffin block in a precisely spaced, array pattern.

4-5 mμ sections from this TEM-block are cut using a microtome. Often serial sections are used. That means that a tissue core of 1mm can produce 250 serial sections. Each section is mounted on a glass slide. The sections can be stained by standard histological techniques, immunohistochemistry for protein analysis or in-situ-hybridization for gene analysis.

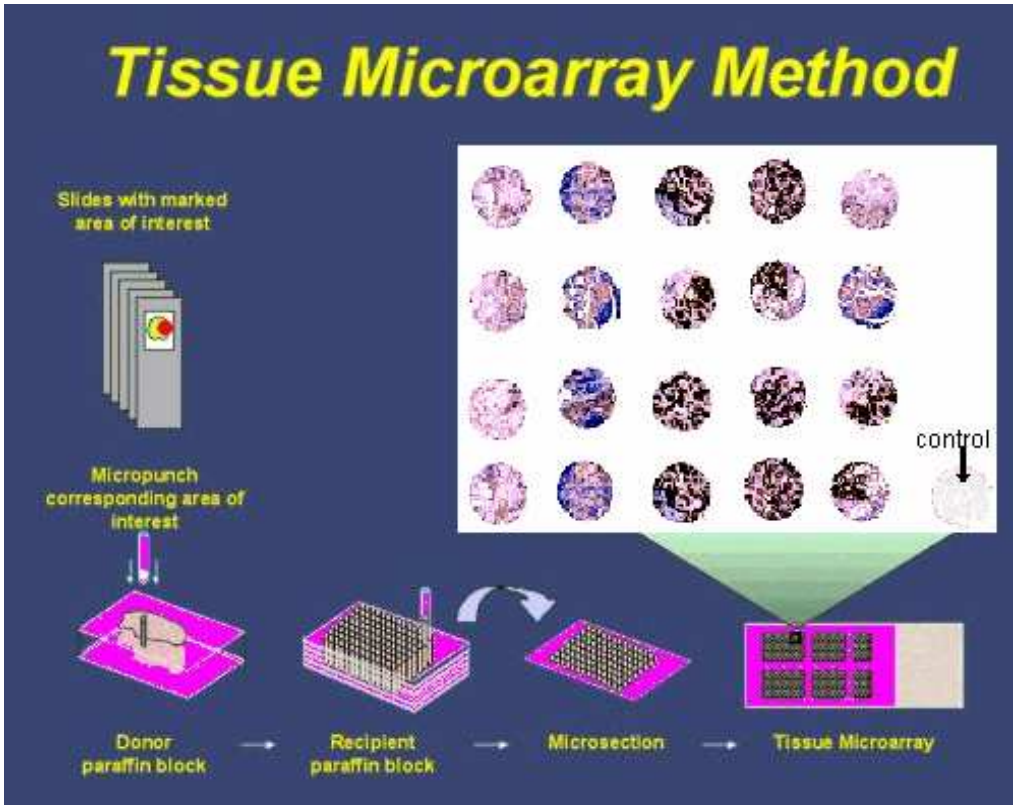


Fig 11. Schematic presentation of the TMA procedure. (Sunnybrook Health Sciences Centre 2006)

3.2 Immunohistochemistry

Immunohistochemistry is a procedure for specific identification of molecules, usually proteins, in a histological tissue section. In principle, the procedure is based on the specific immune reaction, where an antibody specifically will bind to its antigen. An antibody to a specific protein molecule applied to a tissue section will bind to its antigen if it is present in the tissue section. The immune reaction is visualized by labelling of the immune complexes. There are three ways to label immune complexes.

a) **Direct labelling** of the antibody.
b) **Indirect labelling** with a primary antibody first, then adding a secondary antibody that link to the primary.
c) **Indirect labelling with signal amplification**

Different detection systems could be used on the above categories.

The visualization system (labeling system) can be either enzyme based or fluorochrome based.

a) Enzyme-techniques: The labelling system is an enzyme (for instance peroxydase or alkaline phosphatase). By applying a relevant substrate, a water insoluble complex is precipitated and the complex can be seen in the tissue section in an ordinary light microscope. According to the enzyme and substrate chosen, the signal (precipitated complex) can be brown, red or blue.

b) Fluorochrome-technique (fluorescence): The labelling system is a molecule that fluoresces when illuminated by light of a specific wavelength. The signal (fluorescent light) can only be seen in a fluorescent microscope against a black background, without identification of other tissue structures. There are a number of fluorochromes to chose between, giving signals in green, red or blue.

c) Signal amplification: Enhances the sensitivity of immunohistochemical procedures. Many different techniques are used to amplify the signal to antibody ratio. One should avoid too much amplification because of the possibility of amplifying non-specific signals.

According to the fixation of the specimen, antigen in a tissue can be partly or completely or partly concealed. A variety of methods have been designed to maximise the availability of antigen for interaction with a specific antibody. This is called antigen recovery. The most common methods to unmask or “retrieve” the antigens through pre-treatment of the specimen are enzymatic digestion, microwave irradiation and autoclaving/pressure cooking.
A good working dilution for every antibody must be optimised for each individual system. It is important to work out the antibody dilution that gives the strongest specific antigen staining with the lowest non-specific background. Incubation times should be optimised as well.

The most common problems with immunohistochemistry are weak or no signal (staining), alternatively background staining. Different approaches are used to solve these problems. Chemicon international antibodies 101 – an introduction to antibodies

4. Pilot study

The pilot study was part of a research collaboration between the University of Oslo (Centre for Pediatric and Pregnancy-related pathology, Ullevål University Hospital) and University of Melbourne (Pregnancy Research Centre). The selection and identification of the material was done in Oslo prior to going to Melbourne for the homeobox gene-studies.

For the selection and identification procedures in Oslo, we had to learn morphological identification of remodelled spiral arteries and the various cell types in the placental bed. We did that partly in HE-sections, partly in sections stained with cytokeratin (CK7) as a trophoblast marker, Actin as a marker of smooth muscle media in spiral arteries and von Willebrand factor (factor VIII) as an endothelial marker.

4.1 Hypothesis and aim of study

In the pilot study we wanted to test the following hypothesis:

Spontaneous/missed abortions and hydatiform mole are consequences of defect trophoblast function. As homeobox gene expression and chloride channels may play important roles in trophoblast differentiation and proliferation, we predict that the expression of these genes will be different in extravillous trophoblasts from aborted conceptus compared to normal pregnancies.

The aim of the present study was to

1. Characterise expression of a set of homeobox genes (TGIF and HEX) and an intracellular chloride channel gene (CLIC3) in first trimester placental bed tissue harbouring trophoblasts. This has never been done before.
2. Compare gene expression pattern (qualitative) in various cells in the placental bed in abnormal (clinically aborted) and normal first trimester pregnancies.

4.2. Materials and methods

4.2 a) Patient material

Placental bed tissue from first trimester abortions was selected from archive routine material from the files at Department of pathology, Ullevål University Hospital for diagnostic purposes. The aborted tissue was selected from a series of spontaneous abortions, missed abortions and hydatiform mole. Therapeutic abortions served as controls (normal pregnancies). The material was made anonymous, identifiable only by the clinical information of gestational data and type of abortion. The material had been routinely collected by cervical dilatation and curettage of the endometrium for diagnostic purposes.

4.2 b) Methods

The tissue was routinely fixed in neutral buffered four % formaldehyde and embedded in paraffin. Four to five mμ sections were cut in a microtomer and the sections were routinely stained with hematoxylin and eosin (HE). Remodelled spiral arteries and decidua with invading extravillous trophoblasts were identified

and marked in the sections in an ordinary light microscope. The corresponding areas were punched out of the archived paraffin blocks and collected in tissue micro arrays (TMAs). Each TMA contained material from twenty different patients in addition to tissue from tonsils (as control). The TMAs were organized according to diagnosis: spontaneous abortion (SA), missed abortion (MA), hydatiform mole (BA) and therapeutic abortion (TA). The total numbers of TMAs in the pilot study was SA: 3, MA: 3, BA: 1 and TA: 1.

Fifteen serial five mμ sections were cut from each TMA. Sections # 1, 5, 10 and 15 were stained with HE for identification purposes. The remaining 11 were mounted on specially coated glass slides for later immunohistochemistry. Section # 8 was double immunostained in Oslo with cytokeratin CK7 (DAKO, Glostrup, Denmark, 1:300) and smooth muscle actin (DAKO, 1:200). To visualize the immunoreactions, we used standard visualisation techniques (DAB detection kit for CK7 and Fast Red for smooth muscle actin) in a Ventana ES Immunohistochemical Instrument (Tucson, Arizona, USA). The double immunostaining with CK7/smooth muscle actin was used to achieve a more precise identification to the trophoblasts in relation to the remodelling process of the spiral arteries in the placental bed. The CK7 expression is specific for trophoblasts (and glandular epithelial cells) and the DAB staining is dark brown. Smooth muscle actin is quite specific for smooth muscle cells and form a discontinous layer in the walls of remodelled spiral arteries, where trophoblasts induce a fibrinoid replace of the wall.

Seven cut sections on coated slides were left unstained in Oslo (#6, 7, 9, 11, 12, 13 and 14) and brought to Pregnancy Research Centre (PCR) in Melbourne for the homeobox gene and ion channel immuohistochemical studies. The commercial antibodies that we used had not been tested prior to our arrival. Only manual immunohistochemical techniques were used at PRC, see appendix 6.2 and 6.3. The three antibodies used were all from GenWay Biotech, San Diego, California, USA; the TGIF antibody was a rabbit IgG polyclonal one, HEX a mouse IgG monoclonal antibody and the CLIC3 antibody a chicken IgY polyclonal antibody. An indirect avidin biotin technique was used with DAB or AEC as detection reagents (see appendix 6.1 and 6.2).

