

Sexual Dimorphism in Hofbauer Cells

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God helps those who help themselves.

For all my beloved ones; in the past, at this moment, and in the future

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ABSTRACT

Sexual dimorphism is defined as the existing differences between individuals of different genders in features other than gender itself. In addition to well described differences in physical traits, reproductive organs, or hormones the term also indicates the differences which are present in cognition, immunity, and the biological responses to various medical conditions.

Regarding sexual dimorphism in the placenta; although it is known that male foetuses have more severe cases of preeclampsia and cases of gestational diabetes mellitus, on the other hand, female foetuses suffer more HIV infections transmitted from the mother, however, the detailed mechanisms underlying to explain these intriguing facts are unknown.

Hofbauer cells are immune cells located in the placenta that are of fetal origin and can be identified from an early stage of pregnancy - only after 18 days of gestation. They are known to function as macrophages including phagocytosis, antigen presentation, angiogenesis and vasculogenesis, and also known to be involved in several medical conditions during pregnancy such as villitis of unknown etiology and preeclampsia. In spite of their important roles, research focused on Hofbauer cells has been limited, largely due to difficulties in their isolation.

I successfully established a new methodology to isolate Hofbauer cells from whole term placenta with high yield and purity and subjected Hofbauer cells isolated from 6 normal male placentae and 6 normal female placentae to mass spectrometry analysis. From the result of spectrometry analysis, 86 out of 5426 proteins were found to be significantly differentially expressed between male and female placentae ($p<0.05$). These 86 proteins were bioinformatically analysed utilising STRING® and found to connectively form four different pathways engaged in immunity, mitochondrial function, extracellular vesicle functions and transcriptional regulation function. Results from mass spectrometry were validated further using western blotting, which corroborated the proteomics differences.

Furthermore, I extracted RNAs from the cells that were subjected to proteomics and next-generation sequencing was performed. Of 6844 included transcripts, 26 transcripts were significantly up-regulated in male HBCs samples and 23 transcripts were significantly up-regulated in female HBCs samples (adjusted p value<0.05). To identify the signalling pathways between differentially expressed genes, results from RNA-sequencing data was applied to KEGG (Kyoto Encyclopedia of Genes and Genomics) and “cytokine-cytokine receptor interaction pathway” was most related to the functions of

Hofbauer cells as immune cells and had the smallest p value (0.001) and adjusted p value (0.073). From the results, it was clear that although there are some genes which are related to immune functions and are located on X chromosomes, such as IL2RG, are highly up-regulated in female HBCs, male HBCs manage their immunocompetence by regulating the expressions of chemokines, chemokine receptors and several cytokines, cytokine receptors including IL-11, IL23A, IL-1 (IL1A, IL1B, IL1RN and IL1F5), IL22RA1, IL34 and IL2RB, as well as members from the TNF and TGF-β family. Some of them are heavily involved in the pathogenesis of pregnancy related conditions represented by preeclampsia and GDM.

In summary, HBCs exhibit significant differences in proteomic and genetic signature in male and female placentae. It is likely these differences confer functional alterations in immune cell function which may impact a number of critical functions.

Human immune system

Summary of the human immune system

In order to protect the body from various microbes, both pathogenic and non-pathogenic, the human immune system has an extraordinarily complicated network involving the process starting from the recognition of potential antigens, commencement of correspondent immune responses to the eradication of targeted pathogens and creation of immunological memory. This entire series of reactions have to be completed without causing excessive damage to "self-tissues" including favourable commensal microbes. This sophisticated network is composed of two main branches – innate and adaptive immunity. The reactions related to innate immunity occur immediately or within hours after an antigen enters the host. These reactions work as the first line of host defence and the defence mechanisms are encoded in the germline, which can be tracked back as far as 700 million years ago in our ancient ancestors, such as the sponges[1)]. The innate immune responses are not totally nonspecific as they are generally defined to be, in fact, they have to efficiently discriminate between self and non-self antigens and furthermore, they have had to refine their mechanisms of antigen recognition and attack pathways during evolution to acquire different defence systems in maintaining their broad range for targeting and versatile ways of attacking. Our continued survival has proved that they have withstood the test of time.

However, the innate immune system does have its limitations. It lacks immunological memory, meaning even if it encounters the same pathogen which has entered into the host before, it will still arrange a response from scratch, needing time for gradual activation. Also, though it can identify self antigens from harmful non-self antigens by systems which have been acquired through the tides of evolution, it does not have an efficient system for telling beneficial non-self antigens from those dangerous non-self antigens, causing attacks for both allies and enemies, which end in unnecessarily wide engagement into the surrounding tissues and organs, as one can see in the severe inflammations caused by neutrophils after severe injuries and traumas[2)].Thus, we need a more precise and refined defence system – this is adaptive immunity, which our ancestors started acquiring about 450 million years ago[1)].

Adaptive immunity is characterised by its antigen-specific receptors represented on lymphocytes, especially on the so-called T lymphocytes and B lymphocytes, and by antigen-specific antibody production by B lymphocytes. These traits have enabled the host to hold a more specific attack for a particular antigen, and to form the long-term immunological memories resulting in an intensified reaction to the following entry by the same antigen. Unlike the germline-coded recognition

mechanisms in the innate immunity, the information related to forming numerous antigen-specific T cell receptors (TCR) and B cell antigen receptors (immunoglobulin) are encoded by genes which are the outcomes by rearranging different germ-line gene components[3]. This random and imprecise manner of recombination gives both TCRs and immunoglobulins enormous diversity for the antigen repertoire, ensuring they can not only endure a more effective battle but also maintain an extensive range for locking-on to targets.

Details for the human immune system

① Recognition of potential antigens (Innate immunity)

One of the distinctive mechanisms for antigen recognition equipped in the innate immunity is via pattern recognition receptors (PRRs). They include membrane-bound PRRs (Toll-like receptors (TLRs) and C-type lectin receptors (CLRs)) and cytoplasmic PRRs (NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs)). PRRs recognise two types of molecules – pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Of all the PRRs, TLRs play an essential part in initiating immune responses and triggering subsequent downstream signalling, leading to the productions of inflammatory cytokines and activation of related genes.

TLRs were firstly discovered in *Drosophila melanogaster* in 1985. The first sequencing of TLRs in human was published in 1994[4]. It is a family of single membrane spanning proteins expressed not only on the first-line immune cells, such as macrophages, dendritic cells, and neutrophils, but also on the epithelial cells on the boundaries between the environment and the host[5]. So far, 11 different subtypes (TLR1 - TLR11) known as TLR families have been discovered in mammals, with 10 subtypes (TLR1-TLR10) found in humans. TLR1, 2,4,5,6 are mainly responsible for recognising bacteria, while TLR3,7,8 generally identify viruses. TLR9 has ligands for both bacteria and viruses. No ligands have been detected yet in TLR10.

More specifically, TLR2 distinguishes the largest amount of ligands, especially for gram-positive bacteria and yeasts. TLR1 and TLR6 collaborate with TLR2 by forming as a heterodimer in recognition of lipopeptides and peptidoglycans. TLR4 plays an essential role in detecting gram-negative bacteria by recognising lipopolysaccharides (LPS). TLR5 has a sensor for flagellin, an important organ for organisms' mobility, meaning it can detect both gram positive and negative bacteria. TLR3, 7 and 8 function as key players in the first defence line against viruses, identifying double-stranded RNA, single-stranded RNA and GU-rich

single-stranded RNA respectively. TLR9 mediates immune responses against both bacteria and viruses by interacting with DNA in the pathogens[6].

Signalling raised by all TLRs leads to the activation of NFκB (Nuclear Factor kappa-light-chain-enhancer of activated B cells), a pivotal transcription factor whose activation results in the production of pro-inflammatory cytokines such as IL-1,2,6,8,12,TNF-α and also chemokines such as MCP-1, IL-18, RANTES, MIP-2, CXCL-1, CXCL-10. Those pro-inflammatory cytokines will promote the maturation of dendritic cells as well as the activation and differentiation of macrophages, with secreted chemokines recruiting neutrophils to the site of inflammation. Matured dendritic cells obtain the ability of antigen presentation, presenting degraded fragments of proteins from the pathogen to stimulate T cells, orchestrating subsequent immune responses. Activated macrophages also help T cells in activation and differentiation, which in turn get further energised owing to the production of IFN-γ, a most powerful activator for macrophages, by activated T cells[7].

② Various mechanisms for attacking (Innate Immunity)

Recognition of PAMPs and DAMPs using PRRs causes inflammation, a main biological defence response handled by innate immunity, suggesting the commencement of battle against a potential peril to the host. Acute inflammation is defined as having clinical symptoms including pain, redness of affected tissue, immobility of affected tissue, swelling and heat[8]. These symptoms are the result of inflammatory cytokines and chemokines described above and the activation of first-line immune cells, mainly macrophages, dendritic cells and mast cells. This reaction is also mounted by particular endogenous chemical factors - histamine and serotonin, bradykinin, thromboxanes, leukotrienes and prostaglandins - released by mast cells and basophils[9]. Progressed immune responses orchestrated by innate immunity will prompt the activation of adaptive immunity as a result, which will be described later. Here, several different mechanisms endowed in innate immunity to control the invasion of pathogens will be outlined.

1 Phagocytosis

Élie Metchnikoff discovered a phenomenon, which he later termed as `phagocytosis' and the cells involved in the process as `phagocytes' in 1882 using starfish larvae as a model. He placed small thorns from a tangerine tree into the starfish's larvae and observed some unexpected cells start gathering around the thorns. This finding led him to establish a hypothesis that the white blood cells in the circulation take up the bacteria and kill them, aiding in eliminating invading pathogens. This preposition was later further proved and accepted, winning him the Nobel Prize for Physiology or Medicine in 1908 together with Paul Ehrlich, who

concluded the importance of antibodies in the acquirement of immunity and discovered the first effective medication (Arsphenamine) for syphilis.

Phagocytosis is performed by specialised cells as well as by cells who are not specialised for phagocytosis. The former group of cells are generally called professional phagocytes; and comprise neutrophils, monocytes and macrophages. In contrast, the latter group of cells are called non-professional phagocytes, indicating all the other kinds of cells which can perform phagocytosis. Most of them are of epithelial origin, such as epithelial cells, fibroblasts, endothelial cells and mesenchymal cells. They remove the dying cells and certain substances, contributing to the tissue remodelling and the maintenance of homeostasis[10]. Dendritic cells are in a controversial position referring to being called either professional or non-professional phagocytes because their aim for phagocytosis is not to completely destroy and clear the pathogen, but more emphasised in picking up useful information from the consumed organism and carrying out efficient antigen presentation via major histocompatibility complex (MHC) class I and II molecules. This trait can be seen their lessened degradation of a pathogen compared to neutrophils and macrophages[11].

Phagocytosis starts from the migration of primed phagocytes to the site of inflammation and is followed by the adherence to the targeted particles via the interaction between the ligands on the targets and the receptors on the phagocytes. This will induce the ingestion of the target by phagocytes and will develop into forming an endocytic vesicle called a phagosome. A phagosome will be combined with a lysosome of fully contained digestive enzymes to form a phagolysosome so the degradation of the pathogen by enzymes can start. Once the digestion is finished, indigestible materials will be packed into another kind of an endocytic vesicle called a residual body and secreted from the phagocyte (by exocytosis)[12].

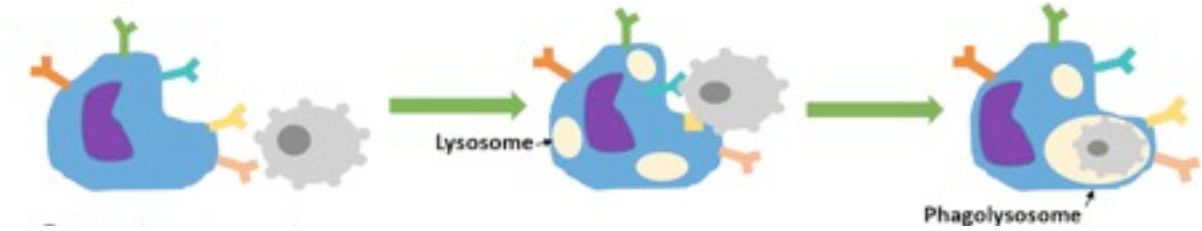


Figure1: The process of phagocytosis - targeted particles will be absorbed into the inside of the macrophages and will be degraded by lysosome inside the phagolysosome.

2 Complement system

The complement system can be activated by three different biochemical pathways - the classical, alternative and the lectin pathway.

The classical pathway begins from the binding of immune complexes (IgM-antigen/ IgG-antigen) to C1q, the first molecule in the cascade, to form an assembly called C1 complex. C1 complex turns on the activation of two serine proteases - C1r and C1s consecutively, causing C1s to split C4 and C2 respectively to form the C4b2b, a C3 convertase. This C3 convertase cleaves C3 into C3a and C3b. The union of C3b with C4b2b converts into a C5 convertase, resulting in C5 divided into C5a and C5b. C5b subsequently combines with C6, C7, C8 and C9 to function as a MAC (Membrane Attach Complex), inducing pore formations into the cell membranes of a bacteria so lysis can occur[13].

In the alternative pathway, C3 activates itself by a process described as 'tickover'. `Tickover' happens in ~1% of total C3 per hour, causing the structure change in C3 so it can bind to Factor B. This combination further allows Factor D to generate Factor Ba and Bb from Factor B. Factor Bb binds to the autoactivated C3 ($C3(H_2O)$) working as a C3 convertase, cleaving C3 into C3a and C3b. C3b forms a complex with Factor Bb and properdin(P), a complement stabiliser protein. As the C3 convertase created in the alternative pathway can cleave several C3 into C3a and C3b, the complex of C3bBbP binds to another C3b, forming the C5 convertase in the alternative pathway. Once the C5 convertase is shaped, the following activation of complements and the constitution of the MAC is the same as occurs in the classical pathway[14].

In the lectin pathway, mannose-binding lectin (MBL), ficolins (Ficolin-1, Ficolin-2, and Ficolin-3), and collectin 11 (CL-K1) bind to certain sugars, such as oligosaccharides which contains terminal mannose, fucose or N-acetyglucosamine, as well as acetylated residues, from bacteria and viruses.

These bindings enable MBL, ficolins and collectin 11 to construct a complex structure with MBL-associated serine protease 1 and 2 (MASP-1 and MASP-2), functioning as a C3 convertase by cleaving C4 and C2 respectively and forming C4b2a. Once the C3 convertase is formed, the following activation of complements and the constitution of the MAC is the same as they occur in the classical pathway[15].

The complement system can be activated in various ways other than those three established pathways and traditional triggers stated above. C-reactive protein is a well-known acute phase protein, which increases in response to inflammation. It binds to the component of bacteria and apoptotic cells, dying cells to activate the classical pathway. Lung macrophages with the use of cell bound serine proteases can create C5a from C5 without relying on any pathways established[13].

Regulating the complement system is also crucial in a way it will not cause excessive inflammatory responses against the host. This includes the involvement of C1-inhibitor, complement decay-accelerating factor (DAF), Factor H and Factor I[16].

3 NK cells

The final prominent defence system present in the innate immune system is a unique surveillance arrangement operated continuously by Natural killer cells (NK cells). Though NK cells share the same lymphoid progenitor with T cells and B cells, they do not have antigen-specific receptors and they express the cell markers of CD16 and CD56, different from those on T cells and B cells[17]. The activation of NK cells is an outcome of the power balance between the activating receptors and inhibitory receptors. The signals from a cell suffering DNA damage, from a tumorigenic environment of uncontrolled cell proliferations, from the discharge of viruses and bacteria, all of these will be recognised by the activating receptors such as NKG2D, boosting NK cells into attack mode[18][19]. On the other hand, NK cells convey MHC class I to distinguish self-cells from non-self and improper killing or attacks against self-cells are prevented by inhibitory receptors, such as killer-cell immunoglobulin-like receptors (KIRs) and CD94/NKG2. These inhibitory receptors prevent NK cells from killing by recognising the MHC class I expressed on the self-cells. Once the MHC class I on the surface of self-cells is absent or the level of expression is decreased, NK cells detect the change via the inhibitory receptors and start killing. The procedures of killing by NK cells are systematic. It starts from the reconstitution of its cytoskeleton so it can form a new immunological synapse to facilitate its docking with the targeted cell. Secretory lysosomes and granzymes (perforin and proteases) are transported to the site of immunological synapse through microtubule organising centre (MTOC) and will be positioned in a tight order. After the connection via the immunological synapse is made even closer by the movement of its plasma membrane, the NK cell releases perforin to form pores into the cell membrane of the targeted cell and also secretory lysosomes and cytotoxic granules into the target cell[20] to perform the killing (either apoptosis or cell lysis). It is well known that cells infected by viruses and bacteria and cells undergoing tumorigenesis will have lessened or lost level of their expression of MHC class I[18], inducing the killing by NK cells.

The range of defence by NK cells is not limited in killing only the self-missing cells. FcγRIII (CD16) receptors expressed on NK cells enable them to bind with antibody bound infected cells. The binding will activate NK cells and cause them to perform the killing by releasing cytotoxic granules. This feature is specifically termed as antibody-dependent cellular cytotoxicity. Medications derived from monoclonal antibodies such as rituximab and abciximab are based on this mechanism, aiming

to bind to a specific surface membrane marker or receptor to trigger the killing of the cell.

The surveillance and regulation carried out by NK cells span to immune cells as well. NK cells are activated by cytokines (Type I IFN, IL-15, IL-12 and IL-18) produced by activated macrophages and dendritic cells. NK cells produce IFN-γ and TNF to promote the maturation of macrophages and dendritic cells, while producing IFN-γ to activate T cells. However, if the dendritic cells fail in maturation or, if the macrophages and T cells become hyperactivated, NK cells will detect these signals via the recognition of MHC class I by inhibitory receptors and start killing as they do with infected cells and cancerous cells[18)].

③ Antigen presentation and activation of T cells (Adaptive Immunity)

Antigen presentations can be performed by professional antigen-presenting cells represented by macrophages, dendritic cells and B cells, as well as by non-professional antigen-presenting cells, which are all the other nucleated cells.

Professional antigen-presenting cells express MHC class II and present both extracellular antigens and internally integrated antigens to naive CD4 T cells[21)]. When the CD4 T cells sense the antigen presented via MHC class II, the T cell receptor (TCR) and its co-receptor (CD3) will bind strongly with the presented antigen. This leads to the activation of LCK (lymphocyte-specific protein tyrosine kinase), resulting in phosphorylation of CD3 and ζ-chains by LCK, particularly their intracellular domains constituting the ITAMs (immunoreceptor tyrosine-based activation motifs). The phosphorylation of ITAMs enables another cytoplasmic tyrosine kinase - ZAP-70, to dock with them. The activation of ZAP-70 induces the stimulation of downstream adapter proteins, such as LAT (Linker for activation of T cells) and SLP-76. Subsequent signalling pathways bring about rearrangement of the actin cytoskeleton, calcium mobilisation and activation of Ras GTPase; all of these contribute to the activation of transcription factors represented by MAPK (Mitogen-activated protein kinase), NF-κB and NFAT (Nuclear factor of activated T-cells). These transcription factors will promote the production of IL2 and the expression of IL-2 receptors[22)].