4.2 c) General information about the composite images (mosaic)

All the tissue samples from the TMAs were studied in an ordinary light microscope. Because of the large quantity of tissue slides, we decided to present the results as mosaic composite representative images. No torn tissue samples were included in the mosaic.

The TMAs are presented in ten mosaics (see appendix 6.3):

1. three from TGIF with two different abortions (MA and SA) and therapeutic abortions as control (see pages 28-30).
2. three from HEX with two different abortions (MA and SA) and therapeutic abortions as control (see pages 31-33).
3. four mosaics from CLIC3 with the three different abortions (MA, SA and BM) and therapeutic abortions as control (see pages 34-37).

Each mosaic from SA, MA and BM contains nine pictures

- three low magnification pictures from the same patient tissue sample (gene-expression, HE and CK7/Actin)
- two other low magnification pictures with expression of the chosen gene.
- one low magnification picture with a tonsil control.
- three high magnification pictures from the marked areas (from the gene-expression, HE and CK7/Actin) (200x)

Each mosaic from the TA's contain seven pictures:

- two low magnification pictures from the same patient tissue sample (gene-expression and CK7/Actin)
- two low magnification other pictures with the gene expression.
- one low magnification picture with a tonsil control.
- two high magnification pictures from the marked areas (from the gene-expression and CK7/methyl-green) (200x)

4.3 Results

We had some technical problems with the immunostaining, mostly related to TGIF. A series of methods for antigen retrieval were tested. We also had some problems with background staining.

The marked areas on the low magnification pictures (see appendix 6.3) show tissue around the spiral arteries in the mosaics, and correspond to the high magnification pictures. The high magnification pictures were chosen as representative examples of the staining pattern we saw in the tissue micro array sections for the corresponding gene expression.

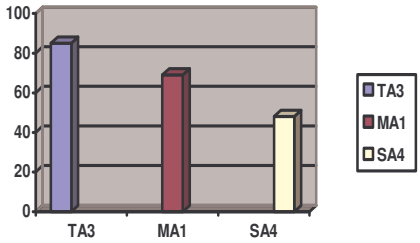
Immunohistochemistry is a qualitative method, but we chose to quantify the different gene expression pattern by counting cells. This was done by counting all stained cells on these marked areas (high magnification pictures in the mosaic). The included cells had an intact nucleus and a high degree of staining in the cytoplasm. Destroyed cells were not included (pyknotic cells and necrotic debris). The quantification was blinded without knowing what kind of tissue we were counting.

The general expression pattern of each gene is summarized in the result section below (4.3 a – 4.3c), and is based on the average staining observed in the different mosaics (se appendix).

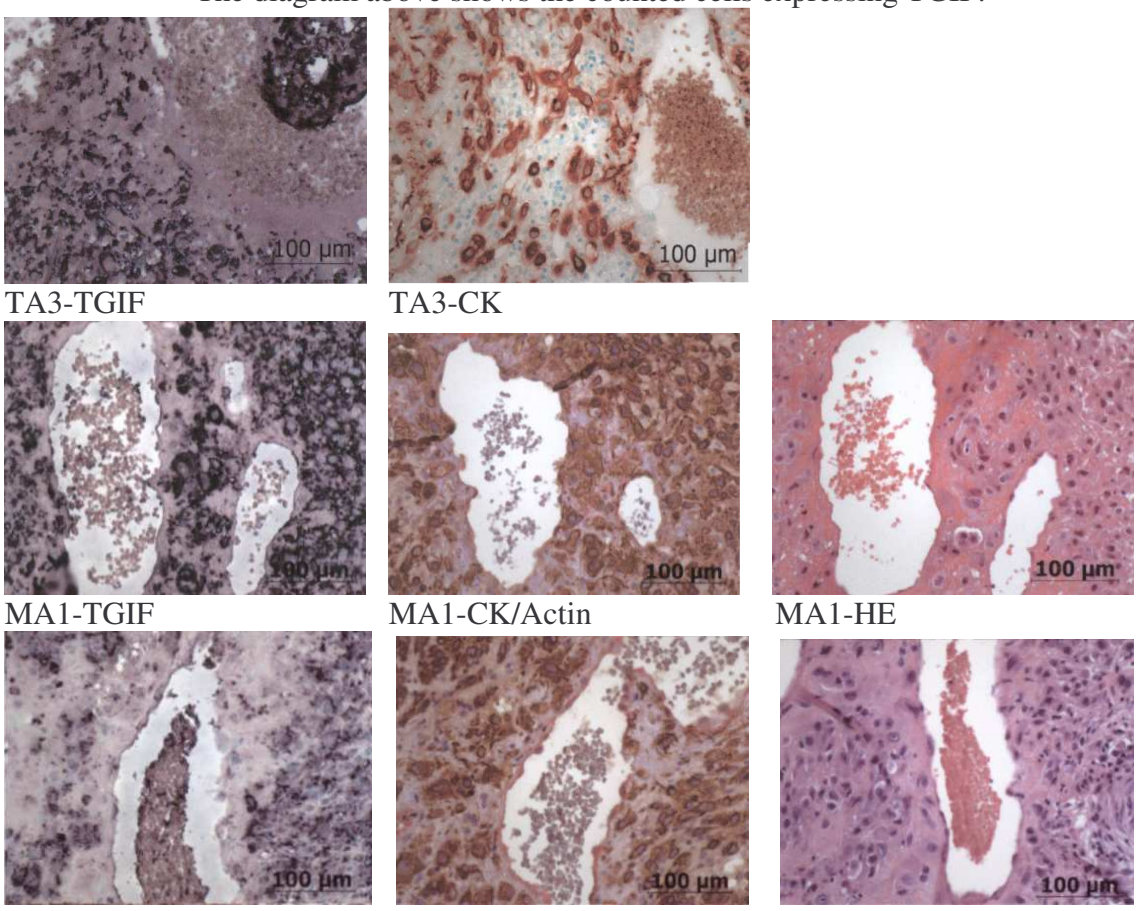
4.3 a) TGIF

Numbers of cells expressing TGIF on the representative high magnification pictures below.

TA3: 85
MA1:69
SA4: 48



The diagram above shows the counted cells expressing TGIF.



TA3-TGIF TA3-CK

MA1-TGIF MA1-CK/Actin MA1-HE

SA4 -TGIF SA4-CK/Actin SA4-HE

Fig 12. Placental bed spiral artery area and TGIF immunopositive cells from the representative marked areas in the mosaics (Magnification x100).

1) TGIF expression in 1.trimester placental bed tissue

The immunereaction pattern in the mosaics was difficult to relate completely to a specific subgroup of cells. i.e. the CK7 positive extravillous trophoblasts, see Fig 12. Some of the positive cells seemed, however, to correlate with trophoblast morphology when visually compared to the CK7 immunostaining, but in a different staining pattern.

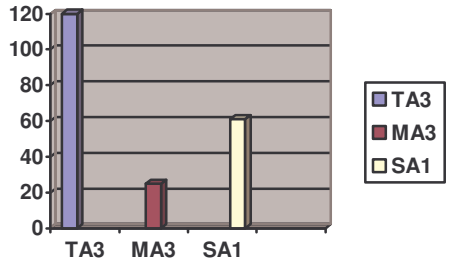
2) Compare TGIF expression pattern (qualitative) in various cells in the placental bed in abnormal (aborted) and normal first trimester pregnancies

There was no large difference in expression of TGIF between the clinical abortions (MA and SA), with the spontaneous abortions with the lowest expression level. Both clinical abortions had reduced expression compared to the control (therapeutic abortions), se columns above.

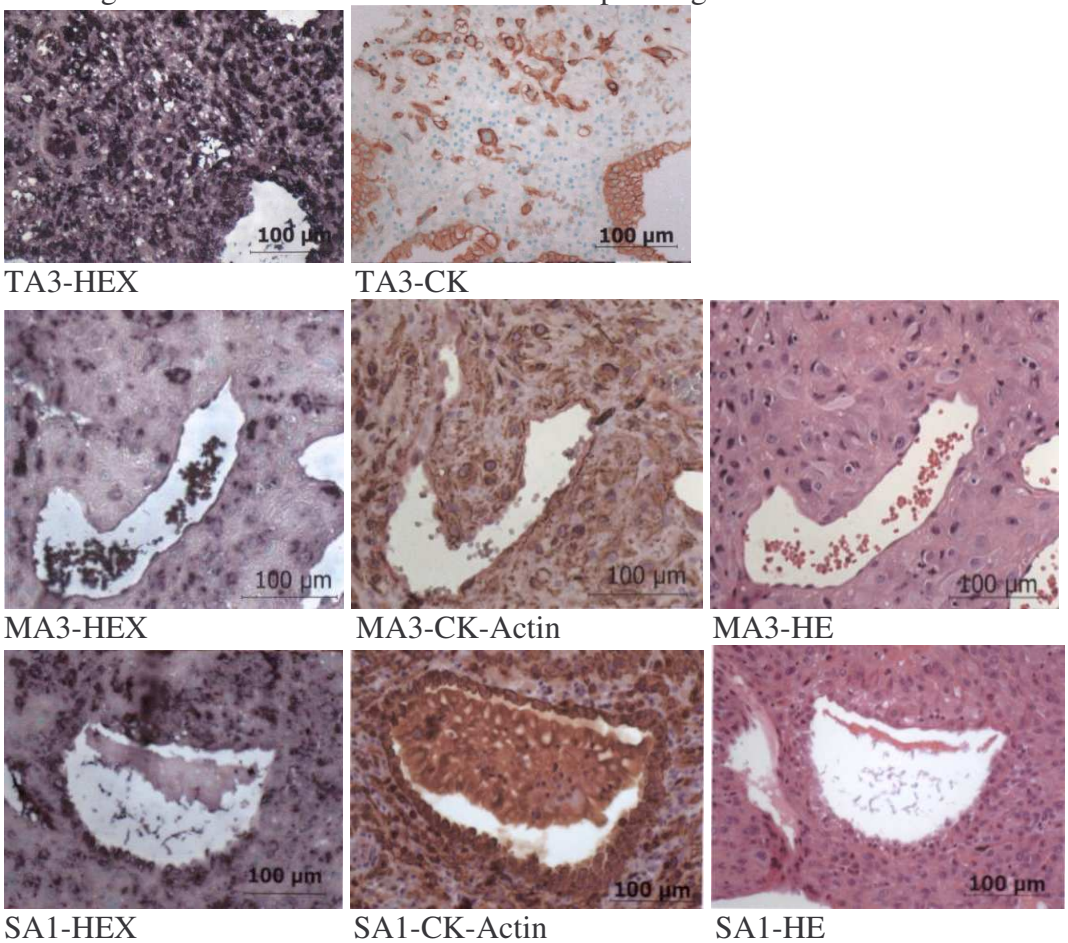
4.3 b) HEX

Numbers of cells expressing HEX on the representative high magnification pictures below.