IL-2, also known as a T-cell growth factor, plays a crucial role in both the activation and differentiation of T cells. IL-2 dominates T cell function by modulating the function of cytosolic tyrosine kinases, mainly via the JAK (Janus family kinase) members. IL-2 receptor complex is composed of three components: IL-2 receptor α (IL-2Rα or CD25), IL-2 receptor β (IL-2Rβ or CD122), and the common γ (γ_c) chain. Activation of the IL-2 receptor complex causes the activation of JAK1 and JAK3, which are located on IL-2Rβ and γ_c respectively. Following the activation of JAK1/3 which phosphorylates the IL-2Rβ domain as a result, downstream signalling

cascade results in the activation of three signalling pathways: JAK/STAT pathway, PI3K (phosphoinositide 3–kinase) AKT pathway, and SHC-RAS-MAPK pathway. The products from the target genes of these three pathways play essential roles in IL-2-dependent activities. Subsequently, CD4 helper T cells increase the expression of IL-12Rβ2, IL-2Rα, IL-2Rβ, and IL-4Rα following the IL-2 signalling. IL-2 and IL-12 signalling promote the expression of T-bet and Blimp-1, forming Th1 cells. IL-2 and IL-4 signalling upregulate the expression of Gata-3, generating Th2 cells. IL-2 and TGF-β signalling induce the expression of Foxp3, aiding the growth of T_{reg} (T regulatory) cells. The development of Th 17 cells is known to be prevented by IL-2 signalling via IL-6Rα and gp130[23)24)].

Following the mechanisms stated above, in response to the antigen presented by professional antigen presenting cells, naive CD4 T cells will go through maturation and become activated either as Th1 or Th2 cells, producing either IFN-γ, or IL4 and IL-5 respectively. Th1 cells orchestrate cell-mediated immunity, utilising IFN-γ for activating macrophages as well as cytotoxic T cells (CD8 T cells), responsible for the defence against intracellular pathogens and the pathogenesis of autoimmune diseases, such as Type 1 diabetes. Th2 cells are in charge of humoral immunity, especially the aspect of using antibodies produced by B cells as their arms for the defence against extracellular pathogens, which is also accountable for the underlying pathologic process of allergy related diseases.

④ B cells and the formation of immune memory (Adaptive Immunity)

Naive B cells are activated via two routes - either with the help from CD4 T cells or without help from T cells but receiving stimulation directly from pathogen elements, both routes occur alongside the recognition of antigen via the B cell receptor (BCR), also known as immunoglobulin (Ig). The interactions between B cells and T cells, specifically follicular T helper (T_{FH}) cells, occur in secondary lymphoid tissues - the lymph nodes and the spleen[25)]. T cells bind with the antigen processed in B cells and presented via MHC class II on the cell surface of B cells. Following this, T cells express CD154 to involve CD40, an essential co-stimulatory molecule on B cells. The engagement between CD154 and CD40 plays a fundamental part in B cells proliferation and differentiation, including the key steps B cells undergo to achieve successful immune responses - isotype switching and Ab (antibody) secretion[25)26)]. Activated B cells become short-lived plasmablasts firstly, secreting IgM antibodies predominantly, which present restricted affinity for the antigen although still remain the main source of protective antibodies produced at an early stage. Subsequently, some of the activated B cells rejoin the B cell follicle, undertaking energetic clonal expansion to form the germinal centre (GC), where the B cells experience further differentiation. In the GC, B cells gain the ability to produce multifunctional and

high-affinity antibodies through the changes on their BCRs - class switch recombination (CSR) and/or somatic hypermutation (SHM)[27)28)].

CSR brings changes in the constant regions of the heavy chain in Ig, allowing B cells to shift their generation of antibodies from IgM to IgG, IgA and IgE class. The antibodies will remain specific to the antigen as CSR is limited to the constant region, which means while the affinity for the antigen will not change, class-switched antibodies will diversify their roles in multidisciplinary ways engaged in immune responses including opsonisation, activation of complement system, antibody-dependent cellular cytotoxicity, and degranulation. On the other hand, SHM creates alterations on the variable regions in Ig, granting B cells the ability to produce antibodies with elevated affinity, a process termed as affinity maturation[27)]. Activated B cells which have successfully been through CSR and SHM become potent antibody-secreting plasma cells, with some of them becoming memory B cells and some differentiating further into long-lived plasma cells (LLPC) in favourable environments such as in the bone marrow and gut-associated lymphoid tissues (GALT)[27)]. Memory B cells and LLPCs perform vital roles in maintaining high-affinity antibody production and prompt responses when encountering re-exposure to the specific antigen, ensuring the secondary immune responses are delivered in a more efficient and accurate manner. The formation of immunological memory is one of the most prominent features observed in adaptive immunity, the effectors of which are not only confined to memory B cells but also extended to memory T cells.

For those B cells that are not activated by the engagement with T cells, mostly marginal zone (MZ) B cells and B1 cells (two minor subsets of B cell populations), these are provoked by specific bacteria-derived circulating antigens, such as Lipopolysaccharides (LPS). These antigens are transported and presented by DCs and macrophages. After receiving essential survival signals from DCs and macrophages, MZB cells and B1 cells evolve into plasmablasts secreting IgM[28)29)].

Macrophages

① The origin of macrophages

The hypotheses regarding the origin of macrophages have been controversial for more than a century since macrophages were firstly described by Élie Metchnikoff in 1884. Until recently, monocytes and macrophages were considered closely related, in a belief that most tissue-resident macrophages are derived from circulating monocytes in the blood stream. This so-called 'mononuclear phagocyte system (MPS)' concept was established by van Furth in 1968 and was generally accepted until more appreciation of monocytes and macrophages were available[30)]. With more data and knowledge gained, it has become clear that

although macrophages can be generated from circulating monocytes, the situation only happens in some specific contexts, such as in the setting of infection. In fact, most tissue-resident macrophages in adults originate from embryonic progenitors that conceive the development of the tissues and organs before birth and will sustain its population by self-regeneration in adults[30) 31)].

Monocyte-derived macrophages share the same precursor with monocytes - haematopoietic stem cells (HSCs) in the bone marrow. Monocytes in human represent approximately 10% of all circulating mononucleated cells and have two main phenotypes categorised by the surface markers they express - either $CD14^{+}$ $^{+}CD16^{-}$ or $CD14^{+}CD16^{++}$. $CD14^{++}CD16^{-}$ type monocytes are also termed as 'classical', having the ability to produce TNF-α and differentiate into monocyte-derived macrophages and monocyte-derived dendritic cells in the tissue and in vitro culture condition[30)32)] .

On the other hand, $CD14^{+}CD16^{++}$ type monocytes are committed to the maintenance of homeostasis in the blood by continuously rolling over on the blood vessel lumen, responsible for the repair of damaged endothelial structures and the recruitment of neutrophils[30)]. Monocyte-derived macrophages contribute to the population of tissue-resident macrophages in the setting of infection or inflammation, however, to what extent they would remain dedicated to tissue-resident macrophages' populations when the inflammation is resolved depends on the type and severity of inflammation. Monocyte-derived macrophages proliferate after severe inflammatory injuries as a substantial loss of tissue-resident macrophages would happen in those cases, and in some known scenarios such as in atherosclerosis, will mix with the tissue-resident macrophages forming an assorted population of macrophages. These phenomena will not be observed frequently in mild inflammations and in cases involving the blood-brain barrier (BBB), where the tissue-resident macrophages will manage to maintain the whole macrophage population[30)]. The main exception to this is in the gastrointestinal tract, where monocyte-derived macrophages constitute the whole population of macrophages[31)].

Tissue-resident macrophages track back their origins to two main roots, primitive haematopoiesis and definitive haematopoiesis. The former indicates the initial formation of endothelial cells and the primitive haematopoietic cells in the blood islands of the yolk sac. The primitive haematopoietic cells further give rise to large nucleated erythrocytes in the majority, with some primitive monocytes/ macrophages and megakaryocytes also being produced[33)]. The latter, definitive haematopoiesis, suggests the creation of HSCs in the aorta-gonads-mesonephros (AGM) and the expansion of those HSCs in haematopoiesis-related organs such as in the liver and the spleen[30)]. Definitive haematopoiesis is a consequence of the

transition of locations for haematopoiesis from the yolk sac to the organ. In the liver, HSCs produce a full-lineage of different blood cells including macrophages.

Whether a tissue-resident macrophage is of primitive haematopoiesis origin (yolk sac) or definitive haematopoiesis origin (liver) varies between tissues. Recent studies utilising fate-mapping have demonstrated in adults, microglia are predominantly of yolk sac origin in comparison with Langerhans cells which have a dual origin, primarily from the liver with some contribution from the yolk sac[30)31)]. Other macrophage populations including alveolar macrophages in the lung and heart macrophages are generally of definitive haematopoiesis origin in an exclusive manner[30)31)]. The proliferation of tissue-resident macrophages is carried out in a self-renewing way in a stable state, regulated by macrophage colony-stimulating factor (M-CSF) whose presence is critical not only for tissue-resident macrophages but also for monocyte-derived macrophages[31)34)].

② M1/M2, and other subtypes of macrophages

Macrophages manage to gain specific functions and produce different profiles of cytokines while surrounded with diverse microenvironments, a process known as polarisation. Several subsets of macrophages have been classified so far - (a) classically activated macrophages (M1 macrophages), (b) alternatively activated macrophages (M2 macrophages) - which are two principal categories, with small subgroups of (c) tumour-associated macrophages (TAM), (d) $CD169^{+}$ macrophages and (e) T cell receptor positive (TCR^{+}) macrophages[32)]. M1 macrophages produce pro-inflammatory cytokines (IL-1β, TNF, IL-12, and IL-18) and are activated through IFN-γ signalling released by NK cells, Th1 cells and cytotoxic T cells and also the stimuli from LPS, virus and granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine capable of inducing the production of pro-inflammatory cytokines[32)35)]. Along with the expression of surface marker of CD68, M1 macrophages express MHC class II at a high level as well as CD80 and CD86, two co-stimulatory molecules which engage with CD28 on T cells and are essential for T cell activation[32)36)]. M1 macrophages play an important role in the defence against infections, especially those caused by intracellular pathogens represented by virus, and some bacteria which reproduce inside the host's cells (e.g. Mycobacterium tuberculosis (M. tuberculosis) and Listeria monocytogenes (L.monocytogenes)). Their trait of being inflammatory also implies their destiny to have unfavourable involvements in certain pathologies characterised by autoimmune diseases and metabolic disorders. M1 macrophages are likely to affect the prognosis of autoimmume diseases not only by their chronic production of pro-inflammatory cytokines but also by their up-regulation of specific proteins, such as suppressor of cytokine signalling 3 (SOCS3), an intracellular regulatory protein which can activate the release of nitric oxide (NO) exacerbating the inflammation in

inflammatory bowel diseases (IBD), interferon regulatory factor (IRF) in systemic lupus erythematosus (SLE), and signal transducers and activators of transcriptions (STATs) in rheumatoid arthritis (RA). In obesity, M1 macrophages are significantly increased in white adipose tissue compared to M2 macrophages and it has been shown that obesity induces the elevated level of gene expression of M1 related cytokines and enzymes[32)37)].

In contrast, M2 macrophages are defined as macrophages which produce anti-inflammatory cytokines the chief of which is IL10 in significant quantities, with IL-12 and TGF-β produced at lower levels[32)35)]. They are activated by cytokines (CSF-1, IL-4, IL-10, TGF-β, and IL-13) as well as stimuli from parasites, fungi and glucocorticoids[31)32)]. M2 macrophages express CD163, a haemoglobin (Hb) scavenger receptor the up-regulation of which in macrophages is a key change suggesting a polarisation towards M2 type[32)38)], together with CD200R membrane glycoprotein and CD206, a macrophage mannose receptor (MMR)[32)]. M2 macrophages play an essential part in the defence against parasites and fungi and in the maintenance of homeostasis including angiogenesis and tissue remodelling[32)39)]. Though M1 macrophages are involved in the progression of autoimmune diseases and metabolic disorders, M2 macrophages are responsible of the exacerbation in allergic diseases. For understanding, four subsets of M2 macrophages are further categorised as M2a subtype activated by IL-4, IL-13 and producing IL-10, IL-1ra, TGF-β; M2b subtype stimulated by immune complexes and IL-1R and secreting IL-1, IL-6, IL-10, TNF-α; M2c subtype evoked by IL-10, TGF-β1 and emitting IL-10, TGF-β1; M2d subtype receiving signals from IL-6, LIF (leukemia inhibitory factor) and producing TNF-α, TGF-β[40)]. M2a subtype is particularly engaged in allergic diseases such as asthma, by releasing IL-13 at high concentration along with chemokines (CCL-17, CCL-18, CCL-22 and CCL-24), which facilitates the activation of Th2 cells and the infiltration of the eosinophils into the lung[39)].

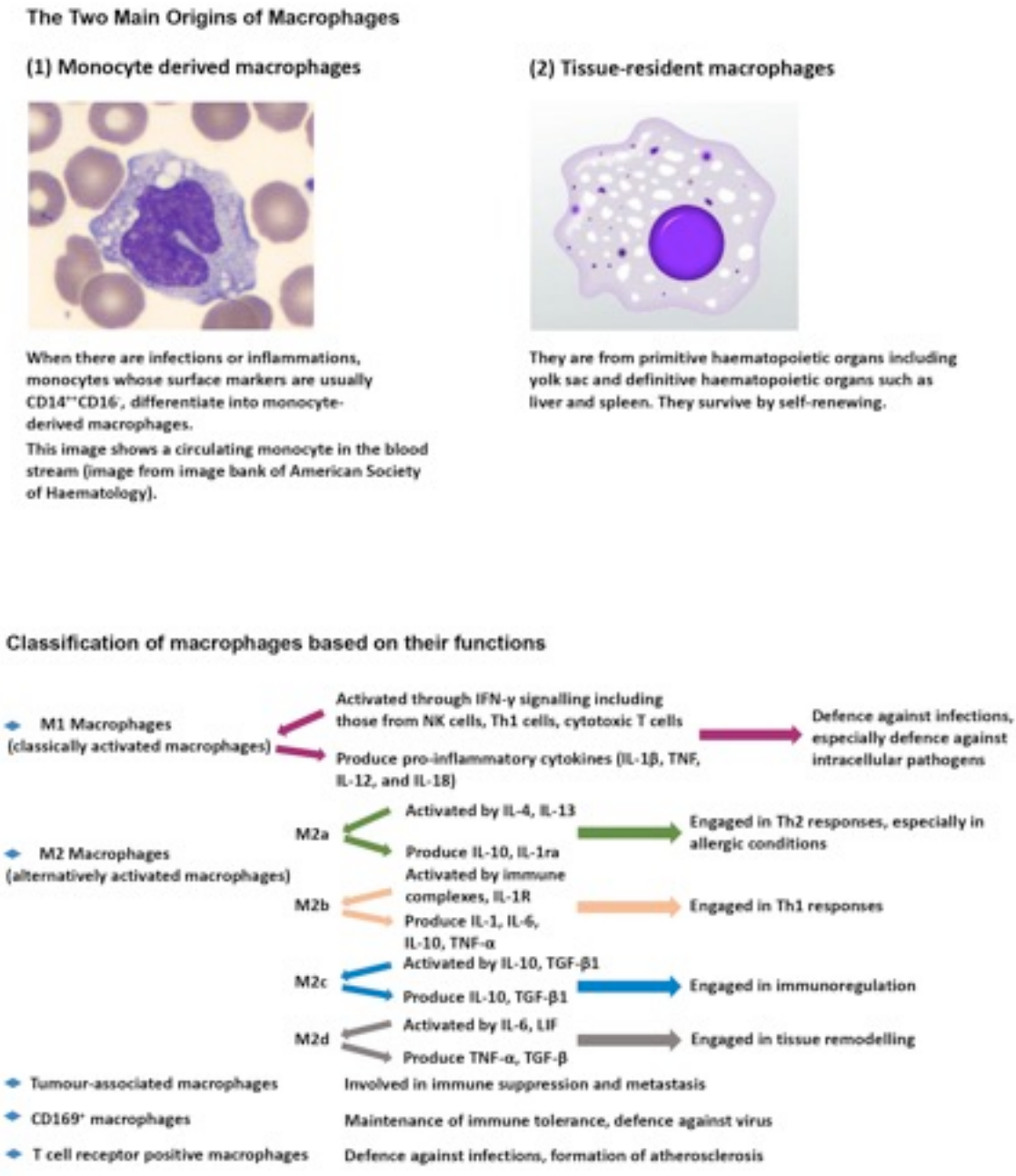


Figure2: The two origins of macrophages and subtypes of macrophages based on their functions.

Tumour-associated macrophages(TAM) are the outcomes of differentiation from circulating monocytes in the surrounding microenvironment observed in tumour tissues, where various chemoattractants are released in recruiting circulating monocytes to the flared tissue[32]. TAM are more similar to M2 type macrophages. They smooth the infiltrate by tumour cells, and contribute to the process of angiogenesis as well as immune suppression and metastasis[32][41]. Chemokine ligand2 (CCL2), CCL5, and IL-10 are highly expressed on TAM accompanied with

surface markers such as MHC class II, scavenger receptor A, macrophage galactose-type lectin-1 (MGL1), and CD81, a profile of mixed markers from both M1 and M2[32)41)]. TAM accelerate the progression of tumour cells by modulating the pathway of signal transducer and activator of transcription 1 (STAT1), a transcription factor heavily engaged in positive adjustment of cell cycles and responses against pathogens, and interferon regulatory transcription factor 3 (IRF3), while expressing chemokine ligands (CCL2, CCL3, CCL5) and secreting cytokines (IL-10 high, IL-12 low). It has been proved that the functions of TAM are however, fundamentally limited in tumour-associated microenvironment as culture of TAM in a normal condition would restore their ability of producing IL-12 and TNF[32)]. Several therapeutic trials have been performed, directed at this plasticity of TAM with an underlying strategy that tries to eliminate TAM or re-polarise TAM into anti-tumoural macrophages by inhibiting their growth factors including colony stimulating factor 1 (CSF1). Effects on the control of glioblastoma, breast and cervical cancers in murine and diffuse-type giant cell tumours in human have been demonstrated[41)]. More committed immunotherapies targeting only TAM but not other beneficial types of macrophages are in development[41)].