TA3:120
MA3:25
SA1:61



The diagram above shows the counted cells expressing HEX



TA3-HEX TA3-CK

MA3-HEX MA3-CK-Actin MA3-HE

SA1-HEX SA1-CK-Actin SA1-HE

Fig 13. Placental bed spiral artery area and HEX immunopositive cells from the representative marked areas in the mosaics (Magnification x100).

1) HEX expression in 1.trimester placental bed tissue and throphoblasts

As with the TGIF, the HEX immunereaction pattern in the mosaics was difficult to relate completely to a specific subgroup of cells, see Fig 13. The localisation of some of the

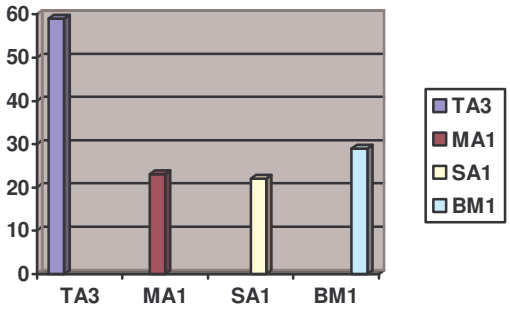
positive cells seemed, however, to correlate with trophoblast morphology when visually compared to the CK7 immunostaining, but in a different staining pattern.

2) Compare HEX expression pattern (qualitative) in various cells in the placental bed in abnormal (aborted) and normal first trimester pregnancies.
The expression of HEX was reduced in the clinical abortions (MA and SA) compared to the controls (therapeutic abortions), with the most marked reduction related to the missed abortions (MA), se columns above.

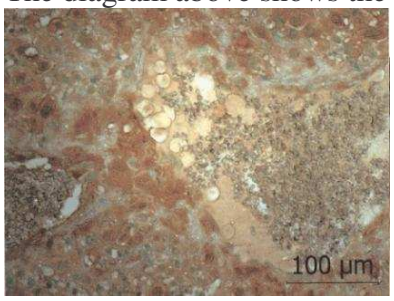
4.3 c) CLIC3

Numbers of cells expressing CLIC3 on the representative high magnification pictures below.

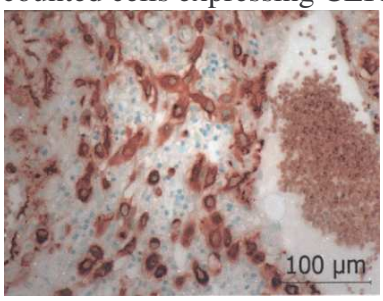
TA3:59
MAI:23
SA1:22
BM1:29



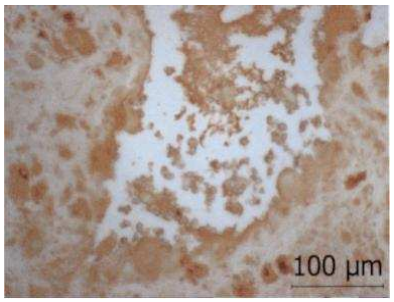
The diagram above shows the counted cells expressing CLIC3



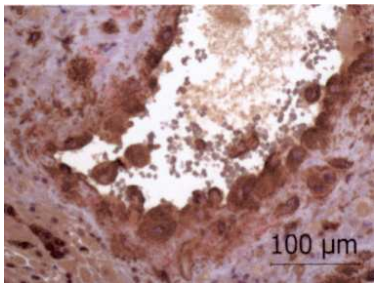
TA3-CLIC3



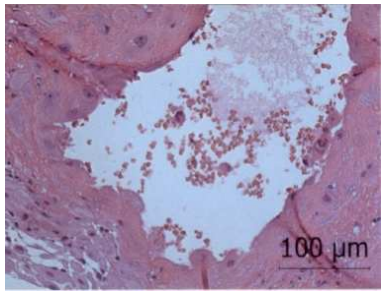
TA3-CK



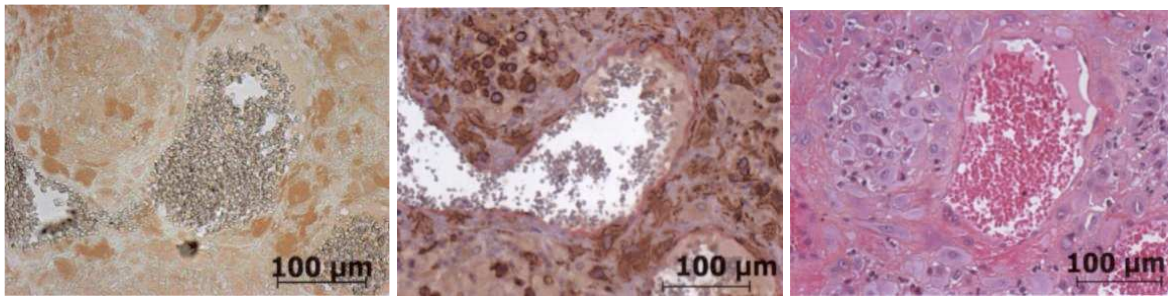
MA1-CLIC3



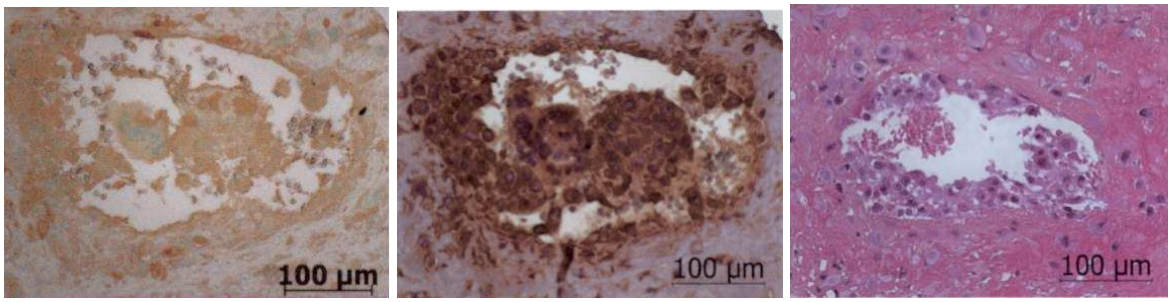
MA1-CK/Actin



MA1-HE



BM1-CLIC3 BM1-CK/Actin BM1-HE



SA1-CLIC3 SA1-CK/Actin SA1-HE

Fig 14. Placental bed spiral artery area and CLIC3 immunopositive cells from the representative marked areas in the mosaics (Magnification x100).

1) CLIC3 expression in 1.trimester placental bed tissue and throphoblasts
It was easier to identify the trophoblast morphology in the CLIC3 immunopositive cells around the arteries and in between in the fibrinoid replacement in the vessel wall. There was also some scattered staining in the placental bed stroma, more difficult to subtype on morphological ground. Some of them might be part of Trophoblasts, when visually compared with the CK7 sections.

2) Compare CLIC3 expression pattern (qualitative) in various cells in the placental bed in abnormal (aborted) and normal first trimester pregnancies.
There was clearly a reduced CLIC3-expression in the trophoblasts in the clinical abortions compared to the controls (therapeutic abortions), but no clear difference between the MA, SA and BM, se columns above.

4.4 Discussion

The expression of Homeobox genes TGIF and HEX and the ion channel protein CLIC3 was reduced in extravillous trophoblasts in clinical abortions in first trimester pregnancies compared to the normal controls (therapeutic abortions). This was shown by a reduced number of immunopositive cells in the placental bed, indicating a defect trophoblast function in the clinical abortions. By visual comparison of the immunoreactivity in the Homeobox and ion channel section with the immunreactivity pattern in cytokeratin 7 immunostained sections from the same patients a difference was seen both in relation to the number of cells but also the pattern of staining in the positive cells and the localisation in the tissue of the positive cells. The visual comparison has its definite limitations, and double immunostaining with the trofoblastmarker CK7 and the investigated gene products would have been necessary as the final proof. The visual comparison technique in serial sections is, however, relatively reliable.

The expression of CLIC3 in the controls (TA) seemed to be higher than in the pathological pregnancies. The meaning of these findings may be related to the functional role of CLIC3 in trophoblasts. Chloride channels are important in many cellular processes like electrolyte transport, pH regulation, water balance, cell potentials and apoptosis. Altered expression level of CLIC3 could imbalance these cellular processes and lead to changes in cell volume, which are known to precede apoptotic events. This could theoretically contribute to abnormal pregnancies. The placental bed tissue from the clinical abortions showed a lower expression of CLIC3, but it is not possible to tell the exact mechanisms of these findings.

The immunostainings with the homeobox genes gave us technical problems and the results were partly difficult to interpret. The expression of TGIF and HEX was partly non specific, diffuse, and we had a persistent problem with background staining. It is uncertain were the proteins of HEX, TGIF and CLIC3 are localized in the cell (nuclear or cytoplasmatic). Our antibody-staining was consistent with a more cytoplasmatic expression. The antibodies were not tested on the actual proteins before our arrival in Melbourne, and it was difficult to optimize the IHC procedures during our short stay. The tonsil control of TGIF and HEX also showed a high degree of diffuse staining. This could indicate that the genes are expressed in the tonsil, alternatively unspecific staining.