$CD169^+$ macrophages are characterised by its surface marker - CD169, also known as siglec-1, or sialoadhesin, a cell adhesion molecule identified on macrophages. $CD169^+$ macrophages are able to bind erythrocytes and are detected in spleen, liver, bone marrow and lymph nodes. Bone marrow origin $CD169^+$ macrophages are discovered to be involved in erythropoiesis by assisting the preservation of HSCs, with further finding established suggesting that removal of G-CSF and $CD169^+$ macrophages will hinder erythropoiesis in the bone marrow but not in the spleen. In kidney diseases, the functions of $CD169^+$ macrophages are contentious. In a model focusing on anti-glomerular basement membrane (anti-GBM), inclusion of $CD169^+$ macrophages were found to be associated with proteinuria and tissue damage. On the other hand, in a model with emphasis on renal ischemia-reperfusion injuries (IRI), the depletion of $CD169^+$ macrophages exacerbated the condition. From the immunological aspects, $CD169^+$ macrophages have positive impacts on the maintenance of immune tolerance by inducing regulatory T cells in the spleen and capturing self-antigen related exosomes and vesicles in the spleen and lymph nodes. $CD169^+$ macrophages also have important roles in the defence against virus - they are a main source producing IFN-γ in the spleen and can recruit NK cells to the site of infection[32)].

Lastly, T cell receptor positive (TCR^+) macrophages are a type of macrophage which can express the T cell receptor either in an αβ subtype or a γδ subtype, a mechanism similar to the traditional expression of TCRs on T cells, largely of an αβ subtype with a small portion of γδ subtype. TCR^+ macrophages are involved in the

defence against infections. Particularly, TCR^+ macrophages contribute to the formation of granulomas in M. tuberculosis infection. This was proved by administrating TNF antibodies, which can suppress the expression of TCR αβ, the shaping of granulomas was found to be inhibited. TCR $αβ^+$ macrophages are also connected to the pathogenesis of atherosclerosis. Large numbers of TCR $αβ^+$ macrophages can be identified in areas of atherosclerosis with low-density lipoprotein (LDL) having the capability of inducing more TCR αβ expression, indicating a new potential therapeutic target in the treatment of atherosclerosis[32].

③ Other distinctive functions of macrophages besides phagocytosis

Since the detail of phagocytosis has been described in the section related to innate immunity, here other distinguished roles of macrophages will be outlined.

1 Muscle regeneration

Injured muscle fibres release various signals (mainly chemoattractants) to attract different types of cells to the site of damage for repair[42]. The significant roles macrophages play in muscle repair are not only because they aid in the clearance of damaged tissue and apoptotic cells together with their production and secretion of growth factors, proteinases and chemoattractants to facilitate the repair, but because they turn to increase the expression of several special molecules which are vital in the process of repair[43]. The urokinase-type plasminogen activator (uPA), a serine protease in charge of cell migration and tissue remodelling[44] by activating the change from plasminogen to plasmin in promoting the degeneration of extracellular matrix proteins, is found to be expressed in macrophages recruited to the wounded site, and in return, uPA is essential in the chemotaxis of macrophages to the damaged muscle[43]. Macrophages also produce meteorin-like, an adipokine from muscle and adipose tissue, to help the expansion of muscle stem cells and redirect themselves from an inflammatory state to a homeostatic shape[45].

2 Wound healing

Though the mechanism that macrophages take part in the process of wound healing has some similarities to what happens in the steps of muscle regeneration, it has more features that mark their irreplaceability. Macrophages are called up to the site of injuries by chemoattractants (as in muscle regeneration) and induce production of crucial growth factors represented by TGF-β and vascular endothelial growth factor (VEGF) along with proteases and protease-inhibitors which modulate the structure of the extracellular matrix (ECM) and the progression of re-modelling[46]. VEGF is especially important in wound healing as, unlike other growth factors, VEGF can solely give influence on several stages indispensable in the process, from angiogenesis, epithelialisation to collagen deposition[47]. Although

keratinocytes located in the epidermis also produce VEGF, the large amounts of VEGF secreted by macrophages are particularly needed in the healing of excisional wounds compared to incisional wounds[46].

Another prominent growth factor of macrophage origin involved in wound healing is platelet-derived growth factors (PDGF). They support fibroblasts' production of osteopontin, a matricellular protein functioning in the formation of scars and at the same time, they are also a key element in the gathering of progenitor cells and further inflammatory cells[46].

3 Iron recycling

Iron is imperative for our body both biologically and physiologically. In addition to being a chief material for the generation of erythrocytes, iron plays an important role in the defence against microbial pathogens. For sites linked with the homeostasis of iron, macrophages, especially those situated in the spleen, and in the liver (Kupffer cells), phagocytise erythrocytes which are impaired or have reached their lifespan and break them down further to bring out heme, ultimately iron. This phenomenon is termed as erythrophagocytosis. Following the circulation of blood to the spleen, each erythrocyte passes through the spleen every 20 minutes[48]. To re-enter the blood stream, every erythrocyte has to go through the narrow interendothelial slit and erythrocytes that fail to show their deformability will be eliminated by macrophages. Erythrocytes with pathogens and immune complexes bound to their surface-expressed complement receptor 1 (CR1) will have macrophages clear up their loadings and those with pathogens inside their bodies as seen in infections caused by Plasmodium will have macrophages tidy out their inclusion bodies without destroying them. It is not clear yet how macrophages decide whether to remove an erythrocyte or just attempt repair due to the limitation of analysing the damaged erythrocytes with a death signal - partly because those are already phagocytosed when a blood sample is taken and also because the portion of those erythrocytes with a removal signal is small[49].

On the other hand, to identify the ageing modifications on erythrocytes efficiently, several recognition pathways have been clarified. First of all, macrophages can detect the changes of the most common erythrocyte membrane protein - Band 3 and its membrane clustering - the alterations of which are sensed by opsonic antibodies and complements, resulting in antibody- and complement-driven phagocytosis. Secondarily, macrophages and endothelial receptors expressed by specific types of cells can identify the expression of phosphatidylserine, a phospholipid, on the outer leaflet of the plasma membrane. Receptors of phosphatidylserine and the opsonisation by phosphatidylserine play important roles in erythrophagocytosis. Last of all, worsened membrane rigidity and ceased

expressions of the CD47 antigen and sialic acid are also related to the recognition of aged erythrocytes. Through all of these possible courses, senescent erythrocytes are destroyed and the iron extracted from degraded heme will be transported via ferroportin, from the cytoplasm of macrophages to the outside[48]. The total amount of iron released by macrophages considerably surpasses the uptake of iron from diets and those stocked in hepatocytes[48].

During infections, to restrain the availability of iron from pathogens, inflammatory cytokines including IL-6 increase the gene expression of hepcidin, a hormone synthesised in the liver and regulating the circulation of iron. Hepcidin makes bonds with ferroportin expressed on macrophages, causing its endocytosis and disintegration. As a result, iron will be trapped inside macrophages without a vehicle for being transported. Along with this fact, iron will continue to be devoured for erythropoiesis, all of which in the end will cause anaemia seen in inflammations[48][49].

The immune system in pregnancy

① General concepts of the immune system in pregnancy and what happens in the first trimester

For a long time, the state of being pregnant had been compared to allotransplantation and the immune system had been considered suppressed to sustain pregnancy. With more progression achieved in the understanding of the immunological interactions between the fetus and the mother, pregnancy is acknowledged to be a condition more complicated than the comparison to allotransplantation, and the fetal-maternal immune responses are appreciated to be taking an active, well-controlled and balanced form with efforts from both sides[50].

In normal pregnancy, the human decidua, which is the mucosal lining of the uterus and forms the maternal part of the placenta, holds a wide variety and large numbers of immune cells. These include dendritic cells, NK cells, macrophages, with T lymphocytes present at a 3-10% rate and the absence of B lymphocytes. During the first trimester, dendritic cells, NK cells and macrophages penetrate the decidua to gather near the invading trophoblast cells from the fertilised egg. Elimination of these immune cells will give severe impact on the shaping of placenta, preventing implantation and development of decidua. In worst cases, it can lead to the cessation of pregnancy - trophoblast cells are not capable of extending to the endometrial circulation without the help from uterine NK (uNK) cells[50]. The theory concerning how fetal antigens keep themselves free from attacks by maternal immune cells had also been contentious. Early researchers suggested that the fetus established a system so it can escape from the scrutiny by maternal immune system, and this hypothesis had been proved to be rather limited. Though there are

certain mechanisms that regulate the maternal immune system to spare unnecessary attacks against the fetus (details related to those mechanisms will be described later), it is more likely a consequence of collective collaborations from the fetus and mother to protect the harmony in between. To begin with, trophoblast cells produce chemokines such as IL8, CCL2, to accumulate immune cells to the site of implantation. Then they initiate the education of these immune cells by stimulating them with regulatory cytokines - primarily macrophage migration inhibitory factor (MIF) and growth-regulated oncogene-α (Gro-α) to shape their differentiation. Refined immune cells then commence to react to the signals from the surrounding microenvironment in a way different from their usual responses and secrete IL-6 and TNF-α[50]. TNF-α, which can be produced from activated macrophages to prevent the growth of tumour cells, plays a unique part in supporting the pregnancy by triggering the manufacture of matrix metalloproteinases (MMPs) which promote the invasion by cytotrophoblasts and allow them to stretch deeper into the spiral arteries within the uterine decidua. TNF-α also encourages the apoptosis of vascular smooth muscle cells near these arteries[51]. Additionally, these pro-inflammatory profiles of cytokines correspond with the background in the early stage of pregnancy, usually the first trimester (week 1-12) and the early second trimester, since successful implantation and placentation are similar to trauma which needs a powerful inflammatory response. More precisely, to achieve successful implantation, aggressive penetrations into the lining of the uterine epithelia and destruction of the endometrial tissues have to be managed by blastocysts. After these have been attained, to ensure proper blood supply in the placenta, trophoblasts have to replace the endothelia and vascular smooth muscles of the maternal blood vessels. As a result, dying cells and debris will occur in these sequential breakthroughs and damaged uterine epithelia will require an appropriate repair. Recruited and educated immune cells, which in 65-70% are uterine-specific natural killer cells, 10-20% are macrophages and 2-4% are dendritic cells, generate pro-inflammatory cytokines (mainly IL-6, IL-8, TNF-α) alongside the endometrial cells and orchestrate the immune state to a Th1 mode, aiding in the removal of dead cells and cellular debris and in the mending of the epithelia[50].

② The fetal immune system becomes active in the second trimester

The fetus undergoes tremendous development during the second trimester (week 13-28) including its immune system. From 5-10 weeks, the liver increases its size in a substantial way, which is followed by the growth of the spleen and thymus - both are liver-derived. By 11 to 12 weeks, stem cells can be identified in the bone marrow and haematopoiesis will begin to be established in the bone marrow replacing the liver[51]. Analysis of the fetal blood around 12-19 weeks would find erythroid

precursor cells as well as granulocytic/monocytic progenitor cells, though granulocytes are not produced in a large quantity until the fetus is born[52]. For populations of macrophages, they can be spotted either in yolk sac or in mesenchyme as early as 4-6 weeks. Those created in yolk sac are generally MHC class II negative with a small portion expressing MHC class II - those can be recognised in the lymph nodes at 11-13 weeks and in the thymic medulla containing T cells by 16 weeks[52]. On the other hand, macrophages of mesenchymal origin are also called Hofbauer cells, which is the main subject of this thesis and will be explained in detail in the next separate section. For dendritic cells in the fetus, a recent study has confirmed that though fetal dendritic cells can respond to toll-like receptor ligation in lymph nodes possibly from 16 weeks, their responses are totally different from the adult ones - instead of conducting an immune response evoking Th1 or Th2 cells, fetal DCs contribute to the immune suppression by stimulating the differentiation of T_{reg} cells, and preventing the production of TNF-α through arginase-2 activity[53]. T cells and B cells also achieve remarkable maturation by this stage. A high ratio of $CD45RA^+$(=Naive) T cells can be detected in the mesenteric lymph nodes between 18-24 weeks and in the spleen, B cells are observable from 13-23 weeks, with the level of immunoglobulin (mainly IgM and IgG) production peaking at 17-18 weeks[52].

The overall immune status cross the placenta is an anti-inflammatory one, optimising the chances for the growing of the fetus and the wellbeing of the mother. The mother usually will not suffer morning sickness anymore in their second trimester and become as energetic as before the pregnancy[50].

③ The third trimester - a pivotal phase bridging the inside and the external world

The third trimester is a crucial time for the maturation of the fetal brain and the integrity of its neuronal network[54]. It has been understood that maternal immune activation (MIA), whether it is caused due to the innate or adaptive immune system and whatever the trigger might be - inflammations, infections, stress and all the other possible causes - will leave impacts on the forming of the fetal brain[54][55]. Research has further corroborated this fact by investigating the correlations between the levels of IL-6 and CRP - two of the most routinely secreted pro-inflammatory cytokines and proteins in response to inflammations - in the maternal blood in the third trimester, and the cognitive functions of the newborn. Maternal high levels of IL-6 and CRP in the third trimester were related to several specific modifications of connections in the salience network, which were associated with the pathogenesis of psychiatric disorders including schizophrenia and autistic spectrum disorders (ASD). High levels of IL-6 and CRP were also positively correlated with the changes in behaviour observed in the fetal and toddler period[54].

As the third trimester is also the final period of pregnancy, the mother has to get ready for the delivery of the fetus, which requires regenerated inflammation. Numerous immune cells will inflow into the myometrium to revive inflammation, resulting in a revitalised pro-inflammatory environment with the immune state in a more Th1-shifted mode. This will ease the labouring steps happening in the birthing, starting from the contractions of the uterus, and to the expulsion of the baby and subsequently, the placenta[50].

④ Several characteristic mechanisms modulating the immunotolerance in pregnancy

1 T_{reg} cells in pregnancy

The activities of $CD4^+CD25^+$ T_{reg} cells can be distinguished following the changes in the estrus cycle in fertile women even before a pregnancy is established. They gather regularly in the uterus and draining lymph nodes when a female is near to estrus. Chemokines represented by CCL3, CCL4 and CCL5 alongside the hormones - estrogen and progesterone - play important parts in the recruiting and retaining of T_{reg} cells[56)57)]. The increase of T_{regs} corresponding with the rise of serum estradiol levels especially in the late follicular phase and the decrease of T_{regs} at the luteal phase suggest their involvement in the allaying of the encounter of paternal antigens and potential implantation.

Once the pregnancy is started, T_{regs} expand themselves from a fairly early stage and from the evidence gained so far, they show specificities to paternally derived cells. The expansion of T_{regs} in pregnancy is not only dependant on the influence from hormones and chemokines, but also relies on the interactions with paternal antigens which are of seminal origin and presented by antigen-presenting cells in lymphoid organs[57)]. Lessened expressions of transcription factor Foxp3 for T_{regs} in endometrial tissue is considered to be linked to infertility[57)58)].

For the maintenance and promotion of pregnancy, T_{regs} function in diverse approaches. Firstly, they elevate their expressions of TGF-β, heme oxygenase-1 (HO-1), and leukemia inhibitory factor (LIF), all of which are fundamental in T_{regs}' protective purposes. Secondly, they directly suppress the activation of effector T cells via the connection with the inhibitory ligands expressed on T cells - chiefly through the programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) pathways. PD-1 and CTLA-4, also known as immune checkpoints, are critical down-regulators of immune responses. In cases of miscarriages, CTLA-4 expressed T_{regs} were found to be as low as those in non-pregnant states, implying their supporting role in averting fetal rejection[57)]. T_{regs} also communicate with DCs and B cells directly to orchestrate their differentiation into an immuno-tolerant phenotype or restrict their proliferation[57)]. Additionally, T_{regs}

increase the expression of galectin-1, an immune regulator that can cause the apoptosis of activated T cells. Finally, T_{regs} produce anti-inflammatory cytokines such as IL-10 and TGF-β to induce an immunosuppressive environment during pregnancy[57]. Taking into account of the versatile roles of T_{regs} in pregnancy, more and more therapeutic trials are trying to utilise T_{regs} as an interventional treatment or a biomarker predicting the prognosis of pregnancy[57)59)].

2 The contributions from B7 family to the immunotolerance in pregnancy

B7 is a membrane protein and serves as a co-stimulatory or inhibitory molecule in adjusting the activation of T cells. Thus far, 10 members of B7 family have been discovered (B7-1 (CD80), B7-2 (CD86), B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7). B7s are normally expressed on antigen-presenting cells (APCs), but can be detected on a great variety of cells in pregnancy. Cells inside the decidua can express B7-1 and B7-2 and further investigations verified that decidual DCs have enhanced expressions of B7-1 and B7-2 compared to peripheral DCs. Culture of these decidual DCs have shown they induce the T cells into a Th2 type polarisation rather than Th1[60)61)].

B7-H1 can be located on lymphocytes, cardiac endothelial cells, and all populations of trophoblast cells as well as APCs. While its function as a co-stimulator is poorly understood, its inhibitory character has been well-examined. B7-H1 binds with PD-1 on T cells, leading to apoptosis or anergy. It also boosts the differentiation of T_{regs} from naive CD4 T cells. Of trophoblast cells, syncytiotrophoblast cells convey more B7-H1 than cytotrophoblast cells and efficiently restrain the production of Th1 cytokines by decidual lymphocytes via B7-H1. B7-DC has a similar role as B7-H1, with a higher affinity for PD-1 but more limited expressions - only on specific APCs under the stimulation from cytokines[61)].

Though other B7 family members such as B7-H2, B7-H3, B7-H4 and B7-H6, have varied pathways in prompting immunotolerance, their parts in pregnancy remain unknown.

3 The significance of placental exosomes related to immunotolerance in pregnancy

Extracellular vesicles (EVs) are secreted from the plasma membranes of cells. Exosomes are the smallest members amongst EVs- often nanometer-sized, and are secreted by most types of cells, operating as a messenger in facilitating cell-to-cell interaction. They are membrane-bound nanovesicles and contain both proteins and multiple species of ribonucleic acids, including messenger RNA (mRNA), micro RNA (miRNA) and transfer RNA (tRNA). During pregnancy, the syncytiotrophoblast creates enormous quantities of EVs including exosomes. Placenta-derived exosomes express tetraspanin CD63 and placental alkaline phosphatase (PLAP)[62)].

In addition to being an agent for transferring cell signals, exchanging genetic information and possibly involved in the pathogenesis of pregnancy-related diseases such as preeclampsia, exosomes have important roles associated with immunotolerance in pregnancy. Exosomes collected from mothers who had normal term births showed inhibitory effects on IL-2 production by activated T cells while those taken from mothers who had pre-term delivery failed to[62]. Culture of T cells with placenta-derived exosomes demonstrated decreased expressions of intracellular domains and tyrosine kinases in T cells, resulting in deficient T cell-mediated responses[62]. Placental exosomes also carry NKG2D ligands and are able to down-regulate the NKG2D receptors on NK cells as well as on cytotoxic T cells and γδ T cells in a concentration-related way[62]. Lastly, pathways leading to apoptosis in immune cells are also adopted by placental exosomes, as they express proapoptotic ligands the major of which include Fas ligand, PD-1 ligand and TNF-related apoptosis-inducing ligand (TRAIL)[62].