In order to reduce the HEX and TGIF background staining, tests were made with varying concentration the antibodies, varying incubation time and techniques for antigen retrieval. The gold standard for IHC studies are fresh frozen or ethanol/acetone fixed material. These procedures leave the epitopes in original state. Formalin fixed paraffin embedded sections show good morphology, but are not ideal for epitope preservation.

In parallel TMA sections we could visually compare the immunostained cells and structures. A more solid proof would have been double or triple immunostainings, identifying two or more proteins simultaneously in the sections. Fluorescence techniques make it possible to detect expression of two or more genes in the same cell simultaneously. Then it is possible to use antibodies against specific markers for trophoblast (CK7) and other genes simultaneously, hence strengthen our hypothesis that the trophoblasts express the actual gene.

There are additional, more general methodological limitations in the tissue material that we used in our study. The collected material consisted of different parts of decidua, villi, embryo, endometrium and myometrium. Remodelled arteries and the presence of extravillous trophoblast cells in the decidua proved that the material collected for the TMAs were from the placental bed. Areas with signs of inflammation or necrosis were abandoned for TMA sampling to standardize the material according to the etiology of the clinical abortion. In the fragmented curettage material, the lack of orientation of the tissue can make it difficult.
Secondly, we base our controls on the assumption that the therapeutic abortions were from completely normal pregnancies, with a normal trophoblast function. There is an obvious possiblility that some of these “normal” controls could have ended in a later clinical abortion.
There is also a variation in the gestational age of the conceptus, varying from 8-12 weeks of amenorrhoe. We put it all together in a first trimester cohort. There might, however, be differences in the gene expression according to gestational age, and, of course

interpersonal, genetic variations. These are, however, mostly general limitations that are present in most clinical studies.

Conclusion

There was a reduction in the expression of Homeobox genes TGIF and HEX and of the ion channel protein CLIC3 in the extravillous trophoblasts in the placental bed in the clinical abortions as compared to the normal controls (therapeutic abortions). The reduced expression was most markedly for HEX in missed abortions. The findings might indicate a defect
trophoblast function in clinical first trimester abortions. The staining pattern in the positive cells varied, especially in the homeobox immunostainings. Further studies are needed.

4.5 Abbreviations

BM- (Norwegian-Blæremola), Hydatiform mole
CLIC- Chloride Intracellular Channel
CK- Cytokeratin
HE- Hematoxylin-Eosin
HEX- Hematopoietically Expressed Homeobox
IHC- Immunohistochemistry
MA- Missed abortion
PRC- Pregnancy Research Center
SA- Spontaneous abortion
TA- Therapeutic abortion
TGIF- Tranforming Growth Factor-Beta-Induced Factor
TMA- Tissue Micro Array
vWF- von Willebrand factor

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http://Chemicon international: antibodies 101 – an introduction to antibodies

6 Appendix

6.1 IHC protocol paraffin sections

1. Dewax for 2x5 mins in histolene or xylene
2. Rehydrate
    i. 2x3 mins 100% Ethanol
    ii. 2x3 mins 90% Ethanol
    iii. 2x3 mins 70% Ethanol
    iv. 2x3 mins 50% Ethanol
    v. 3 mins H20
    vi. 3 min PBS.
3. Circle sections using wax pen.
4. Add 5% hydrogen peroxide in methanol (200µl pr slide) to block endogenoud peroxidases.
5. 3 x5 min washes in PBS.
6. Add blocking solution A (zymed kit) for 30 min, at room temperature.
7. 3x5 washes in PBS.
8. Add primary antibody made up in 1%BSA/PBS overnight at 4C°.
9. 3x5 min washes (solution B zymed kit).
10. Add secondary antibody for 30 min at room temperature.
11. 3x5 min washes.
12. Add HRp (solution C from zymed kit) for 30 min at room temperature.
13. 3x5 min washes.
14. Add detection reagent (either DAB made up in PBS or AEC made up in H20) for 10 min.
15. Ad counterstain (either heamatoxalin or methyl green).
16. With AEC – coverslip using 80% glycerol or with DAB rehydrate
    a. 100% EtOh, 90% EtOH, and histolene
17. corverslip using D.P.X moutant.

6.2 IHC protocol for CLIC 3

1. Dewax in histolene 5 min
2. Dewax in histolene 5 min
3. 100% ethanol 3 min
4. 100% ethanol 3 min
5. 90 % ethanol 3 min
6. 5% H2O2 in meoh 200 microl 30 % H2O2+1ml meoh
   Waxpen, damp box, R.T 30 min
7. Wash 1 x PBS 3 min
8. Block 30 min R.T. 200 microliter (10% normal sheep serum, %5 FCS, 1 x PBS
9. Dilute 1 %? Ab's in 5% FES/1xPBS
    a) normal chicken ig Y 1/500
    b)CLIC3 9/9/04 old batch 1/5000
    c)CLIC3 11/05 new batch 1/500 1 microliter-500 microliter, 1/1000 100microliter(1/500)+100microliter
10. Add 150 microliter appropriate Ab dilution to section. O/N 4 degrees celcius
11. wash 3 x 10 min PBS/0,1% tween 20, shaking

12. 2°Ab Biotin chicken IgY6/4/05 1/1000
    µl→1000µl PBS/5% FCS 200µl 1 section 60 min R.T.
13. Wash 2 x PBs/0,1%tween 20
    1 x PBS
14. Add 200 µl?section 20 min R.T
15. Wash 3 times 5 min 1x PBS
16. Make up DAB 1 x tablet DAB + 1x tablet urea in 1 ml mq
17. Add 200 µl / section- 4 min
18. Wash in tap water (running) 3 min
19. Counterstain with Methyl green (1% in PBS)
    5 min R.T 200 µl /section Tap off.
20. Mounted on coverslips in 80% glycerol

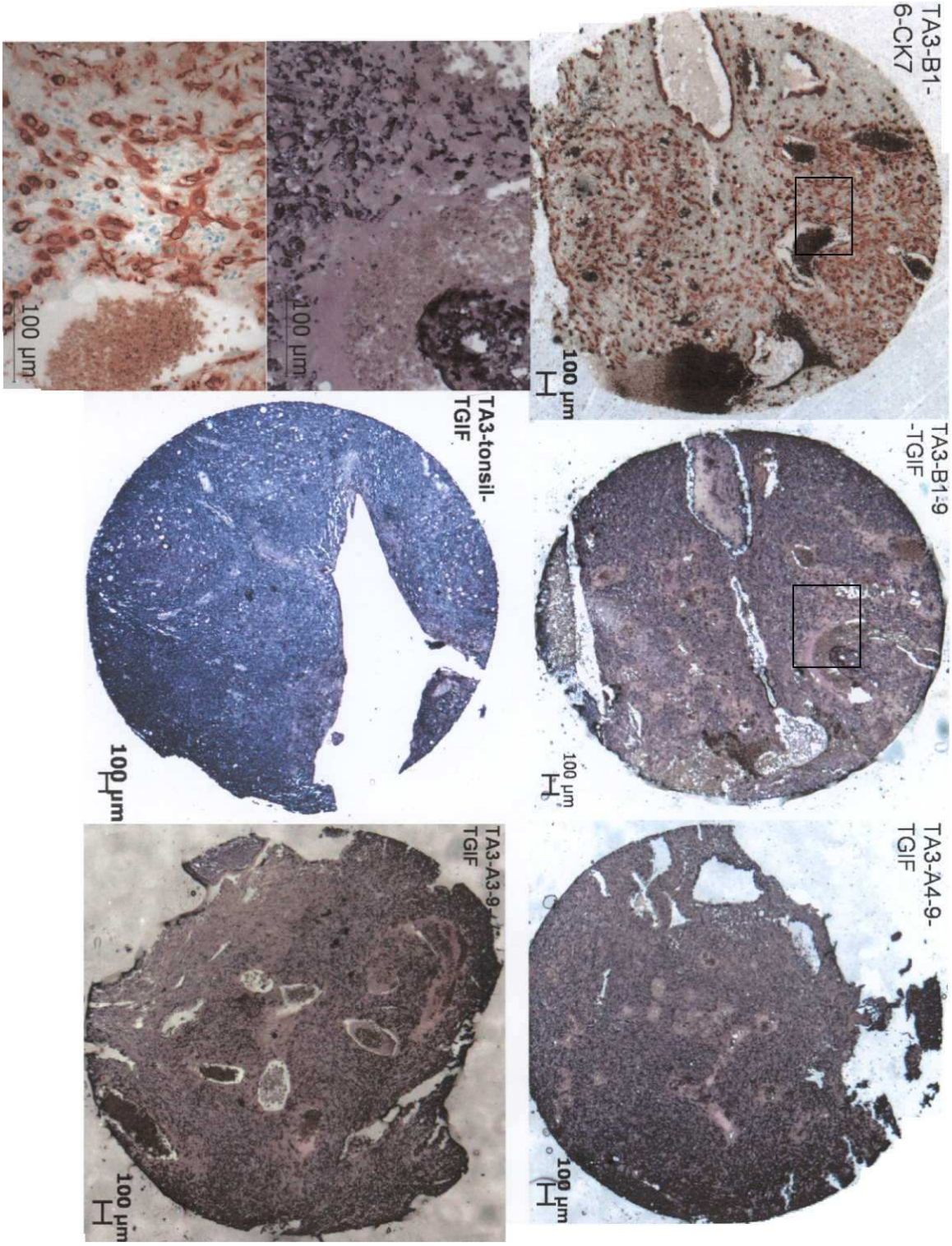
Sealed slides of coverslips with clear nailpolis\ IHC CLIC3
Tissue array trial

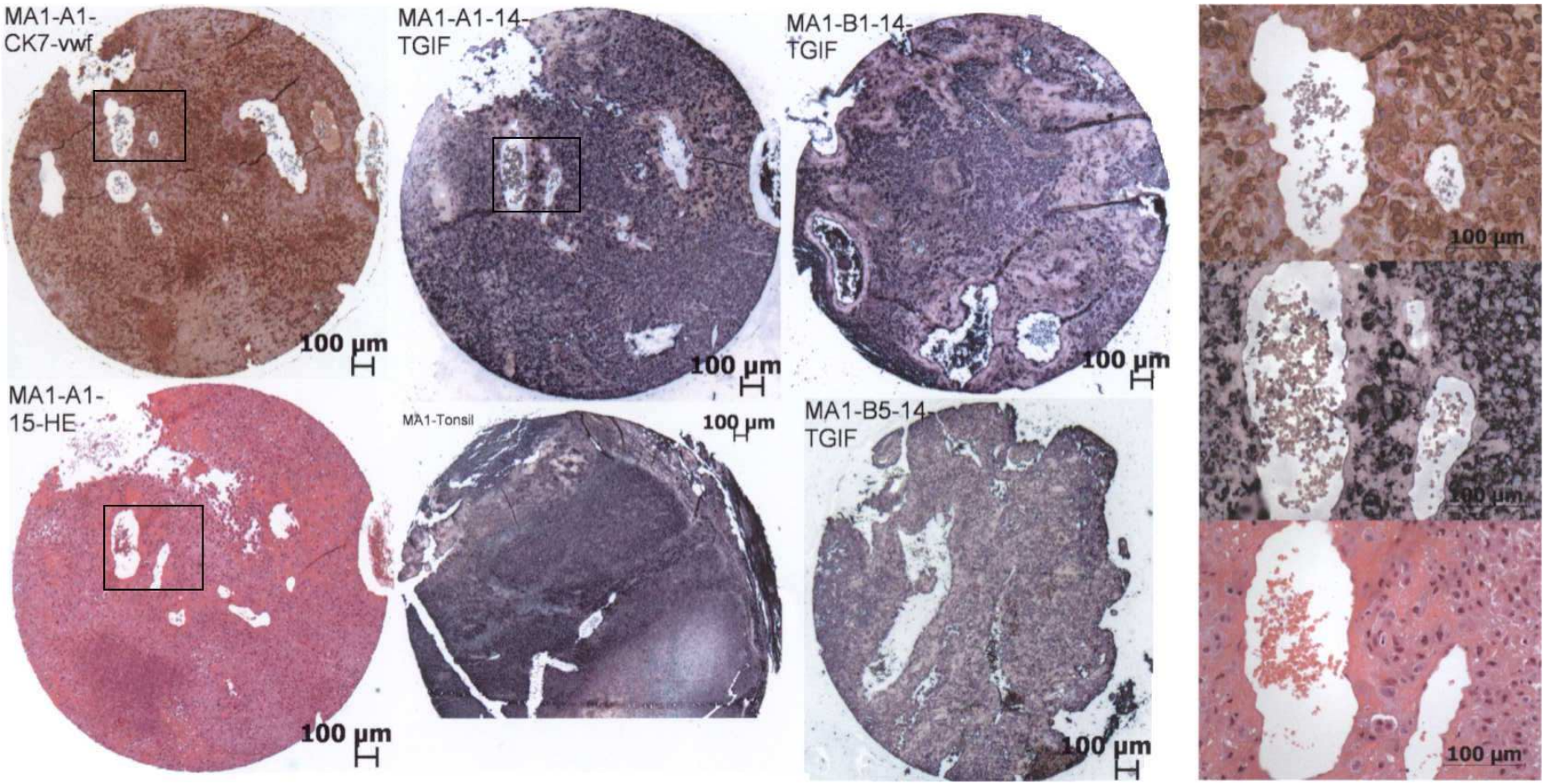
Tissue array sections:

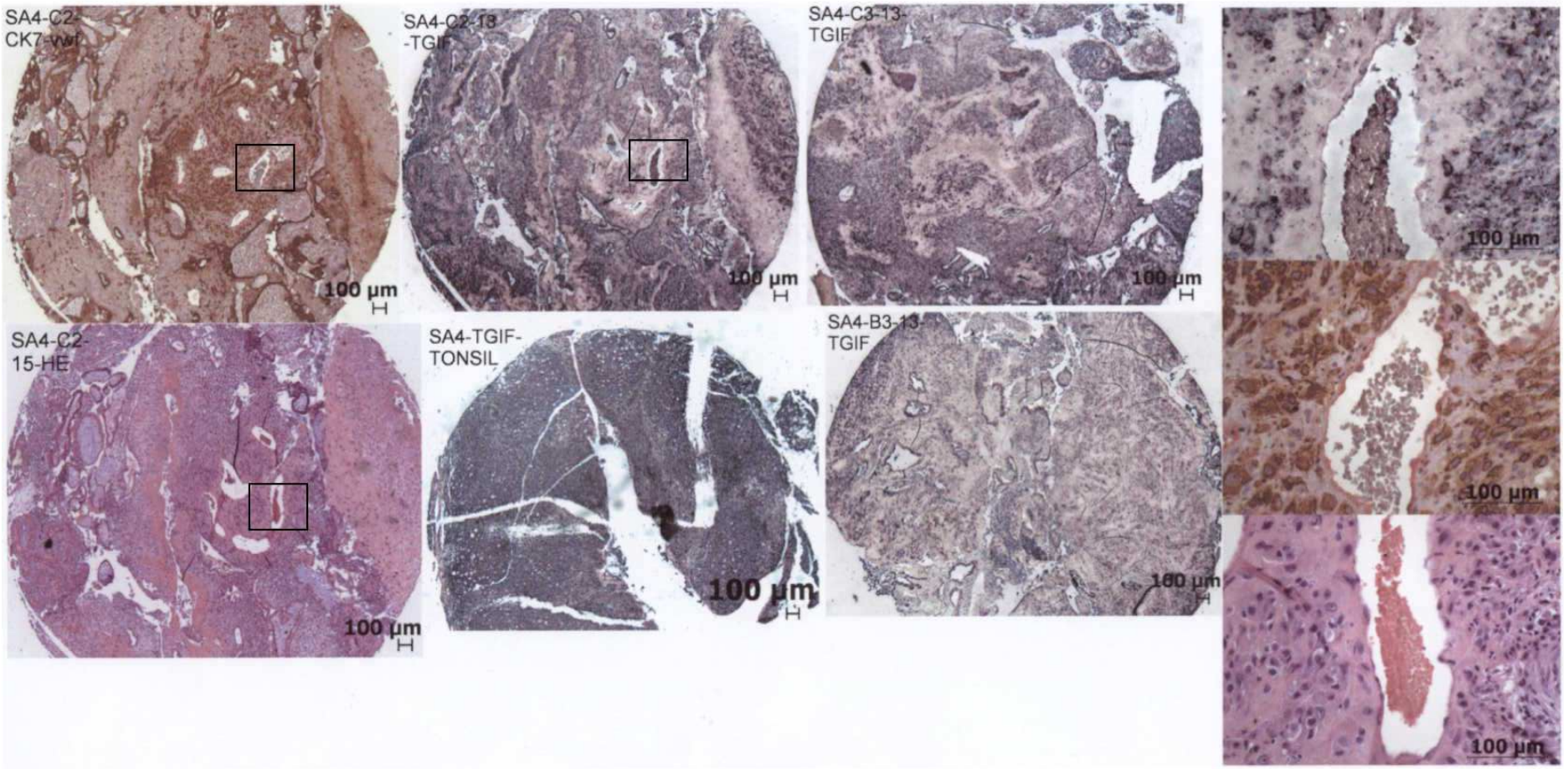
1. MA-1 Ahus 6	non immune IgY	1/4000
2. MA-1 Ahus 7	CLIC3	1/4000
3. TA-3 18	CLIC3	1/4000
4. SA-1 Ahus 6	CLIC3	1/4000
5. BM-1 Ahus 6	CLIC3	1/4000
6. P2 20/2/02 Top	non immune IgY	1/4000
bottom	CLIC3	1/4000