Hofbauer cells

① From its discovery to identification of its multifaceted roles

Back in the middle of the 19th century, the presence of Hofbauer cells (HBCs) were firstly described as large round shaped cells identified in the stroma of chorionic villi of the human placenta. American gynaecologist J. Hofbauer was the first to perform an extensive characterisation of these cells from the aspects of morphology and functions at the beginning of the 20th century and the term of `Hofbauer cells' was acknowledged since then. HBCs were depicted as pleomorphic cells in the villous stroma bearing round, spindle or star shaped body. The sizes of HBCs are varied between 10 to 30 μm in diameter and the cytoplasm is filled with various vacuoles which are membrane-bound and luminous under microscopy and granules with diversified density[63]. As more investigations related to the development of HBCs have become available, it is now understood that HBCs usually appear on the 18th day of gestation and increase gradually until week 18 of the pregnancy[64][65]. However, because of the physical pressure from the villous stroma, the number of HBCs decreases subsequently after the 18th week and by the time of birth, the amount of HBCs are extremely limited[64][65]. By applying Y chromosome-specific probes in male fetuses, HBCs were confirmed to be of fetal origin[64]. During the early period of pregnancy, HBCs are generated from mesenchymal cells in the villi, but this changes as the time passes by - in the late phases of pregnancy, HBCs are mainly derived from recruited fetal monocytes[64]. Now, HBCs are used to define any placental macrophages of fetal orgin that can be located in the placental villi, amnion and chorionic lavae[66].

From the classifications for macrophages, HBCs are of an M2 phenotype[66)67)40)]. Though in general, macrophages are highly plastic and can polarise to a specific phenotype following the stimulations from cytokines and pathogens in an according microenvironment (details should be referred to the section of "macrophages", especially the part of `M1/M2, and other subtypes of macrophages'), HBCs remain an M2 phenotype even in the presence of placental inflammation such as chorioamnionitis[67)] and also in other chronic inflammatory states (e.g. gestational diabetes mellitus[40)]). Regarding the level of their expressions of surface markers as M2 macrophages - this includes Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN also known as CD209), CD206 and CD163, it has been proved that there are no significant differences observed between HBCs which were collected from the placentae of allergen-sensitised mothers and those from non-sensitised mothers[67)], suggesting that HBCs are not involved in Th2 responses actively such as in the pathogenesis of allergic diseases. To detect HBCs correctly, proper knowledge about the surface markers specific to HBCs is indispensable. Both CD163 and CD64 are constitutively expressed on HBCs of all possible subtypes, although the expression of CD68, which is a traditional standard marker for macrophages, varies between different timings of pregnancy. The expression of CD68 reaches its maximum by the second trimester and then substantially diminishes as pregnancy progresses[66)], indicating it is not a suitable candidate for identifying HBCs.

Though HBCs function as M2 type macrophages, their important roles in the defence against infectious antigens are not disrupted. HBCs are capable of expressing TLR2, TLR3 and TLR4 to a remarkable level in response to LPS and polyinosinic-polycytidylic acid[68)]. HBCs are also able to express MHC class I and II molecules and produce IL-1, which underlines their full potential for antigen presentations as the release of IL-1 reflects efficient antigen presentations[69)]. HBCs perform phagocytosis and retain nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-dependent enzymes - principally inducible nitric oxide synthase (iNOS) and NADPH oxidase. iNOS creates NO, which is a valuable agent in infections and inflammation showing anti-bacterial and cytotoxic activities. Studies have also demonstrated that HBCs possess enormous glucose-6-phosphate dehydrogenase (G6PD) activities. G6PD is an enzyme in charge of the transformation from glucose-6-phosphate to 6-phosphogluconate and two sub-products - NADPH and ribose 5-phosphate. This fact reveals that by maintaining abundant G6PD in their cytoplasm and endoplasmic reticulum membranes, HBCs secure their constant contributions to anti-inflammatory actions through NADPH-iNOS-NO pathway and to the synthesis of RNA and DNA via generation of ribose 5-phosphate[70)].

HBCs are further characterised by their significant involvements in vasculogenesis and angiogenesis[71]. They develop VEGF in large quantities[66], with extended studies having uncovered their possible exact roles in the mechanisms. The process of vascularisation starts around 21st day in pregnancy. Endothelial progenitor cells evolve into cords under the trophoblastic layers forming angiogenic cell cords (ACC), which successively differentiate and shape primitive vascular tubes (VT), a primary lumen structure. Substantial numbers of HBCs have been observed to be located in adjacent to ACC and VT, with plenty HBCs just amongst the cells of ACC and inside the primary lumen. More surprisingly, the amount of HBCs is in a positive correlation with the number of vascular structures, all of these implying the close interactions between HBCs and ACC, VT and the engagement of HBCs in the process of angiogenesis and vasculogenesis[71]. Another distinct attribute that exemplifies HBCs' part in the development of the chorionic villi is in the placenta, they exclusively express sprouty (Spry) proteins, a type of membrane-associated proteins that regulate receptor tyrosine kinase (RTK) signalling. RTK signalling exists as a trigger for the start of mitogen activated protein kinase (MAPK) pathway, which is modulated by various growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and VEGF. In particular, Spry-2 proteins can either promote RTK signalling or inhibit it by collaborating with c-Cbl, a ubiquitin ligase that works towards the down regulation of RTK[72]. HBCs convey both Spry proteins and c-Cbl, indicating their expected function as a regulator in the migration of trophoblast cells and in the branching of the villous tree. This disclosure is especially important since it also proposes the possibility that the dysfunction of HBCs can be responsible for preeclampsia and intrauterine growth restriction (IUGR), both related to poor development of the chorionic villous tree[72].

HBCs have another major duty during pregnancy - to adjust the level of human chorionic gonadotrophin (hCG) through phagocytosis. HBCs are known to be positive with hCG in immuno-histochemical staining, and since macrophages are not capable of producing hCG, hCG within the HBCs are concluded to be absorbed by HBCs via phagocytosis. The reason HBCs have to modify the concentrations of hCG in the placenta is noteworthy. Maternal hCG gets into the fetal circulation via the mesenchyme of the placental villi. HCG facilitates the sexual differentiation into a male pattern, consequently extra hCG can do harm for the development of genitals. It has been verified by using phorbol 12-myristate 13-acetate (PMA) treated THP-1 cells, which is an established human monocyte-macrophage lineage cell line, the shaping of vacuoles are confirmed to be induced by hCG, the same phenomena of which may explain the formations of plentiful vacuoles in HBCs. Considering the concentration of total hCG in fetal plasma is just 3% of the maternal

plasma level, it is not difficult to imagine the considerable part HBCs play in maintaining an appropriate level of hCG cross the placenta[73].

Other key functions HBCs hold are as a source for the production of prostaglandin E_2 (PGE_2), an effective vasodilator as well as thromboxane A_2 (TXA_2), a potent vasoconstrictor. Though HBCs can synthesise both, the amount of PGE_2 created is more predominant than that of TXA_2[74]. Finally, HBCs also take up collagen supporting the extracellular matrix (ECM) remodelling in ensuring the durability of the villi in pregnancy[65].



Figure3: Hofbauer cells in the villi (photo from embryology.ch)

② HBCs in pregnancy-related diseases

In the division above, the reason why HBCs can be considered to be involved in the pathogenesis of preeclampsia and IUGR is stated. More studies have supported this by confirming that in preeclampsia, which is characterised by pregnancy-induced maternal hypertension[75] and as a result, widespread endothelial damage[76], the gene expression of HBCs is reduced along with a decrease in the number of HBCs. Besides, the level of folate receptor-β (FR-β), a key carrier for folate in macrophages, is also lessened in HBCs in preeclampsia[75]. This discovery matches with the fact that folate is not only essential in satisfactory development of the fetus, but also have a role in the prevention of preeclampsia - higher dietary folate intake and the usage of folic acid supplement during pregnancy are associated with lower risks of suffering preeclampsia[77].

Villitis of unknown etiology (VUE), a common type of placental injury occurring in 5% to 15% of all placentae, remains an important cause for IUGR and repeated pregnancy loss. Outsized fetal vessels are a remarkable feature in some cases of VUE and are linked to the occurrences of neonatal encephalopathy and cerebral palsy[78], which can have catastrophic effects on newborns. It is known that VUE is

not caused by infectious agents but rather considered to be an outcome of the overreactions of maternal immune responses against the fetal antigens, especially the involvement of maternal $CD8^+$ T cells[64)78)]. In VUE, HBCs are significantly increased compared to normal placentae, and this is regarded as a consequence of enhanced antigen presentations by HBCs via their MHC class II molecules. It is suspected that T cells may have gone through priming before the current affected pregnancy, either by fetal MHC antigens in previous pregnancies or by autoantigens or foreign antigens that cross-react with fetal alloantigen. This prior sensitisation theory is backed by the observations that VUE is often a repetitive medical condition and more common and dangerous in multigravidas[78)].

Histological chorioamnionitis (HCA) is caused by pathogens in the genital tract, mostly the ascending infections caused by *Ureaplasma urealyticum*, *Mycoplasma hominis*, and *Gram-negative anaerobes*[79)], resulting in inflammation which are often exacerbated by the infiltration of neutrophils in the chorionic plate and amnion[64)79)]. It affects 2-4% of full term pregnancies, with a higher morbidity in preterm pregnancies - an estimated 40-70% of women suffer this condition in premature deliveries[79)]. Like in VUE, HCA is associated with increased risks of developing neurological disorders in the fetus, such as cerebral palsy, which leads to critical neonatal mortality and severe disability in the lifetime[64)79)]. An expansion of HBCs, the number of which is as high as two to three fold compared to the normal placenta, has been confirmed in the placentae with HCA, accompanied by a rise of the levels of TNF-α converting enzyme (TACE) in the placenta and fetal membranes in cases of HCA, points to the inflammatory role of TNF-α from the TACE pathway in HCA[64)]. The pattern of the change in the number of HBCs is different in HCA from the one in VUE - though the number of HBCs in the villi is increased in HCA, the total number of HBCs in the placenta may not vary from the normal or even fall. The background rationale for this needs more exploration, however, how HBCs are involved in the inflammatory processes induced by infections in HCA has become evident. A higher expression of Toll-like receptor 4 has been detected in HBCs in HCA[64)]. As described in the section "recognition of potential antigens (Innate immunity)", TLR-4 is specialised in sensing gram-negative bacteria by recognising lipopolysaccharides (LPS). Additionally, HBCs collected from placentae with infections had enlarged phagosomes with elevated level of acid phosphatase, suggesting their active execution of functions as macrophages[64)].

Other infections also have their own ways of presentations in relation to HBCs. The Zika virus is categorised as one of the TORCH agents (Toxoplasma, Others, Rubella, Cytomegalovirus, Herpes) and is a mosquito-borne flavivirus. Like other pathogens belonging to TORCH, the infected mother can be asymptomatic or only

has mild symptoms, while the transmissible agents can be passed on to the fetus via the placenta causing devastating defects including microcephaly or even the death. Zika virus infection of the placenta is not associated with severe inflammatory villitis as seen in other TORCH infections, but is more characterised by a widespread infectious scale involving a wide range of cells in the placenta especially in the first trimester. HBCs as well as cytotrophoblasts, fibroblasts, endothelial cells and the fetal leukocytes are the targets for the Zika virus and the entries into these cells are usually via the expressions of the viral entry cofactors such as tyrosine-protein kinase receptor3 (TYRO3), receptor tyrosine kinase (AXL) and mucin domain 1 (TIM1) on those cells. HBCs start proliferation in the Zika virus-infected placenta and therefore a hyperplasia of HBCs has been proved to be a key finding in the placenta infected with the Zika virus. In experimental studies, it also has been revealed that after the Zika virus gets HBCs infected, the virus will replicate themselves in HBCs and the virus obtained from the HBCs also showed contagious traits[80)]. In contrast to the circumstances in the Zika virus-related infections in the placenta, although human immunodeficiency virus 1 (HIV-1) infect HBCs as well as T cells, HBCs contain the virus in intracellular virus-containing compartments (VCCs). VCCs are unreached from the external environment, but can be accessed from the cell surface. Binding with neutralising antibodies (VRC01 and 4E10, VRC01 binding with the envelope glycoprotein GP120 of HIV and 4E10 with the glycoprotein 41, another envelope glycoprotein on HIV mediating its entry[81)]) via FcγRI on HBCs have demonstrated inhibiting effects on viral replications, which could explain why the risk of transmission of HIV in the uterus is less than 7%[82)].

Apart from their roles in circulatory diseases and infections, HBCs play an important part in metabolic diseases as well. Adipsin, an adipocyte-released protease, is in positive correlation with the levels of obesity and the body mass index (BMI). It also serves as a rate-limiting enzyme in the process of forming acylation stimulation protein (ASP), an element dedicated in lipid storage in the adipose tissue. Both adipsin and ASP increase in pregnant women with obesity compared to the lean controls, and the placenta may intensify these conditions through the production of adipsin and ASP by HBCs - it has been confirmed that not fibroblasts or cytotrophoblasts, but only HBCs in the placenta give rise to adipsin and ASP. The obesity in the mother raises the risks of suffering gestational diabetes to a higher degree, considering that pregnancy is already an insulin-resistant state. High levels of adipsin and ASP in the mother may also contribute to the obesity of the newborn[83)].

HBCs are also suggested to be related to other metabolic disorders such as GM1 gangliosidosis, type VII mucopolysaccharidosis, and β-glucuronidase deficiency,

as the number of HBCs usually rise in these metabolic storage disorders and HBCs themselves show frothy appearances, but the exact mechanisms how HBCs are involved in these disorders remain unidentified[64].

Sexual dimorphism

① Sexual dimorphism in general

Sexual dimorphism is defined as the existing differences between individuals of different genders in features other than gender itself[84]. Sexual dimorphism in humans involves differences observed in reproductive organs, the hormonal system, basic physical traits - height, weight, and the composition of blood cells, also the divergences of the cognitive system resulting from the deviations in aspects related to the morphology and function of the brain. So, it is natural to accept the fact that sexual dimorphism is distinctive in immune responses as well. It is widely believed and proved that females are more immunocompetent than males in response to infectious diseases and cancers - the risks of men dying from malignant cancers are twice as high as women[85], while females are likely to be more vulnerable regarding autoimmune disorders compared to males - 80% of patients with autoimmune disorders are females[86]. For a long time, this tendency was considered to be the outcome of the influence by hormones, especially by testosterone, the major male sex hormone that was regarded as immunosuppressive. More studies have been done to investigate the interactions between testosterone and white blood cells, and it was shown that the administration of high doses of testosterone did show slight constraining effect on the proliferation of lymphocytes in rats, however, the overall interpretation with lymphocytes remains a mixed one[86]. Clearly, more associated mechanisms need to be clarified.

From the prospects of innate immunity in humans, some sex differences may be germline encoded. To appreciate this fact better, knowledge about sex chromosomes which decide our fates either be a male or female is important. In most mammals including humans, females normally have two sex chromosomes of the same type - XX, while males generally have two sex chromosomes of different kinds - XY. Many genes on the X chromosome are involved in the modulating of immune responses. The final protein products of these genes range from PRRs including TLR7 and TLR8 to receptors for cytokines, such as IL2RG, a subunit can be seen in the receptors for several interleukins, IL-3 receptor-α chain, and IL13RA2, a subunit for the IL-13 receptor, and also transcriptional factors, like FOXP3. The human X chromosome carries over 1100 identified genes, comprising nearly 5% of the human genome. On the other hand, the human Y chromosome holds roughly 100 genes, in which the testis determining factor SRY gene and some

genes functioning as regulators for autoimmune disorders and infectious diseases are included. As a result, X-linked genes have more determining influences in driving different immune responses between sexes. As females contain two copies of the X chromosome and to avoid the possibilities of double level of the X-related genes, one copy of the X chromosomes has to be silenced to guarantee that only a singular copy operates in each sex. This process of inactivation is performed by the X-inactive specific transcript (XIST) gene. An estimated 15% of X genes in humans manage to escape X inactivation and accordingly these genes see a higher copy number in females compared to males. As to those X-linked genes which are destined to be inactivated, the procedure is conducted in a random manner, either the copies from the maternal X chromosome or paternal X chromosome, giving rise to a mosaic pattern in female X chromosomes, not in the males[85)]. So concluding from these findings, it is not surprising to observe that TLR7 whose gene is coded on the X chromosome has a higher expression in females and in vitro, exposing the peripheral blood mononuclear cells (PBMCs) to TLR7 ligands induces higher secretion of IFN-α in cells from females than males[85)87)]. However, concerning the genes not coded on sex chromosomes, situations are various. In human, neutrophils from males have elevated expressions of TLR4 and produce more TNF than those from females. Males also have a larger number in NK cells compared to females. Nonetheless, neutrophils and macrophages from females show enhanced phagocytic activities and antigen-presenting cells are also more capable of antigen presentations in females[85)].

In adaptive immunity, though males have increased numbers of CD8 T cells, females have higher levels of CD4 T cells and higher CD4/CD8 ratios[85)]. In addition, cytotoxic T cell activities are proved to be higher in females compared to males[85)]. Females always present more efficient secondary immune responses than males including the reactions caused by vaccines. It has been demonstrated that females administrated with a half amount influenza vaccine can attain the same level of antibodies as the males who are vaccinated with a full dose vaccine, which is corresponding with the fact that irrespective of age, females always have greater numbers of B cells and increased titres of immunoglobulins[85)].

As stated above, though generally androgens (dihydrotestosterone and testosterone) show immunosuppressive traits, the characterisation of their functions needs further careful investigation. In females, oestrogen broadly shows encouraging effects on the differentiation of immune cells and boosting impacts on immunity[85)]. However, another essential female sex hormone - progesterone, has distinct anti-inflammatory functions including down-regulating the activation of MAPK and NF-κB[85)88)]. Besides hormones, other factors like microbiota and nutritions also have important roles in immune responses and can display a huge

variety between sexes and even individuals. Immune responses also differ between males and females at different ages. As a fetus, males are more inclined to have less flexibility responding to the surrounding stresses and tend to be more inflamed compared to females. More details related to the sexual dimorphism in the placenta will be stated in the next subsection. During toddler years, males develop their immunity exponentially and show more eminent pro-inflammatory responses than females. This trend continues until puberty. After then, females in general show stronger pro-inflammatory reactions than males through their lives. Lastly, males and females also see their immunities behave in different ways in different medical conditions. Although it is commonly recognised and verified that females are more immunocompetent in infections, women are more likely to suffer acquired immune deficiency syndromes (AIDS) from HIV compared to men who have the same viral loads, despite the fact that women have more energetic activities of CD 8 T cells when compared with men having the same viral loads[85]. Higher rates of deaths are detected in women infected with influenza A viruses while males are more exposed to these viruses[85]. In contrast, men have higher mortalities for Ebola and Middle East Respiratory Syndrome (MERS)[85]. During the current pandemic caused by Coronavirus (COVID-19), males have also borne more cases of critical conditions and suffered more deaths than females[89]. More scrutiny is demanded in identifying the reasons for this beyond the factors of life habits such as smoking and drinking and underlying conditions including obesity and others.