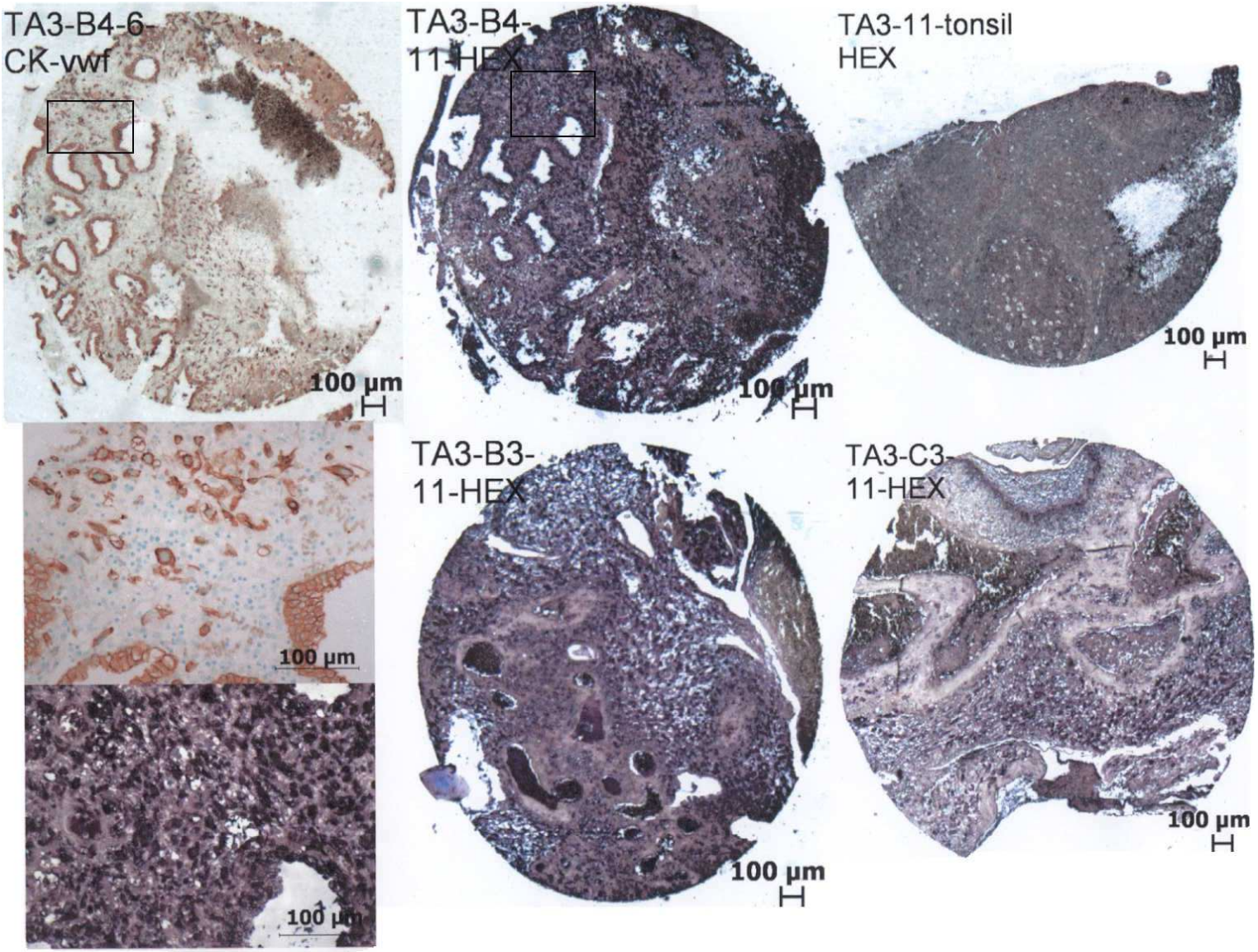
1) non immune normal chicken IgY 1/500 dilution from 14/12/05 diluted further 1/8
   50µl(1/500) + 350 µl FCS/PBS ⇒ 1/4000
2) CLIC3 old 9/6/04 1/500 dilution from 12/12/05, diluted further 1/8 150 µl(1/500)
   + 1050 µl FCS/PBS ⇒ 1/4000

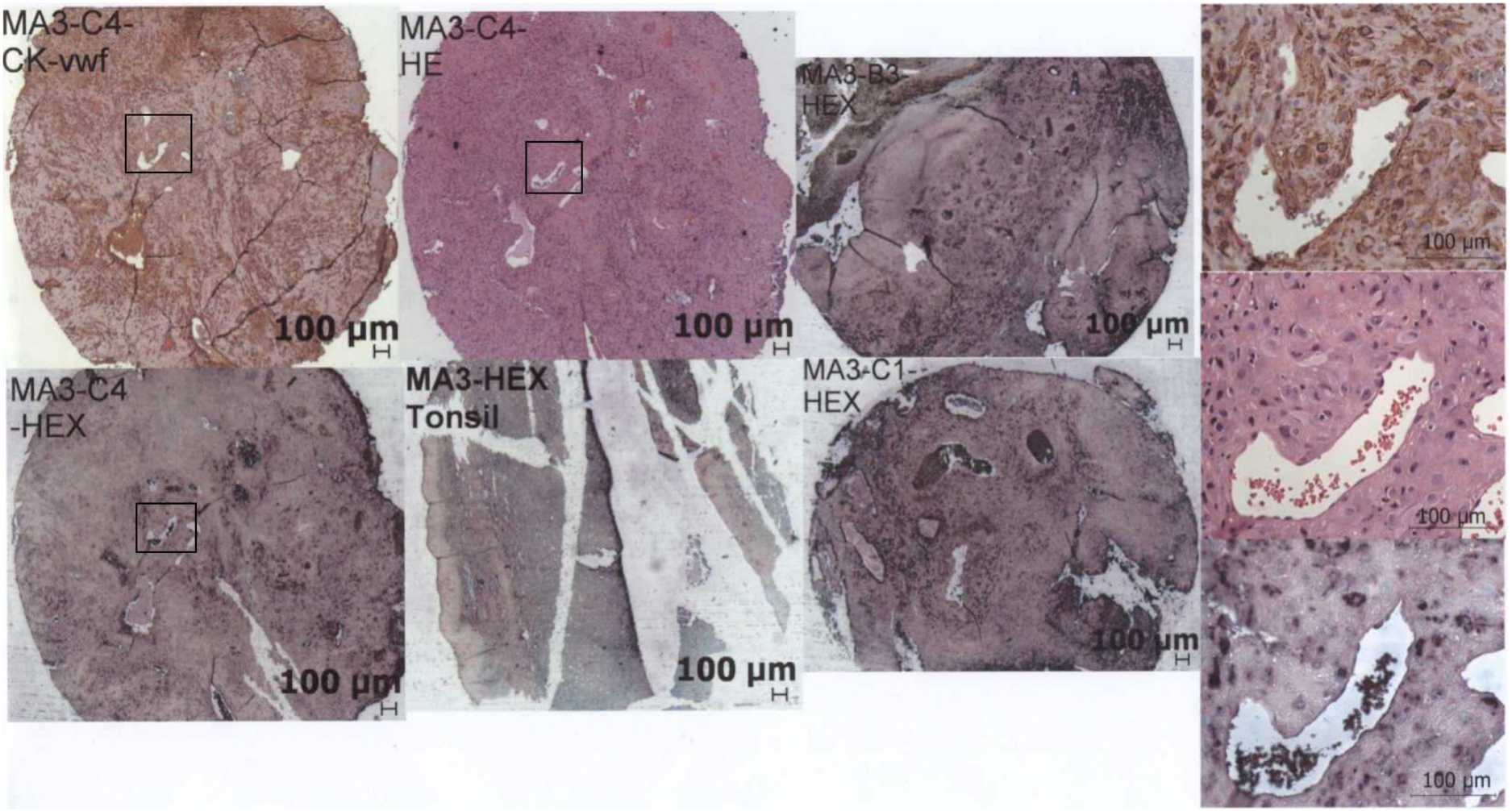
6.3 Mosaic pictures from the TMAs




MA1-A1-
CK7-vwf
100 µm
MA1-A1-14-
TGIF
100 µm
MA1-B1-14-
TGIF
100 µm
MA1-A1-
15-HE
100 µm
MA1-Tonsil
100 µm
MA1-B5-14-
TGIF
100 µm
100 µm
100 µm
100 µm


SA4-C2-
CK7-vwf
100 µm
SA4-C2-13
-TGIF
100 µm
SA4-C3-13-
TGIF
100 µm
100 µm
100 µm
SA4-C2-
15-HE
100 µm
SA4-TGIF-
TONSIL
100 µm
SA4-B3-13-
TGIF
100 µm
100 µm


TA3-B4-6-
CK-vwf
100 µm
TA3-B4-
11-HEX
100 µm
TA3-11-tonsil
HEX
100 µm
100 µm
100 µm
TA3-B3-
11-HEX
100 µm
TA3-C3-
11-HEX
100 µm