② Sexual dimorphism in placentae

Firstly, from the genome and gene levels, different gene expressions between sexes can be detected cross the placenta and these genes play central roles in the development of the placenta and the maintenance of pregnancy. As in other human tissues, genes demonstrating sex differences are not only coded on sex chromosomes but also on autosomes. In fact, from studies, more than 60% of the genes showing sex-biased expressions were found to be coded on autosomes. Interestingly, many of the genes that were found to be sex-biased in the placenta have not been recognised as sex-biased in other tissues, such as blood, liver, or brain, implying the originality of the sexual dimorphism in the placenta. For genes coded on sex chromosomes, those which were discovered to have higher expressions in female fetus placentae were vastly the results of the escape from X chromosome inactivation and these genes coded on X chromosomes mainly have regulatory functions including dephosphorylation and histone demethylation. For genes coded on autosomes, female fetus placentae have higher expressions of the genes of hCG and luteinising hormone (LH), both contributing to the expansion and vasculogenesis of the placenta[90]. Female fetus placentae also display enhanced expressions of immune responses related genes. These are JAK1, a critical

regulator in IFN signalling pathway; IL2RB, a subunit for IL2 receptor; latent TGF-β binding protein and clusterin, a protein responsible for the clearance of apoptosis and cellular debris[91]; IL1RL1 (IL1 receptor, type1) and CXCL1(Chemokine ligand 1). The higher expressions of these genes in female placentae reflect the fact that female fetuses are generally more immunocompetent in response to various surrounding stimuli including infections[92]. From these findings, another noteworthy aspect is female fetus placentae are more devoted to extra-embryonic tissue growth, from the development of the placenta, the vascularisation of the placenta to the immune construction of the placenta, while the male fetus placentae are more committed to the growth of the fetus itself[90]. This matches with the observation that males often have greater growth in utero than females even if the weight of the placentae are the same[93], and this was confirmed to be independent of the influence from androgens in mice[94]. This tendency has been verified from a different approach by observing the fetuses in pregnant women with chronic asthma. Female fetuses repress their growth to adapt to the unfavourable environment induced by chronic maternal asthma and manage their growth at a level not to have intrauterine growth restriction (IUGR). On the contrary, male fetuses develop normally even in the environment of chronic maternal asthma and only when the asthma encounters an acute worsening, their growth will be restricted, which can lead to severe outcomes including IUGR, premature birth or even stillbirth[95].

In pregnancy-related conditions, such as in preeclampsia, though female fetuses tend to have more preeclampsia cases than males, male fetuses are more likely to have severe preeclampsia events. In severe preeclampsia, male fetus placentae were found to have more dead cells, higher expressions of pro-apoptotic markers and inflammatory components including NFκB p65 with lower angiogenic factors such as VEGF compared to female fetus placentae[96]. For the markers to detect and monitor potential preeclampsia, female fetus placentae have increased soluble fms-like tyrosine kinase 1 (sFlt-1) and sFlt-1/PlGF (placental growth factor) ratios in both healthy and preeclamptic pregnancies compared to male fetus placentae[97]. Male fetuses suffer more gestational diabetes mellitus (GDM) and pregnancy induced hypertensive disorders (PIHD) compared to female fetuses[98]. Surprisingly, though female fetuses are usually considered to be more immunocompetent in dealing with infections, female fetuses are more susceptible to HIV infection from the mother than the male fetuses regardless of the maternal viral levels and delivery factors (odds ratio 2.14; 95% CI: 1.56 to 2.95). The potential underlying mechanisms for this remain unknown[99].

Hypothesis and Aims

Hypothesis

As described in the section of HBCs, as HBCs are of fetal origin, it is reasonable to consider that HBCs can demonstrate sexual dimorphism in various functions including physiological and immunological responses.

The differences between sexes observed in HBCs may have significant contributions to pregnancy-related conditions such as preeclampsia and other medical conditions occurring in pregnancy.

Aims

① Establish a methodology of isolating HBCs with high yield and purity, also consistent repeatability.

② Identify the sexual dimorphism in HBCs utilising the established methodology to isolate HBCs for proteomic analysis.

Validate the data from proteomic analysis using adequate techniques.

③ Identify the sexual dimorphism in HBCs at RNA-sequencing level and find potential pathways for explaining the roles of HBCs in placental sexual dimorphism.

Materials and Methods

Isolation of HBCs

The details for the isolation of HBCs should be referred to results chapter 1.

In brief, healthy placental samples (whole placentae) from mothers without infections and underlying medical conditions such as gestational diabetes and preeclampsia are acquired within 30 mins after caesarean sections and processed immediately. The process starts from mincing the tissues and digesting the tissues using trypsin solution, which is followed by digestion in collagenase solution (please refer to appendix 1 for the details of the contents for trypsin solution and collagenase solution). Tissues are centrifuged and obtained cells are loaded on to the Percoll gradient or Ficoll/lymphoprep and centrifuged further. To obtain Hofbauer cells with a high yield and purity, either positive selection or negative depletion is applied.

Mass Spectrometry Analysis

Mass spectrometry analysis was used to identify the sexual differences in Hofbauer cells at protein levels. Details for mass spectrometry analysis and data analysis should be referred to results chapter 2.

In summary, samples were run on the Thermo Fusion Lumos mass spectrometry which was coupled to a Dionex Ultimate3000 LC system. Relative intensity value was recorded for each ionised peptide, and data was analysed using Maxquant (version 1.6.3.3) searching against the human database based on default settings, protease trypsin, carbamidomethylation of cysteines as fixed modifications, and oxidation of methionines , acetylation of the protein N-term as variable modifications. Peptide and protein FDR (false discovery rate) were set to be 1%, which is a commonly accepted value. Then for the quantification for individual protein, label free quantification was performed. Quantitative analysis was performed and the values obtained for each protein were transformed into log2 form, and the median was subtracted. Finally, to compare the differences between HBCs from males and females, student t-test was applied.

Western Blotting

Western blotting was applied to validate the data obtained from mass spectrometry analysis.

For the details for preparation of protein lysates and procedures of western blots, please refer to appendix 2 and 3.

Next-generation sequencing

To identify the sexual differences between male and female Hofbauer cells at RNA levels, next-generation sequencing was applied.

Please refer to appendix 4 for the details of preparation of RNA samples and results chapter 3 for the details of next-generation sequencing.

To identify the signalling pathways between differentially expressed genes acquired from the sequencing data, results from RNA-sequencing data were applied to KEGG (Kyoto Encyclopedia of Genes and Genomics) by comparing all significant genes (adjusted p value<0.05) to all the genes obtained in the analysis and pathways overrepresented were identified.

Results Chapter 1

Establishing HBC isolation methodology

Background

Pure cell isolation is essential for cell line culture and further cell analysis. Identifying the differences existing in the expressions of proteins and RNA transcriptomes, DNA genomes between different kinds of cells are key entries in resolving the questions for diversified mechanisms behind in the fields of science, especially in immunology, developmental and stem cell biology, and oncology[100]. Despite its importance, the process of isolating cells can always be challenging from the prospects of techniques. A successful isolation has to guarantee a high productivity, in order to get sufficient cells for further analysis, and high purity, to avoid influences from contaminants and other cells that are not of interest. If the isolated cells are to be cultured, the recovery of the cells from the process of separation is also crucial[100][101].

Though HBCs were discovered more than 100 years ago, there is no consensus as to the optimal isolation of these cells with high yield and purity. In the process of establishing the methodology of isolating HBCs, two papers were carefully read through as references[74][102]. However, the types of enzymes used were not stated in a clear way and varied between papers. Importantly, many critical details including the steps of filtering the cells and information related to centrifugations were omitted. So, the final version of the protocol of isolating HBCs shown here has been optimised many times from the successes and failures of every experiment and has become an original and effective one.

Results

To establish the methodology of isolating and obtaining a pure population of HBCs, several trials have been made.

Trial1

Until extracting the pure HBCs

① Healthy placental samples (whole placentae) from mothers without infections and underlying medical conditions such as gestational diabetes and preeclampsia were acquired within 30 mins after caesarean sections and processed immediately. A total wet weight of 30g of placental tissue were finely minced with surgical scissors and washed with PBS until the supernatant was clear.

② After the digestion of the minced tissue with the trypsin solution for 15 to 20 minutes, tissue was further digested in the collagenase D solution for 1.5 to 2 hours to generate a single cell suspension.

Please refer to the Appendix 1 for the details of the contents for trypsin solution and collagenase D solution.

③ Obtained cells were loaded onto a discontinuous Percoll gradient (40%/20%) and centrifuged for 20 minutes at 400g without brake.

④ Cells from the 40%/20% Percoll interface were washed with RPMI medium to remove Percoll and reloaded onto a second discontinuous Percoll gradient (35%/ 30%/25%/20%) and centrifuged for 30 minutes, at 400g without brake.

⑤ Cells from 20%/25% to 30%/35% interfaces were combined and washed to remove Percoll.

Obtaining pure HBCs – positive selection by MS (Magnetic Separation) columns

⑥ Cells were filtered using 70µm strainer and were washed with ice cold MACS buffer.

⑦ Cells were resuspended in MACS buffer at a concentration of 10 million/100µl. Then cells were incubated with CD163-Biotin antibody for 10 minutes at 4°C.

⑧ Cells were washed with MACS buffer to remove the unbounded antibodies and resuspended in MACS buffer at a concentration of 10 million/90µl.

⑨ Cells were incubated with streptavidin micro-beads at a concentration of 10 million/10µl for 15 minutes at 4°C.

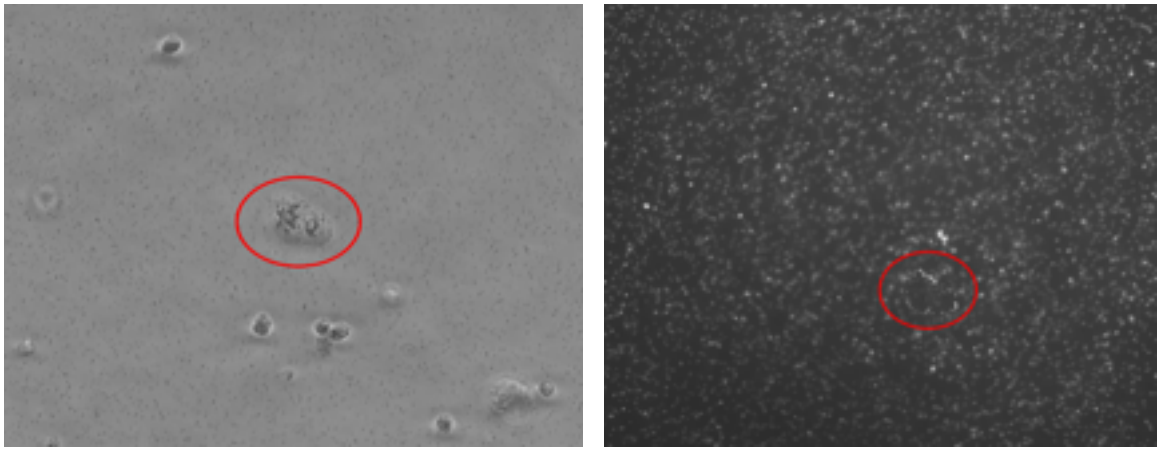
⑩ Cells were washed with MACS buffer and resuspended in MACS buffer at a concentration of 10 million/500µl.

⑪ The column was washed three times using MACS buffer and then cells were loaded onto the MS column placed in a Miltenyi Mini magnet.

⑫ After finishing loading the cells, the column was washed three times and cells were collected using the plunger after the positive selection.

The images of HBCs obtained by this methodology.

Figure 1



(A) Bright Field (x20) (B) Fluorescent Field (x20)

Figure1: Several HBCs can be confirmed in one field with one large HBC circled in red line (A). Beads (latex beads coated with fluorescently-labelled rabbit IgG) can be located to have been phagocytosed into the HBCs 2 hours after the beads were supplied. One HBC with multiple beads inside is circled in red line (B).

Overview for trial 1

Although a small quantity of HBCs were able to be acquired using the methodology from trail 1, digestions were incomplete and not efficient enough to get a sufficient amount of single HBCs for further analysis. Secondly, preparations for the discontinuous Percoll gradient were tedious and difficult in spite of that the outcomes of separations by the discontinuous Percoll gradient were not always clear. Furthermore, the cell numbers obtained by this methodology were limited since the large sizes of HBCs resulted in them getting stuck in the MS columns.

Trial2

Until extracting the pure HBCs

① The selections of placental samples were with the same criteria with the trial 1.

② After the digestion of the minced tissue with the trypsin solution for 15 to 20 minutes, tissue was further digested in the collagenase IA-S solution for 1.5 to 2 hours to generate a single cell suspension.

The contents of trypsin solution were the same with the one generated in Trail 1.

The contents of collagenase IA-S solution were replacing collagenase D with collagenase IA-S but maintained the same concentrations. Other contents in the collagenase IA-S solution remained the same with the one created in the trial 1.

Following the digestion in the collagenase IA-S solution, the tissue was centrifuged for 30s at 300g and passed through a 100µm sieve to separate cells from undigested solid tissues.

③ Obtained cells were loaded on to the Ficoll/lymphoprep and centrifuged for 20 minutes at 400g without brake.

Obtaining pure HBCs – by depletion of contaminating cells using anti-EGFR, anti-CD10 antibodies

④ Antibody conjugated beads were prepared by incubating mouse anti-human EGFR and mouse anti-human CD10 antibody respectively with goat anti-mouse IgG antibody conjugated Dynabeads at a concentration of 1µg antibody to 1mg beads for 1 hour.

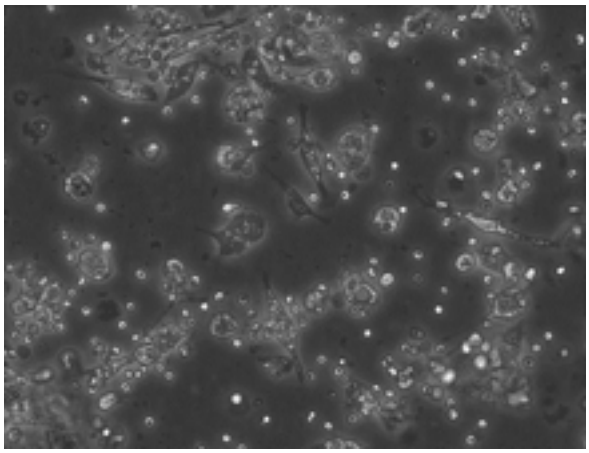
⑤ Beads were then exposed to a magnet (DYNAL Magnetic Particle Concentrator-M) and washed three times to remove unbounded antibodies. Antibody conjugated Dynabeads were resuspended using MACS buffer.

⑥ After ③, cells were collected and washed. Antibody conjugated beads were resuspended and added to the population of cells at a bead-to-cell ratio of 30:1. Cells with beads were incubated for 30 minutes at 4°C under gentle tilting and rotating.

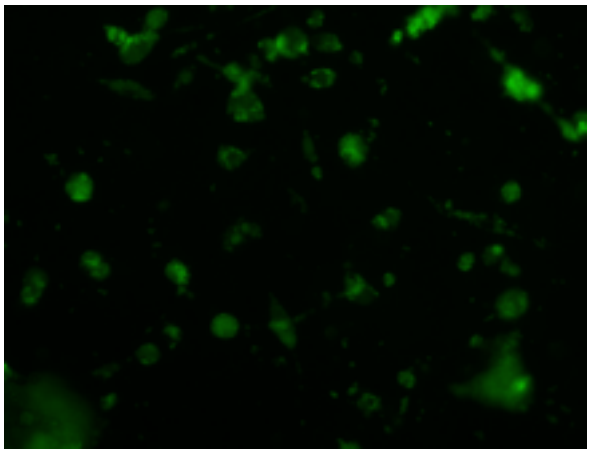
⑦ The tube containing the cells was placed in a magnet (DynaMagTM-50) for 10 minutes and the supernatant was transferred to a new tube.

The images of HBCs obtained by this methodology.

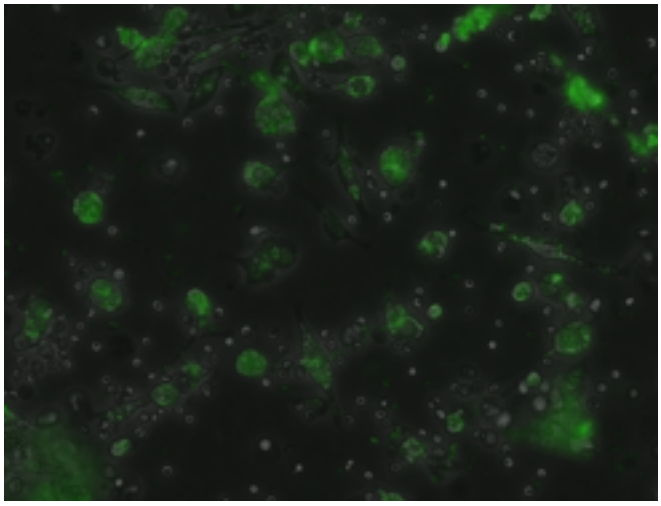
Figure 2



(A) Bright Field (x20)



(B) Fluorescent Field (x20)



(C)Merged image (x20)

Figure2: More HBCs can be identified in one field compared to figure 1 along with various other types of cells, such as endothelial cells (A). HBCs demonstrated phagocytic ability after the beads (latex beads coated with fluorescently-labelled rabbit IgG) were supplied (B). When image (A) and image (B) were combined, it is clear that the number of HBCs obtained was satisfactory although the purity of HBCs was not good enough to pursue further analysis (C).

Overview for trial 2

To correct the insufficiency of digestions, for the collagenase solution, collagenase IA-S was substituted for collagenase D. Though collagenase D and collagenase IA-S are both produced from the anaerobic bacteria *Clostridium histolyticum*, they prove different effects on digesting the connective tissues. In this project, both types of collagenases were the products from MERCK®. However, as the compositions of each type of collagenase were almost identical between companies, here, the explanations related to the specific functions of each collagenase were cited from Worthington Biochemical Corporation® as more information were available. Collagenase IA-S belongs to type 1 collagenase and has balanced enzymatic activities from various digestive enzymes including collagenase, clostripain (a proteinase), caseinase and trypsin. Contrarily, collagenase D is a type 4 collagenase designed to have extremely low tryptic activity to restrain the damage to membrane proteins and receptors[103]. Considering HBCs are located in the midst of the connective tissues in the villi, it was crucial to choose the collagenase with higher tryptic activity to break down the surrounding layers. The necessity for this transition was evident in trial 1 and the correctness of it has been validated in trial 2.

Instead of the discontinuous Percoll gradient in trial 1, Ficoll/lymphoprep was adopted. Percoll and Ficoll are both density gradient media utilised for separation of

cells. Percoll comprises colloidal silica particles (15-30 nm diameter)[104] and has a density of 1.131 g/cm^3. Continuous or discontinuous Percoll gradient can be adjusted from the original density. On the other hand, Ficoll, composed of polysaccharides with high molecular weights, has been especially preferred for separating lymphocytes from other components in blood[104]. Its density is 1.077g/cm^3 and usually unsuitable for remodifying. As stated above, preparations for the discontinuous Percoll gradient are complicated. In addition to this, for the comparisons of the efficiency of Percoll and Ficoll, experiments aimed at isolations of human mesenchymal stem cells have demonstrated that significantly higher numbers of cells were obtained with Ficoll compared with those with Percoll and the colony-forming efficiency was also higher in Ficoll[104]. This result was corresponding with the isolation of HBCs as well, with Ficoll resulting in higher numbers of HBCs.

From trial 1, it has been observed that micro-beads and Miltenyi's MS columns are not suited for collecting HBCs. For trial 2, negative depletion has been applied. Extended pros and cons of positive selection and negative depletion will be discussed in the final discussion for this chapter. In general, negative depletion leaves cells of interest in a relatively unactivated condition. Anti-Epidermal growth factor receptor (EGFR) and anti-CD10 antibodies were chosen for removing contaminating cells. Anti-EGFR antibody was designed at depleting cytotrophoblasts, while anti-CD10 antibody was intended to get rid of both cytotrophoblasts and fibroblasts[102]. The purity of HBCs after depletions with these two kinds of antibodies was around 70%, at a maximum of 80%, which was not satisfactory enough to proceed to the proteomics and RNA-sequencing analysis for HBCs alone. The behind reason for this is considered as the existence of other types of cells remaining after the sequential digestions and cell separations. They are suspected to be some endothelial cells, other mesenchymal stromal cells, also cytotrophoblasts and fibroblasts whose expressions of EGFR and CD10 are not strong enough to be extracted by anti-EGFR and anti-CD10 antibodies.

Trial3

Until extracting the pure HBCs

Same with trial 2.

Obtaining pure HBCs – positive selection using anti-CD163 antibody conjugated Dynabeads

① Beads were prepared by incubating mouse anti-human CD163 antibody with goat anti-mouse IgG antibody conjugated Dynabeads for 1 hour at a concentration of 1μg antibody to 1mg beads.

② Beads were then exposed to a magnet (DYNAL Magnetic Particle Concentrator-M) and washed three times to remove unbounded antibodies. Antibody conjugated Dynabeads were resuspended in MACS buffer.

③ After cells were collected from Ficoll/lymphoprep and washed, cells were resuspended in RPMI 1640 medium and mixed well by pipetting. Resuspended antibody conjugated Dynabeads were added to the population of cells at a bead-to-cell ratio of 30:1.

④ Cells with beads were incubated for 30 minutes at 4°C under gentle tilting and rotating.

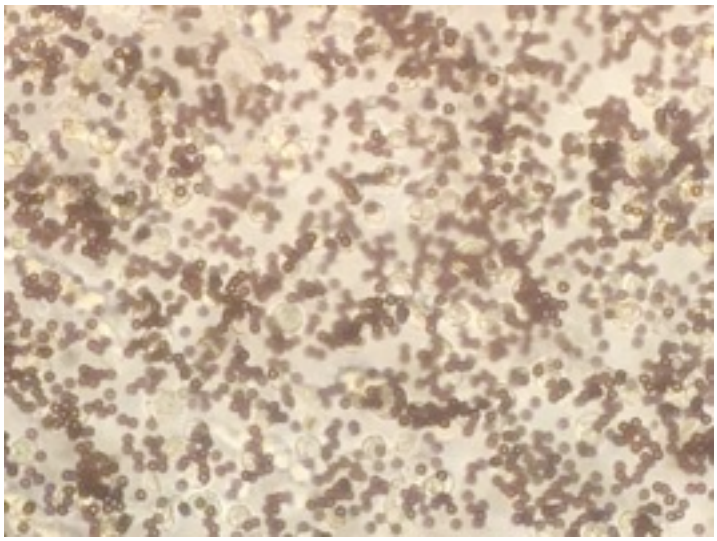
⑤ To prevent HBCs from phagocytosing antibody conjugated Dynabeads, magnet (DynaMagTM-50) was placed in ice and the tube containing cells was put into the magnet and kept for 10 minutes.

⑥ The supernatant in the tube was discarded. The tube was removed from the magnet quickly and cold PBS was added. The contents in the tube were well mixed by vortex. The tube was placed back into the magnet and held for another 10 minutes.

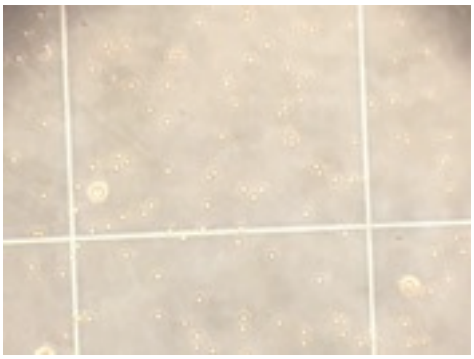
⑦ The supernatant was discarded again. The tube containing cells attached with anti-CD163 antibody conjugated Dynabeads were collected and counted.

The images of HBCs obtained by this methodology.

Figure3



(A) Bright Field (x20)



(B) Bright Field (x20)

Figure3: HBCs are attached with brown-coloured anti-CD163 antibody conjugated Dynabeads. The purity of HBCs is high (>90%), with few contaminating cells can

be detected(A). Placed on the haemocytometer, only HBCs can be identified in the field(B).

Overview for trial 3

From trial 2, it has been verified that due to the diversity of cells existing in the placental tissue, it is extremely unlikely to get pure populations of HBCs with the aid of antibody conjugated beads to deplete other major kinds of cells, such as fibroblasts and cytotrophoblasts. For the positive selection of HBCs, CD163 has been chosen as a stable marker for HBCs in trial 1 and trial 3. As stated in the section of Hofbauer cells, especially in the subdivision - from its discovery to identification of its multifaceted roles, though CD68 is generally regarded as a traditional marker for mononuclear phagocyte lineage cells represented by monocytes, macrophages, osteoclasts, and tissue macrophages including Kupffer cells[105], regarding HBCs, the expressions of CD68 on HBCs reach their peak by the second trimester and substantially diminish as pregnancy proceeds. In contrast, the expressions of CD163 on HBCs are consistently maintained in all subsets of HBCs through pregnancy[65], making them suitable markers for identifying HBCs.

The cell numbers obtained by the methodology established in trial 3 were at least 5 millions from 30g of wet placental tissue (ranging from 5 million to 15 million), which was enough to progress to further analysis.

Final discussion for results chapter 1

It took almost 8 months to go through every iteration to establish the final methodology of isolating HBCs from the whole placenta. Even from the very beginning, as to which side of the placenta should be chosen to collect the wet placental tissue, several times of investigations were required. Though HBCs are of fetal origin[63](as described in the section of `Hofbauer cells'), more HBCs were acquired from the tissue collected from the maternal side of the placenta than from the fetal side. This sustains the fact that HBCs are villous macrophages, predominantly located in the cores of the villi and functioning as macrophages at the maternal-fetal interface in the placenta.

For the sequential enzymatic digestions, though digestive enzymes, such as collagenase, DNAase and trypsin, are usually utilised for isolating single cells from solid tissues, the exact types of these enzymes, the concentrations of these enzymes and the time period of exposing the cells to these enzymes are pivotal factors for successful cell isolations. Incorrect kinds or concentrations of enzymes and inadequate time of the exposure to enzymes can cause incomplete digestion or excessive digestion which can lead the cells to be incapacitated or even to die, as these enzymes digest not only the extracellular matrix but also the important proteins that the cells of interest have[106]. Although it was not dictated in this chapter, there were multiple times of trials made to manage without the initial digestion in the trypsin solution as it was feared that trypsin can damage the proteins of HBCs. However, those trials ended in failures in getting single cell suspensions. The reason for this is considered to be the connective tissue cells within the placental stroma which are extraordinarily strengthened because they are of heterogeneous origins, creating various connective tissue fibres to improve the physical stableness of the villous core[107]. As a result, to obtain HBCs which are positioned in the cores of the villi, it was necessary to use the trypsin solution at first to break down the connective layers, which is followed by the corresponding collagenase solution also with a considerable tryptic activity as stated in the overview for trial 2.

Generally, for isolation of intensely purified cell populations, fluorescence activated cell sorting (FACS) is widely introduced[100]. Unfortunately, the access to FACS was not available all the time and as the placenta samples come at a random time frame, it was not realistic to rely on FACS to obtain pure HBCs. Magnetic-Activated Cell Sorting (MACS) was the alternative decision. For MACS, either positive selection or negative depletion is commonly adopted. Positive selection is carried out by directly extracting the cells by labelling the cells with certain surface protein. The isolated cells can be highly purified. However, they will be bound with antibody conjugated beads so will not be suitable for some further analysis such as by flow cytometry. In

addition, the cells are considered to be activated to some degree through the process. Consequently, cells collected via positive selection are not suited to be investigated for some particular physiological functions immediately. To scrutinise the sexual differences between HBCs, the guarantee of a high purity of cells had to be prioritised. So positive selection was firstly performed in trial 1 using micro-beads and Miltenyi's MS columns. The reason that micro-beads were selected was because micro-beads are believed to activate the cells to the least level compared to other types of beads. Negative depletion was applied in trial 2. Instead of labelling the target cells, undesired cells are supposed to be pulled out by antibody conjugated beads, leaving the aimed cells untouched. Isolated cells are not bound with magnetic beads, so preferable for various further investigations. However, to gain pure populations of HBCs by negative depletion was demonstrated to be impossible in trial 2 and the details related to this should be referred to the overview for trial 2. Besides the cell types mentioned as contaminating cells in the overview for trial 2, red blood cells were another major source left after the negative depletion. They were not completely removed by cell separation using Percoll or Ficoll/lymphoprep and red blood cell lysis buffer was supplied for several times in order to get rid of them, which proved to be unsuccessful. The underlying reason for this is contemplated as there is a substantial quantity of nucleated red blood cells (nRBCs) in the fetal circulation (in the new born, the ratio of nRBCs is around 0.1% and this ratio is higher in the early stage of pregnancy[108]), and nRBC cannot be eradicated by red blood cell lysis buffer, the main composition of which is ammonium chloride.

Summarily, since obtaining pure HBCs was the first priority for planning further experiments, positive selection utilising MS columns and CD163-biotin antibody was chosen firstly. Considering the possibility of application for further functional analysis, negative depletion utilising anti-EGFR, anti-CD10 antibodies was performed as the second trial. However, as it was impossible to get pure HBCs by negative depletion (reasons are stated in the overview for trial 2), positive selection utilising anti-CD163 antibody conjugated Dynabeads was opted in the end. Among these trials, changes of digestive enzymes were also made.

Results Chapter 2

Investigating sexual differences between males and females in HBCs

1. Methodology

To investigate traits related to sexual dimorphism in HBCs, HBCs were isolated from 6 normal male placentae and 6 normal female placentae using the methodology established in the third iteration of HBC isolation as stated in the results chapter 1.

The cell numbers of HBCs obtained by each placenta ranged from 5 million to 15 million from 30g wet placental tissue. The purity of HBCs acquired were >90% determined by microscopic observation.

Table1 shows clinical characteristics of the mothers who provided placentae. Data are expressed as mean±SEM (standard error of the mean). BMI stands for body mass index.

Table1

	Male placentae (n=6)	**Female placentae** (n=6)
Maternal Age (year)	35.8±1.8	35.8±1.7
Gestational Age (week)	38.6±0.3	39.0±0.1
BMI (kg/m^2)	28.7±2.2	25.8±1.2
Birth Weight (g)	3637.5±133.2	3291.8±183.6

Cells (1 million cells from each sample) obtained were further subjected to mass spectrometry analysis.

Mass Spectrometry Analysis

This was performed at the Target Discovery Institute, who analysed these samples.

Principle of Mass Spectrometry and the methodology for data analysis

Briefly, MS is an important tool contributing to proteomics, a key discipline in science aiming to disclose cellular roles at protein levels. Utilising MS for proteomics has become more and more well-recognised and paramount in identifying complicated protein samples[109] .The basic principle of MS is to separate electrically charged species (ions) in the gas phase. So in the process operated by MS, samples have to be transferred into multiple ionised fractions. This is usually done by the equipped ion source in MS devices. Ionisation converts the solid or liquid targeted sample into the gas phase[110]. Successively, these ions are

transported into the mass analyser, where the mass-to-charge ratio (m/z) of the ionised peptides will be measured. In most MS, the ions generated are positive ions. So the m/z of a cation in MS is equivalent to the mass of the cation divided by its charge, and as the charge of a cation created in MS is basically always 1, the m/z of a cation is normally equal to the mass of the cation[111)]. The mass analyser sorts the ions by their m/z, deflecting the ions through a magnetic field. The outcomes of deflections depend on the m/z of the ions, with lighter ions influenced more than heavier ions. This will raise different outflows of ions according to their weights. And the ion detector will record the number of ions at every m/z scale by altering the force of the magnetic field. Electric signals brought out by the detector will be captured to generate the mass spectra, which delivers the information of the number of ions at each m/z value in the form of histogram[109)110)].

In this project, samples were run on the Thermo Fusion Lumos mass spectrometry which was coupled to a Dionex Ultimate3000 LC system. Relative intensity value was recorded for each ionised peptide, and data was analysed using Maxquant (version 1.6.3.3) searching against the human database based on default settings, protease trypsin, carbamidomethylation of cysteines as fixed modifications, and oxidation of methionines , acetylation of the protein N-term as variable modifications. Peptide and protein FDR (false discovery rate) were set to be 1%, which is a commonly accepted value. Through this process, acquired spectra were matched with the theoretical spectra including the exact fitness between them. Then for the quantification for individual protein, label free quantification was performed.

Quantitative analysis was performed and the values obtained for each protein were transformed into log2 form, and the median was subtracted. Finally, to compare the differences between HBCs from males and females, student t-test was applied.

Data

Data was returned as an Excel file which contained the following parameters.

Data was analysed by comparing log2 fold change.

2 Results

Figure 1 shows the Volcano plot data indicating the differential expression of proteins in HBCs between males and females.

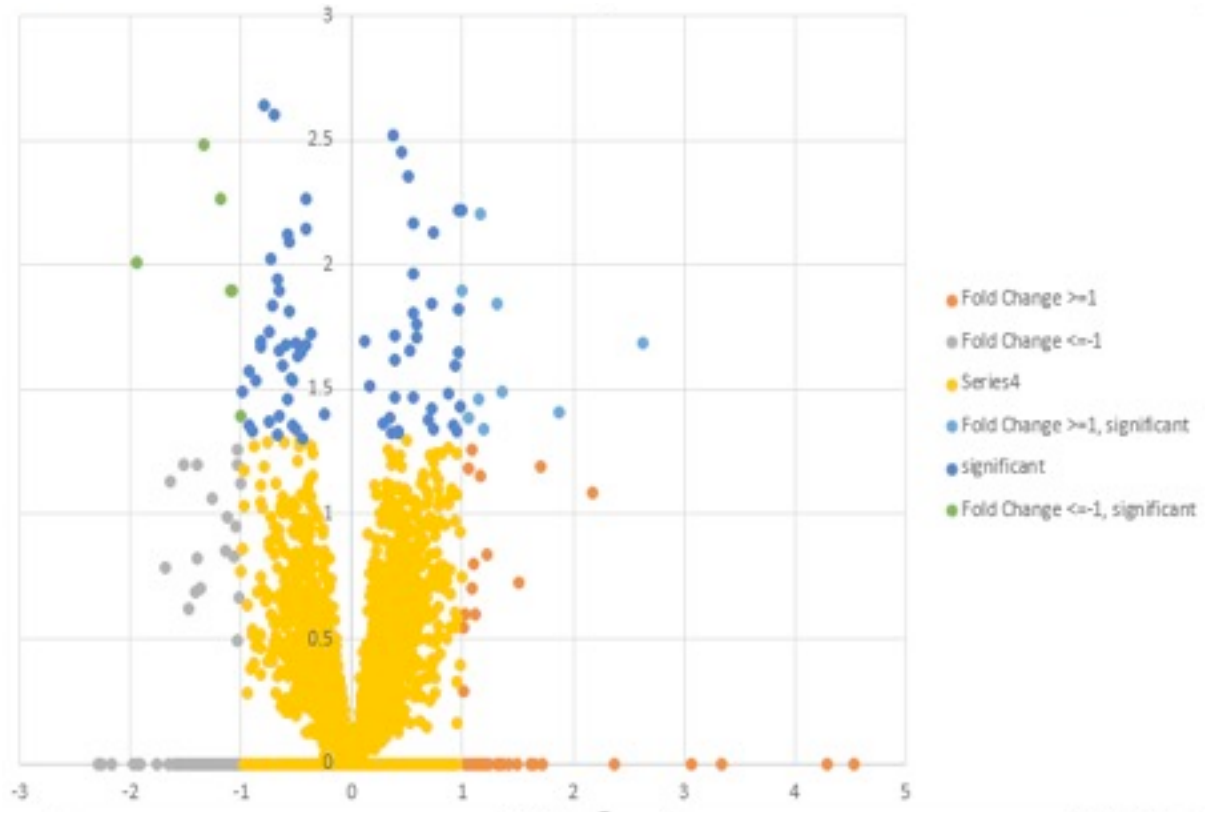


Figure1: Of 5426 proteins, 86 proteins were found to be significantly differentially expressed between males and females ($p<0.05$).

X axis: log2 fold changes Y axis: -log10(p value).

10 proteins (marked as light blue 🔵) were highly expressed in male placentae ($p<0.05$) with a log2 fold change ≥1.

5 proteins (marked as dark green 🟢)were highly expressed in female placentae ($p<0.05$) with a log2 fold change ≥1.

Another 71 proteins which were differentially expressed between male and female HBCs ($p<0.05$) with a log2 fold change between 0 and 1 are labelled as dark blue 🔵.

For all 86 proteins which were significantly differentially expressed between males and females in HBCs, to examine the potential signalling pathways between them, these proteins were input to the biological database STRING® for analysis.

Figure 2 shows the pathways identified between them (proteins are represented by their gene names).



Figure2: Significantly differentially expressed proteins were analysed by STRING®.

There were four main pathways with respective functions that were detected.

① Pathways related to immune functions

Figure 3 shows the pathways (enclosed with blue line) where all the proteins are involved in immune functions.