MA3-C4-
CK-vwf
100 µm
MA3-C4-
HE
100 µm
MA3-B3-
HEX
100 µm
100 µm
MA3-C4
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Tonsil
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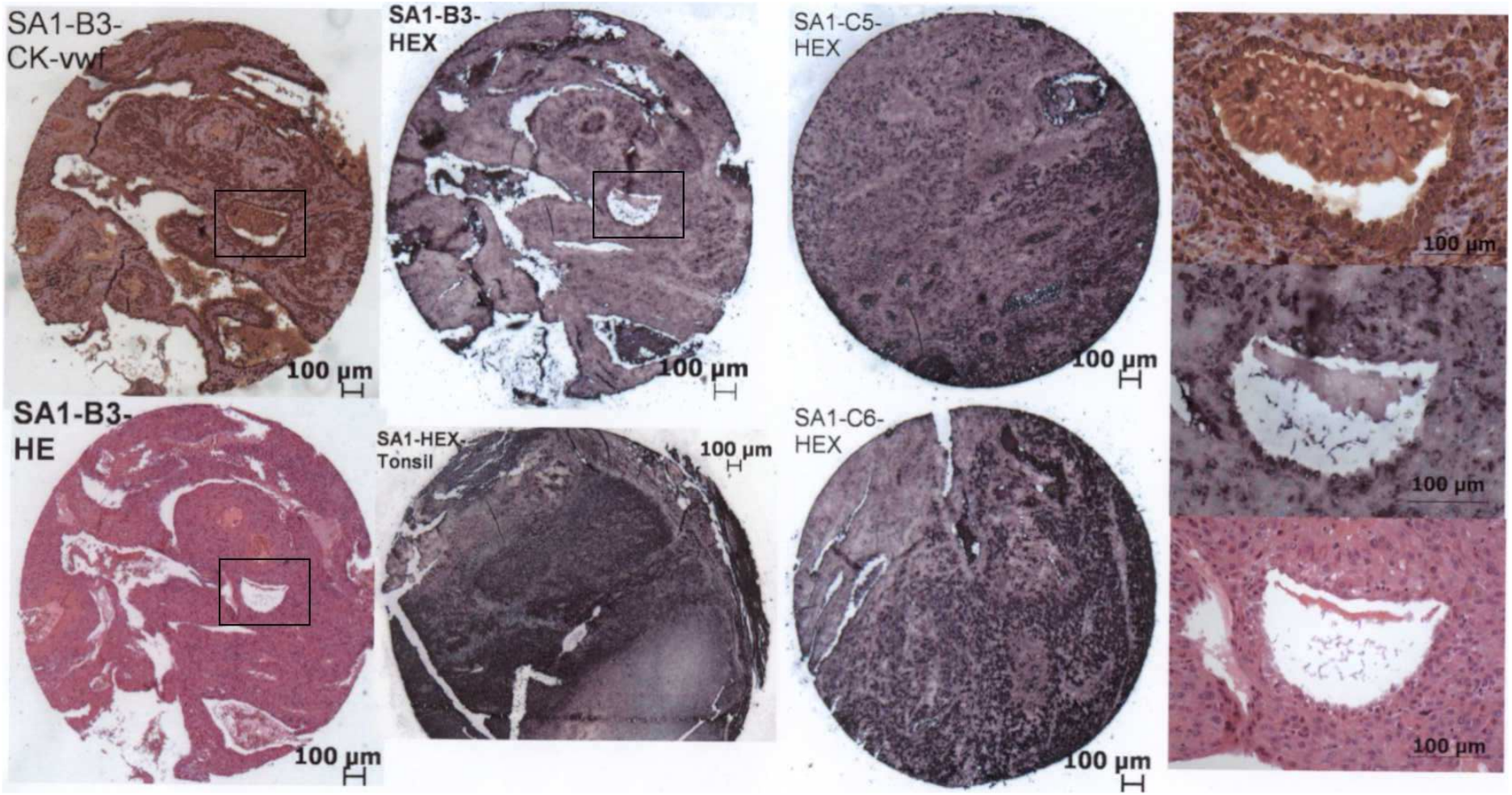
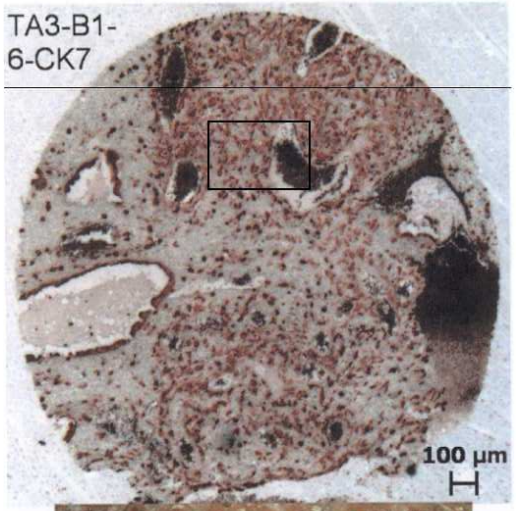
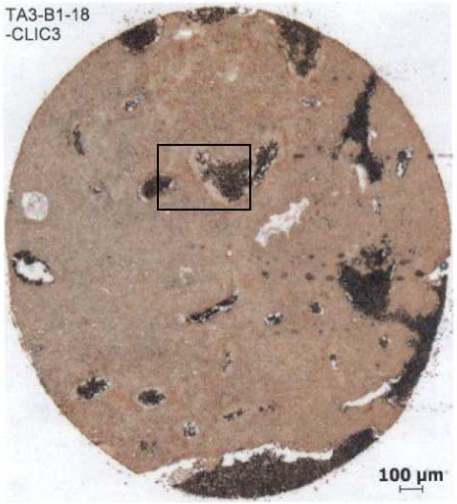
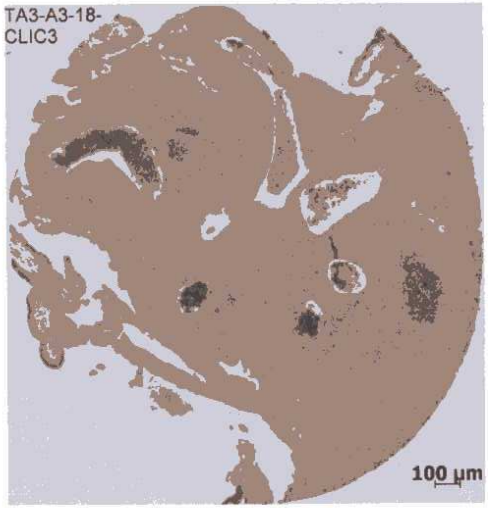
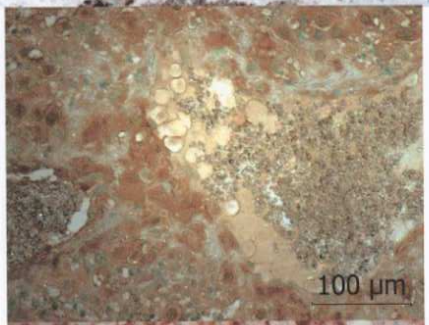
MA3-C1-
HEX
100 µm
100 µm
100 µm


SA1-B3-
CK-vwf
100 µm
SA1-B3-
HEX
100 µm
SA1-C5-
HEX
100 µm
100 µm
SA1-B3-
HE
100 µm
SA1-HEX-
Tonsil
100 µm
SA1-C6-
HEX
100 µm
100 µm
100 µm


TA3-B1-
6-CK7
100 µm


TA3-B1-18
-CLIC3
100 µm


TA3-A3-18-
CLIC3
100 µm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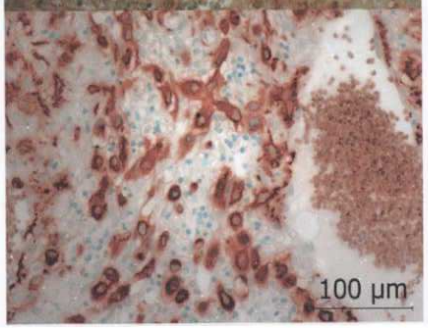
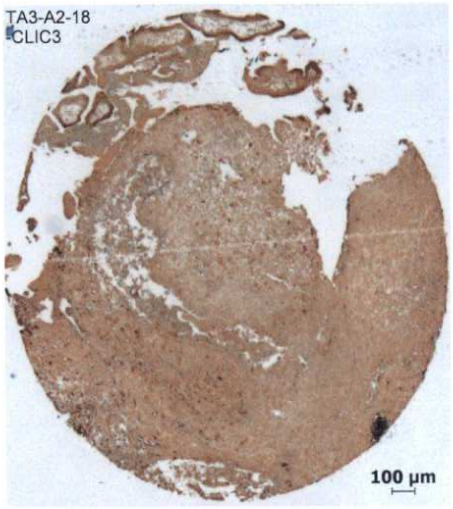
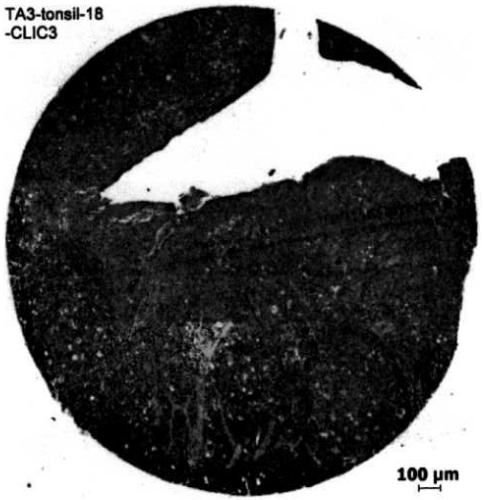
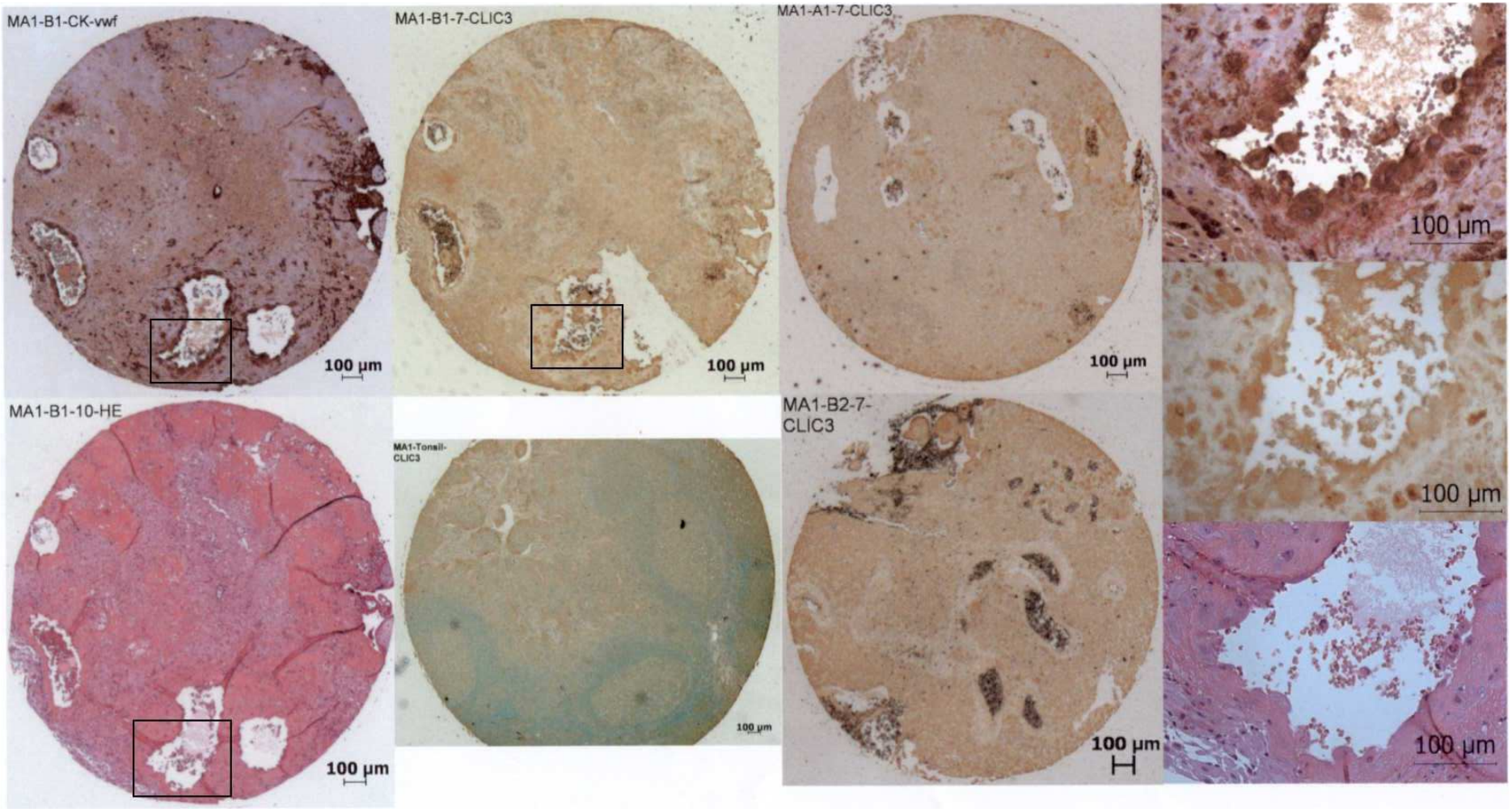
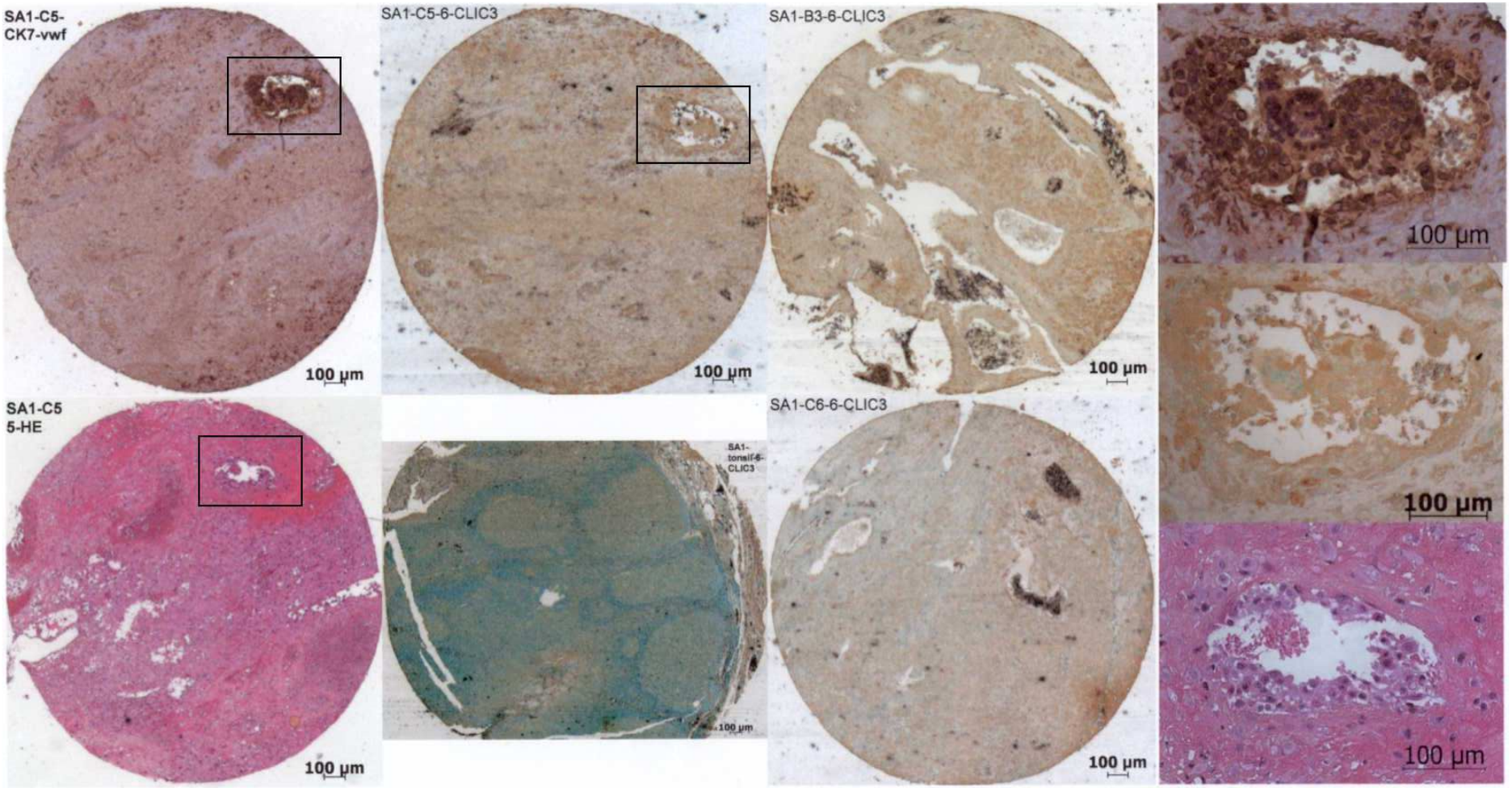
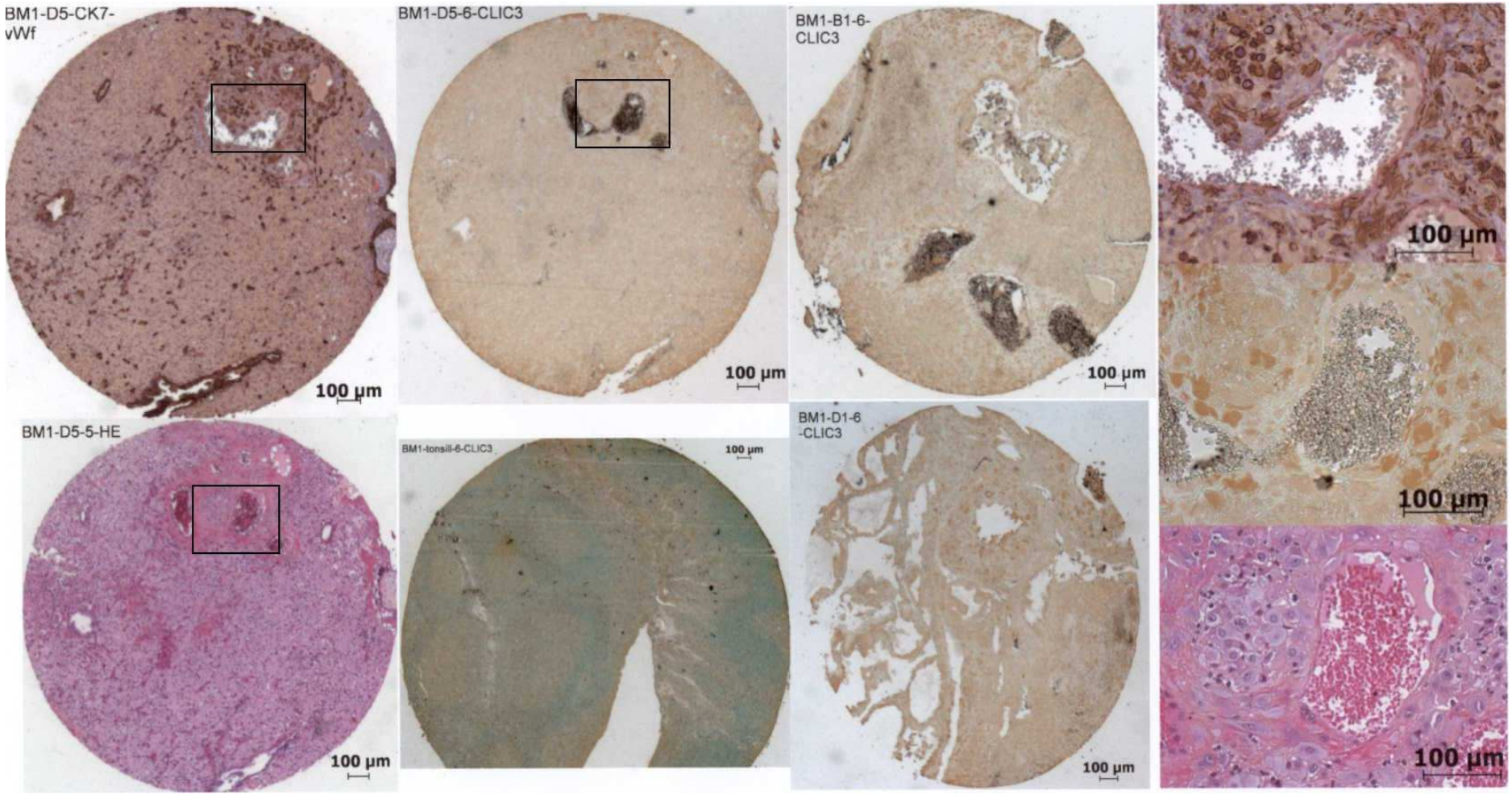

100 µm


TA3-A2-18
CLIC3
100 µm


TA3-tonsil-18
-CLIC3
100 µm


MA1-B1-CK-vwf
100 µm
MA1-B1-7-CLIC3
100 µm
MA1-A1-7-CLIC3
100 µm
100 µm
100 µm
MA1-B1-10-HE
100 µm
MA1-Tonsil-CLIC3
100 µm
MA1-B2-7-CLIC3
100 µm
100 µm


SA1-C5-
CK7-vwf
100 µm
SA1-C5-6-CLIC3
100 µm
SA1-B3-6-CLIC3
100 µm
100 µm
SA1-C5
5-HE
100 µm
SA1-
tonsil-6-
CLIC3
100 µm
SA1-C6-6-CLIC3
100 µm
100 µm
100 µm


BM1-D5-CK7-vWf
100 µm
BM1-D5-6-CLIC3
100 µm
BM1-B1-6-CLIC3
100 µm
100 µm
100 µm
100 µm
BM1-D5-5-HE
100 µm
BM1-tonsill-6-CLIC3
100 µm
BM1-D1-6-CLIC3
100 µm