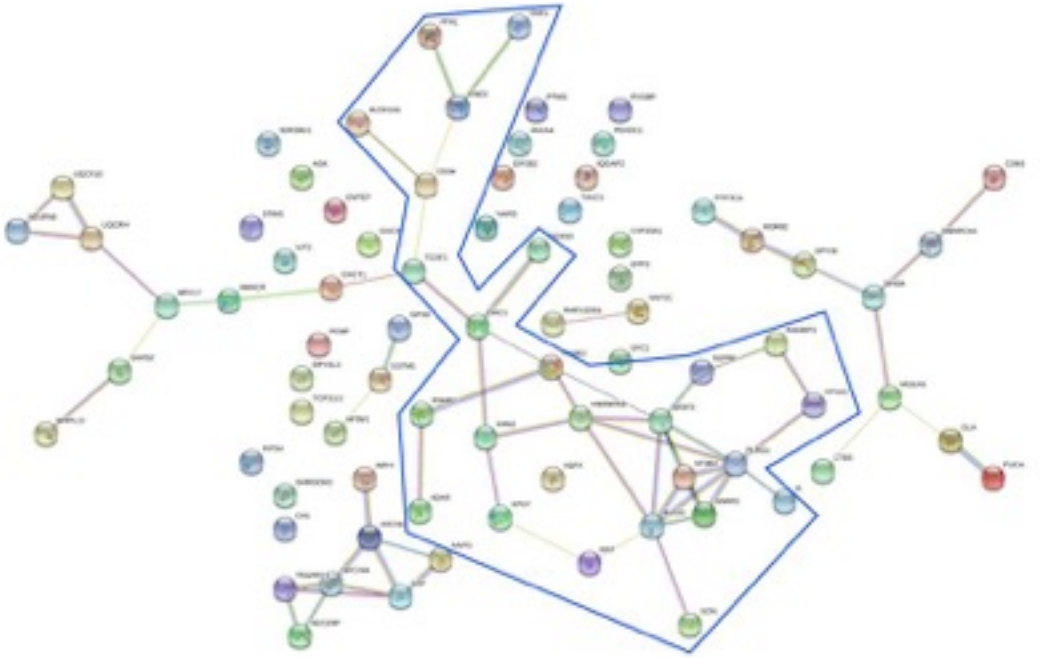


Figure3: 25 proteins were discovered to be engaged in immune responses. Of 25 proteins, 7 proteins were found to be significantly highly expressed in male HBCs, and 18 proteins were found to be significantly highly expressed in female HBCs.

Table2 shows the names of proteins and their gene names involved in immune function that were significantly highly expressed in male HBCs with the fold changes (compared to female HBCs) and p values.

Table2

Name of Protein (Gene Name)	Fold Change	P value
ATP-dependent 6-phosphofructokinase, liver type (PFKL)	1.34	0.046
1,4-alpha-glucan-branching enzyme (GBE1)	1.50	0.017
Retinal dehydrogenase 1 (ALDH1A1)	6.18	0.021
Exportin-7 (XPO7)	1.91	0.025

Name of Protein (Gene Name)	Fold Change	P value
NAD(P) transhydrogenase, mitochondrial (NNT)	2.00	0.013
Proteasome activator complex subunit 3 (PSME3)	1.43	0.004
Proteasome subunit beta type-2 (PSMB2)	1.39	0.0035

Table3 shows the names of proteins and their gene names involved in immune function which were significantly highly expressed in female HBCs with the fold changes (compared to male HBCs) and p values.

Table3

Name of Protein (Gene Name)	Fold Change	P value
Enolase2 (ENO2)	1.90	0.044
Haematopoietic progenitor cell antigen CD34 (CD34)	2.14	0.013
Treacle protein (TCOF1)	1.59	0.048
H/ACA ribonucleoprotein complex subunit 4 (DKC1)	1.45	0.044
ATP-dependent RNA helicase DDX50 (DDX50)	1.64	0.014
5-3 exoribonuclease 2 (XRN2)	1.19	0.040
Protein BUD31 homolog (BUD31)	1.58	0.013

Name of Protein (Gene Name)	Fold Change	P value
U1 small nuclear ribonucleoprotein C (SNRPC)	1.60	0.012
Splicing factor 3B subunit 4 (SF3B4)	1.42	0.046
Serine/arginine-rich splicing factor 3 (SRSF3)	1.33	0.021
Nuclear pore complex protein Nup88 (NUP88)	1.50	0.035
RAN-binding protein 3 (RANBP3)	2.27	0.005
Importin subunit alpha-1 (KPNA2)	1.57	0.022
Pleiotropic regulator 1 (PLRG1)	1.47	0.015
Heterogeneous nuclear ribonucleoprotein A/B (HNRNPAB)	1.41	0.021
Protein Red (IK)	1.41	0.023
Double-stranded RNA-specific adenosine deaminase (ADAR)	1.33	0.005
Protein SON (SON)	1.37	0.0498

Important functions of the proteins identified and their potential roles in pathways will be discussed later in this chapter. This will apply for other proteins involved in other pathways.

② Pathways related to mitochondrial functions

Figure 4 shows the pathways (enclosed with pink line) where all the proteins are involved in mitochondrial functions.

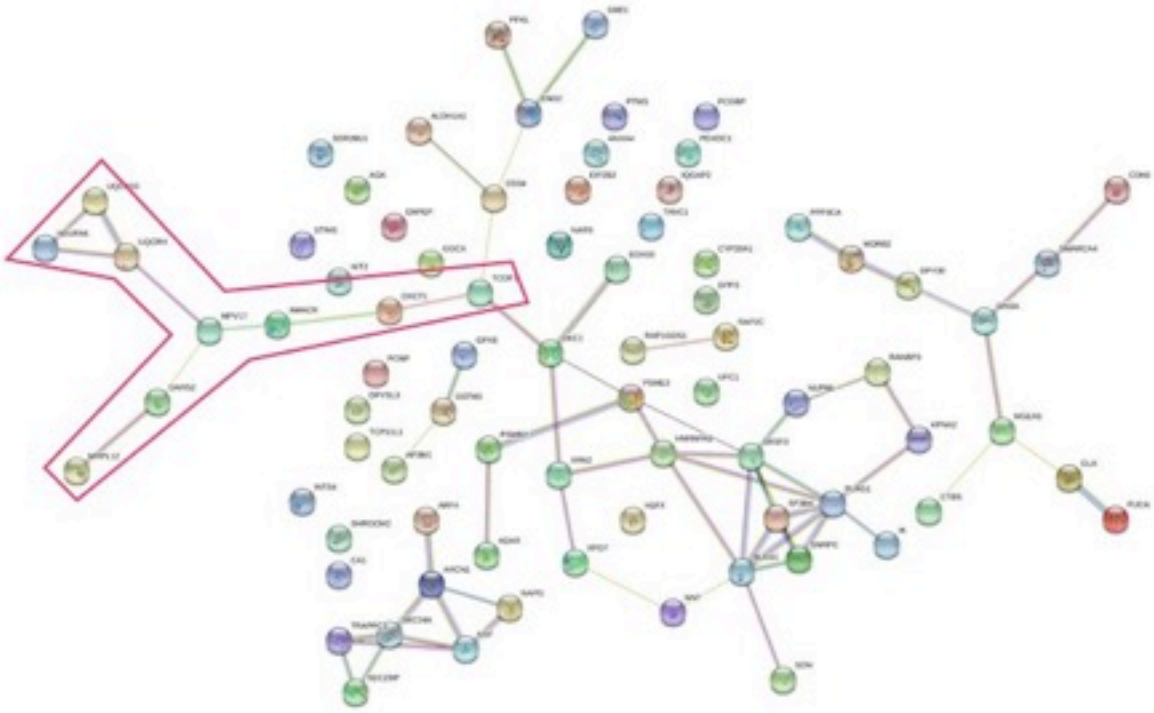


Figure4: 9 proteins were found to be engaged in mitochondrial associated responses. Of 9 proteins, 6 proteins were found to be significantly highly expressed in male HBCs, and 3 proteins were found to be significantly highly expressed in female HBCs.

Table4 shows the names of proteins and their gene names involved in mitochondrial function which were significantly highly expressed in male HBCs with the fold changes (compared to female HBCs) and p values.

Table4

Name of Protein (Gene Name)	Fold Change	P value
Cytochrome B-C1 complex subunit 9 (UQCR10)	1.94	0.022
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 (NDUFA6)	1.31	0.034
Aspartate-tRNA ligase, mitochondrial (DARS2)	1.66	0.038

Name of Protein (Gene Name)	Fold Change	P value
Protein Mpv17 (MPV17)	1.67	0.046
Alpha-methylacyl-CoA racemase (AMACR)	2.29	0.046
Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial (OXCT1)	3.64	0.039

Table5 shows the names of proteins and their gene names involved in mitochondrial function which were significantly highly expressed in female HBCs with the fold changes (compared to male HBCs) and p values.

Table5

Name of Protein (Gene Name)	Fold Change	P value
Cytochrome b-c1 complex subunit 6, mitochondrial (UQCRH)	1.54	0.025
39S ribosomal protein L12, mitochondrial (MRPL12)	1.58	0.040
Treacle protein (TCOF1)	1.59	0.048

③ Pathways related to vesicle functions

Figure 5 shows the pathways (enclosed with brown line) where all the proteins are involved in vesicle functions.

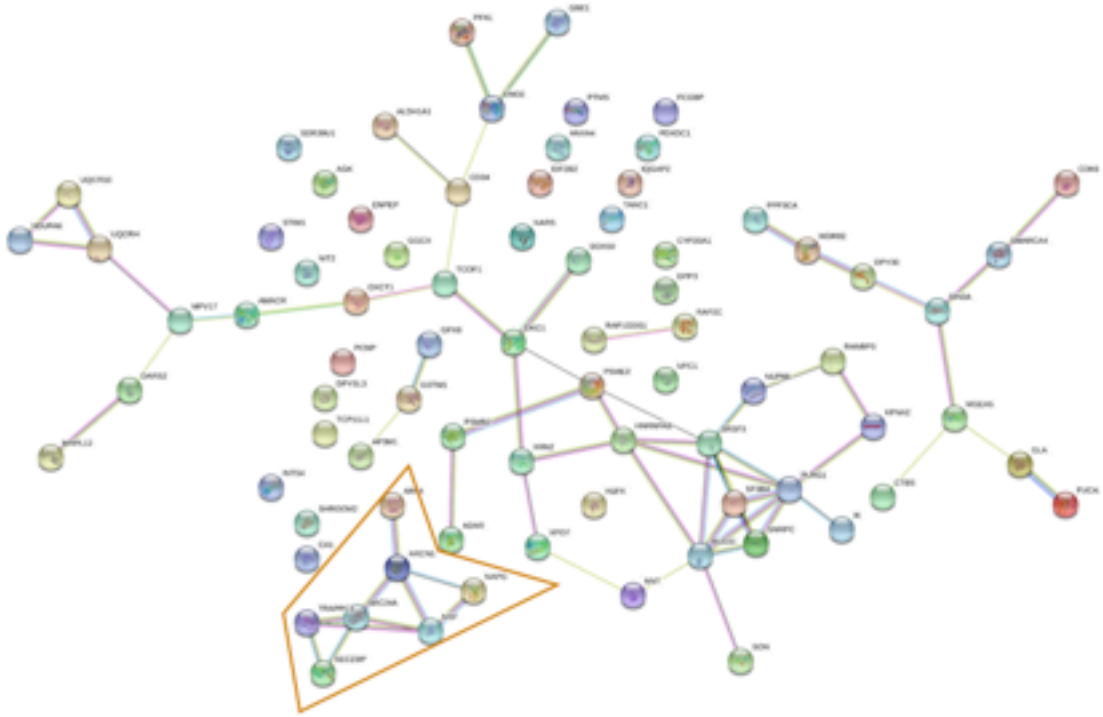


Figure5: 7 proteins were found to be engaged in vesicle related responses. Of 7 proteins, 6 proteins were found to be significantly highly expressed in male HBCs, and 1 protein was found to be significantly highly expressed in female HBCs.

Table6 shows the names of proteins and their gene names involved in vesicle function which were significantly highly expressed in male HBCs with the fold changes (compared to female HBCs) and p values.

Table6

Name of Protein (Gene Name)	Fold Change	P value
ADP-ribosylation factor 4 (ARF4)	2.08	0.041
Coatomer subunit delta (ARCN1)	1.29	0.047
Protein transport protein Sec24A (SEC24A)	1.51	0.019
Trafficking protein particle complex subunit 3 (TRAPPC3)	1.34	0.047
SEC23-interacting protein (SEC23IP)	1.47	0.007

Name of Protein (Gene Name)	Fold Change	P value
Vesicle-fusing ATPase (NSF)	1.30	0.003

Table7 shows the name of the protein and its gene name involved in vesicle function which were significantly highly expressed in female HBCs with the fold changes (compared to male HBCs) and p values.

Table7

Name of Protein (Gene Name)	Fold Change	P value
Gamma-soluble NSF attachment protein (NAPG)	1.45	0.029

④ Pathways related to transcription functions

Figure 6 shows the pathways (enclosed with grey line) where all the proteins are involved in transcription functions.

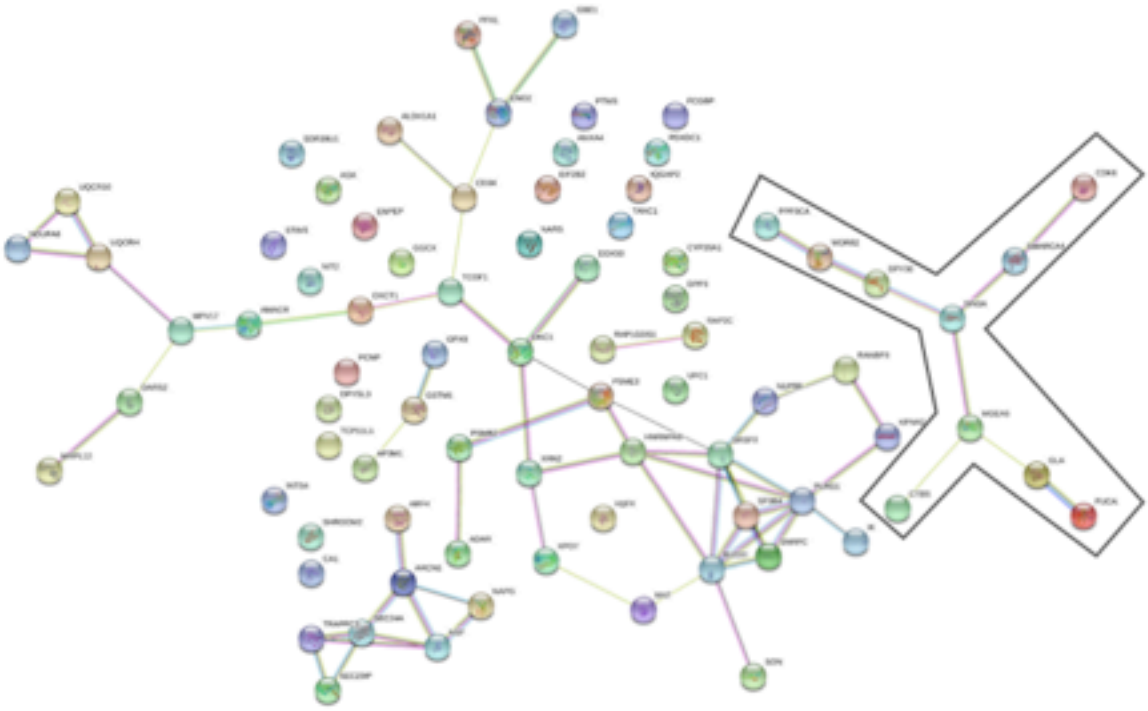


Figure6: 10 proteins were found to be engaged in transcription associated functions. Of 10 proteins, 5 proteins were found to be significantly highly expressed in male HBCs, and 5 proteins were found to be significantly highly expressed in female HBCs.

Table8 shows the names of proteins and their gene names involved in transcription function which were significantly highly expressed in male HBCs with the fold changes (compared to female HBCs) and p values.

Table8

Name of Protein (Gene Name)	Fold Change	P value
Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform;Serine/threonine-protein phosphatase (PPP3CA)	1.89	0.044
WD repeat-containing protein 82 (WDR82)	1.08	0.020
Di-N-acetylchitobiase (CTBS)	1.83	0.033
Alpha-galactosidase A (GLA)	2.23	0.006
Plasma alpha-L-fucosidase (FUCA2)	2.49	0.014

Table9 shows the names of proteins and their gene names involved in transcription function which were significantly highly expressed in female HBCs with the fold changes (compared to male HBCs) and p values.

Table9

Name of Protein (Gene Name)	Fold Change	P value
Protein dpy-30 homolog (DPY30)	1.48	0.008

Name of Protein (Gene Name)	Fold Change	P value
Paired amphipathic helix protein Sin3a (SIN3A)	1.28	0.019
Transcription activator BRG1 (SMARCA4)	1.50	0.008
Cyclin-dependent kinase 6 (CDK6)	1.87	0.047
Protein O-GlcNAcase (MGEA5)	1.52	0.021

3 Validations for results from mass spectrometry

To validate the results from mass spectrometry, another 12 normal placentae were recruited. HBCs were isolated from 6 normal male placentae and 6 normal female placentae using the methodology established in results chapter 1.

The cell numbers of HBCs obtained by each placenta were ranging from 5 million to 15 million from 30g wet placental tissue. The purity of HBCs acquired were >90% confirmed by microscopic observation.

Table10 shows clinical characteristics of the mothers who provided the placenta. Data are expressed as mean±SEM (standard error of the mean). BMI stands for body mass index.

Table10

	Male Placentae (n=6)	Female Placentae (n=6)
Maternal Age (year)	36.8±1.0	31±1.5
Gestational Age (week)	39.3±0.10	39.0±0.18
BMI (kg/m^2)	24.7±2.10	23.8±1.66
Birth Weight (g)	3447.5±196.60	3369.17±226.99

To prepare for the protein lysate, proteins from each sample were processed with lysis buffer. Please refer to the appendix 1 for the components of the lysis buffer

and the detailed procedures for preparing for protein lysates. To decide the protein concentration of each sample, a Qubit 3.0 fluorometer was utilised. Western blots were performed to detect the targeted proteins which were chosen from the pathways verified by STRING® described above. The criteria based on which the proteins were selected were mainly from the availability of the antibodies for the proteins. Please refer to the appendix 2 for the detailed methodology for western blotting.

Due to the number of wells of the gel for western blotting, three samples were randomly selected from the 6 recruited placental samples for each sex. And the cells of these 6 samples from both sexes were consistently used for validation by western blotting.

① Validation for pathways related to immune functions

Proteasome activator complex subunit 3 (PSME3) and proteasome subunit beta type-2 (PSMB2) were chosen from the pathways related to immune functions, and western blots were performed.

Image 1 shows the results of western blotting.

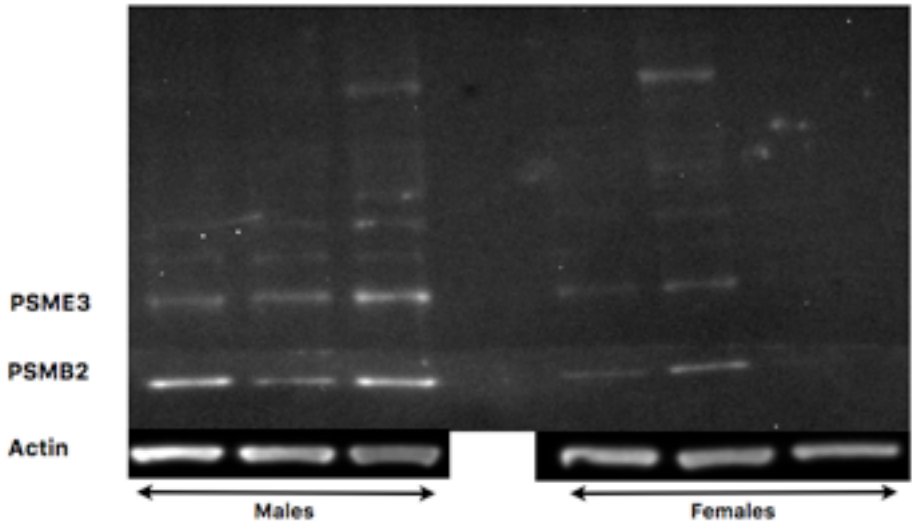


Image1: Male samples showed stronger bands compared to female samples both in PSMB2 and PSME3, which were corresponding with the results from mass spectrometry. Quantification of equal protein loading was justified by actin bands. Anti-proteasome subunit beta type 2 antibody (rabbit monoclonal, abcam®, ab166628) and anti-proteasome activator 28 (PA28=PSME3) antibody (recombinant rabbit monoclonal antibody, invitrogen®, cat no.700180) were used for the detection of targeted proteins.

② Validation for pathways related to mitochondrial functions

Treacle protein (TCOF1) was chosen from the pathways related to mitochondrial functions, and western blots were performed.

Image 2 shows the results of western blotting.

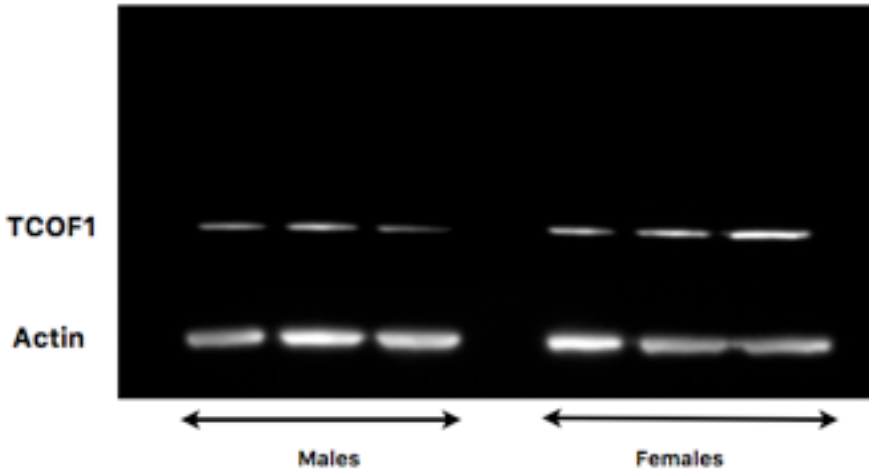


Image2: Female samples showed stronger bands compared to male samples for TCOF1, which was corresponding with the results from mass spectrometry. Equal protein loading was justified by actin bands.

Anti-TCOF1 antibody (rabbit polyclonal, abcam®, ab65212) was used for the detection of the targeted protein.

③ Validation for pathways related to vesicular functions

Protein transport protein Sec24A (SEC24A) was chosen from the pathways related to vesicle functions, and western blots were performed.

Image 3 shows the results of western blotting.

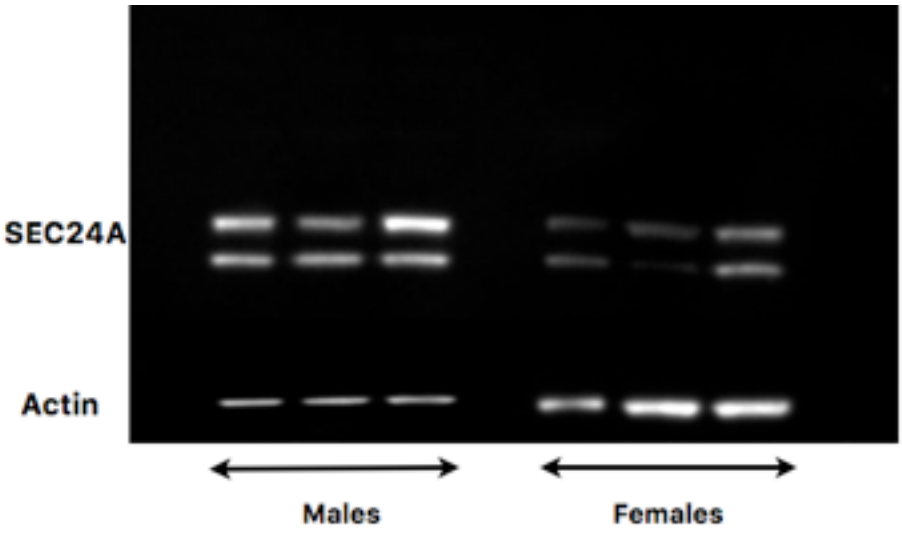


Image3: Male samples showed stronger bands compared to female samples in SEC24A, which was corresponding with the results from mass spectrometry.

The quantifications of protein loading were justified by actin bands.

Anti-SEC24A antibody (rabbit polyclonal, abcam®, ab102660) was used for the detection of the targeted protein.

④ Validation for pathways related to transcription functions

Transcription activator BRG1 (SMARCA4) was chosen from the pathways related to transcription functions, and western blots were performed.

Image 4 shows the results of western blotting.

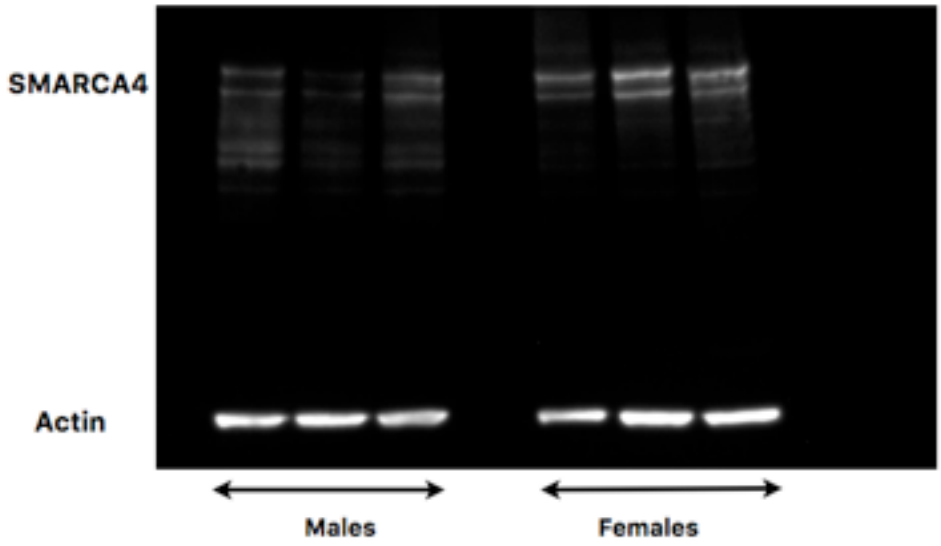


Image4: Female samples showed stronger bands compared to male samples in SMARCA4, which was corresponding with the results from mass spectrometry.

The quantifications of protein loading were justified by actin bands.

Anti-BRG1 antibody (rabbit monoclonal, abcam®, ab110641) was used for the detection of the targeted protein.

Discussion for results chapter 2

Proteins related to immune functions

For proteins involved in immune functions, 7 proteins were significantly up-regulated in male HBCs compared to female HBCs. Phosphofructokinase, liver type (PFKL) is an enzyme in charge of the conversion of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate, a crucial step in the process of glycolysis[112]. It is the

liver isotype of phosphofructokinase 1(PFK-1), an enzyme existing as a collective form combined from three main subunits - liver type (L), muscle type (M) and platelet type (P or C). The composition of PFK-1 differs between tissues, with the liver, placenta, and some other tissues predominantly having the liver type[113]. In glycolysis, most glucose and fructose will be turned into fructose 1,6-bisphosphate after their entry into the cells[114][115], and this step is irreversible. It is well known that glycolysis plays important roles in the metabolic pathways for producing energy for fundamental cell activities, however, its role in immunity is generally less well known. Actually, activation of macrophages and DCs requires the reactions of glycolysis, and pro-inflammatory stimuli can boost the glycolytic responses promoting the invigoration of macrophages and DCs. Activated macrophages have higher expression of glycolysis-related enzymes, suggesting an elevated level of glycolysis occurring in these cells. Interestingly, the products of glycolysis in activated macrophages and DCs will be metabolised to lactate via pyruvate, without pyruvate contributing to the citric acid cycle (TCA) creating oxidative phosphorylation (OXPHOS). This phenomenon which is termed as the Warburg effect was originally observed in cancerous cells, where the microenvironment prefers anaerobic glycolysis to produce energy rather than relying on the oxidative phosphorylation pathways in mitochondria as seen in most normal cells[116]. Despite the presence of ample oxygen for macrophages and DCs to execute oxidative reactions, the reason they choose glycolysis as their main energy source for activations is mitochondria has other vital work to perform. Decreased mitochondrial respiration functions as a critical part in the production of reactive oxygen species (ROS) for the host defence[117], killing bacteria in the phagosomes[118]. Another protein which was highly expressed in males, 1,4-alpha-glucan branching enzyme (GBE1), is also engaged in the metabolism of glucose. It helps generate regular α-1,6-glucosidic branches from α-1,4-linked glucose chains, increasing the solubility of the glycogen polymer which is composed of α-1,4-linked glucose units with branches of α-1,6-glucosidic bonds[119], serving as an important storage of glucose.

The other two proteins which were highly expressed in male HBCs in immune related functions - exportin-7 (XPO7) and NAD(P) transhydrogenase, mitochondrial (NNT), are proteins focused more on the movements inside the cells. Exportin-7 aids the transport of specific proteins (nuclear export sequences, NES) from the nuclei through nuclear pores to the cytoplasm by shuttling the cargos containing NES in a RanGTP-dependent manner. Among the numerous NES, signal transducer and activator of transcription 3 (STAT3) is one of the molecules whose functions are mainly regulated by exportin-7. STAT3 in the nucleus binds to DNA, modulating gene expressions. Then STAT3 is exported outside of the nucleus with the assistance of exportin-7 through the nuclear pores into the cell cytoplasm,

where it will be reactivated by kinases existing in the cytoplasm, restoring its transcriptional activity and preparing for retranslocation into the nucleus. Disruptions of the engagement between STAT3 and exportin-7 will cause the nuclear retention of STAT3, which jeopardises the downstream gene expression adjusted by STAT3[120]. Genes regulated by STAT3 are incredibly diversified, including those involved in immune functions. STAT3 works as both immune-promoter and suppressor, playing pivotal roles in the development of dendritic cells[121] and Th17 cells, while hindering macrophage inflammatory signalling[122]. On the other hand, NAD(P) transhydrogenase, mitochondrial (NNT) is an enzyme located on the inner membrane of mitochondria, mediating the reaction of transhydrogenation between NADH and $NADP^+$ to produce NADPH ($NADH + NADP^+ \rightarrow NAD^+ + NADPH$)[123]. High $NADH/NAD^+$ ratio is known as a preferable environmental factor for mitochondria to generate reactive oxygen species(ROS)[124], so from the prospects of sustaining the balance of redox and diminishing the damage to surrounding tissues by ROS, the role for NTT as an antioxidative enzyme is extremely important[123][125]. However, from the aspects of immunological defence against pathogens, ROS is indispensable and it has been demonstrated that high levels of expression of NTT in macrophages can lead to reduced levels of ROS and nitric oxide, resulting in impaired pro-inflammatory responses against LPS[126].

Retinal dehydrogenase 1 (ALDH1A1) was highly regulated in male HBCs and is known as a modulator of the retinoid signalling. ALDH1A1 is an isotype of the aldehyde dehydrogenase1 (ALDH1) proteins, catalysing the oxidation of retinal from retinol (vitamin A) to retinoic acid (RA). After the release of RA in the cytosol, RA enters into the nucleus and binds with its nuclear receptor (retinoic acid receptor alpha (RAR-α) and retinoid X receptor (RXR)), regulating the transcription of genes involved in cell differentiation, proliferation to apoptosis[127]. In immunity, RA itself works in various ways contributing to the immunological homeostasis. For immunotolerance, it induces the differentiation of naive T cells into T_{reg} cells, and prevents the differentiation of naive T cells into Th17 cells. Nonetheless, in infections and autoimmune diseases, it alleviates the functions of effector T cells, promoting the progression of immune responses[128].

The final two proteins which were significantly up-regulated in male HBCs involved in immunity are both proteasome associated proteins.

Proteasome activator complex subunit 3 (PSME3) and proteasome subunit beta type-2 (PSMB2) are subunits constituting the proteasome, a kind of multi-catalytic proteinase complex which are responsible for downgrading intracellular proteins[129][130]. The cascade starts from the labelling of the targeted proteins with ubiquitin, which functions as an initiating signal for degradation. By processing

regulatory proteins and proteins in abnormal forms, the ubiquitin-proteasome system (UPS) governs every aspect of the cellular routines, from the cell cycle, signal transduction, to quality control and expansion of proteins, metabolism and immune responses[130]. In immunity, proteasomes produce antigen peptides from intracellular pathogens, an essential part in the sequential process of presenting processed antigens via MHC class I. The efficiency of cleaving the proteins into antigenic peptides and the mechanism of presenting antigens via MHC class I are extremely important in the defence against intracellular microorganisms and tumours. The fact that male HBCs have more intense expressions of PSME3 and PSMB2 may suggest that male HBCs can produce antigens presented by MHC class I more diversely and effectively, which might explain why female fetuses are more susceptible to infection from the mother than the male fetuses (as seen in HIV[99]).

Of all 18 proteins which were highly expressed in female HBCs and engaged in immune responses, multiple proteins are involved in the procedures of editing RNAs, such as the splicing approach and pre-mRNA processing. MYC(c-MYC) is one of the oncogenes coding for transcription factors which up-regulate the expressions of various genes with versatile functions, resulting in the proliferation of cells and tumour progression. MYC-led transcription needs the conversion of pre-mRNAs to mature mRNAs, during which introns must be eliminated by core spliceosomes to secure the creation of mRNAs and their stability. Protein BUD31 homolog (BUD31), which was found to be highly modulated in female HBCs, is a type of spliceosome related protein, interacting with spliceosomes and ensuring the catalytic competence of spliceosomes. It has become clear that eliminating the function of BUD31 shows a fundamental effect in restraining the growth of MYC-dependent cells[131]. From these facts, it can be regarded that in immunity, BUD31 is more immunosuppressive in contrast to its oncogenic contributions. There are no confirmed studies showing that BUD31 operates in a way that benefits anti-oncogenes. Other proteins which are also specialised in splicing and highly up-regulated in female HBCs are U1 small nuclear ribonucleoprotein C (SNRPC), splicing factor 3B subunit 4 (SF3B4), serine/arginine-rich splicing factor 3 (SRSF3), pleiotropic regulator 1 (PLRG1) and protein SON (SON). Closely related to BUD31, all of them contribute to the regulation of cell cycles, with some of them having been confirmed to be accountable for the development and progression of cancers[132][133][134] - SNRPC is a tumour marker for breast cancers[132] and SF3B4 is a diagnostic marker for detecting early-stage hepatocellular carcinomas[133]. SRSF3 not only stimulates the proliferation and transformation of cancer cells[135], it directly suppresses the translation of pro-inflammatory signals arisen in microglia in response to LPS challenge by binding the 3′ untranslated region (UTR) of mRNA, which indicates its straight repression of the innate immune response and the

activation of microglia[136]. PLRG1 and SON can be favourable for both normal cells and cancerous cells. While they do promote the growth of tumour cells, PLRG1 is essential for cell proliferation and loss of PLRG1 in mouse embryos caused cell cycle arrest and apoptosis[137]. SON is invaluable in maintaining the homeostasis of cells, with the ability of repairing the damaged DNAs and restoring their integrity[134]. Heterogeneous nuclear ribonucleoprotein A/B (HNRNPAB) is another protein concerned with pre-mRNA and m-RNA processing, from alternative splicing, nuclear export of mRNA to its translational regulation[138][139]. As many other proteins related to splicing, HNRNPAB is also considered oncogenic and its overexpression has been revealed to accelerate the epithelial-mesenchymal transition, which eases the metastasis of cancers. However, in newborns, it inhibits the occurrence of colorectal carcinoma[139]. Protein Red (IK), the last protein functioning in the nucleus, separates the nucleolus from other components[140]. Isolated IK can suppress the expression of MHC class II induced by IFN-γ, which can be a therapeutic approach for autoimmune diseases as excessive expression of MHC class II is associated with autoimmune diseases and their severity[141]. The biological function of IK is unknown[140].

Another three proteins, which were found highly up-regulated in female HBCs and give influence to immunity, were proteins all involved in the transport between the nucleus and cytoplasm. Nuclear pore complex protein Nup88 (NUP88) is one kind of the chief component proteins (Nucleoporins) for nuclear pore complexes, which are massive multiprotein channels, dedicated for the transport between the nucleus and cytoplasm. NUP88 itself is oncogenic, the overexpression of which facilitates the growth of multinucleated cells. It has been identified as a tumour marker, predicting the progression of oncogenesis. In fact, high levels of NUP88 has been linked with the metastasis and increased mortality rates in colon cancer and poor prognosis in breast cancer[142]. The second protein, RAN-binding protein 3 (RANBP3), is a co-factor for exportin 1 (XPO1) which is a main executor mediating nuclear export, particularly the export of Rev and U small nuclear RNAs (U snRNAs)[143]. Rev, which stands for regulator of expression of virion proteins, is an essential regulatory protein in the protein expression of HIV. Rev ties with the Rev responsive element (RRE) in HIV Envelope polyprotein (Env) sequence and XPO1 to export all partly spliced and unspliced HIV viral mRNA to the cytoplasm for translation[144]. Without Rev, mRNAs from HIV will be limited in the nucleus, failing to get involved in viral reproduction. From this striking fact, it is reasonable to consider that higher expression of RANBP3 in female HBCs compared to males' (fold change: 2.27, p value:0.005) could have enhanced the export of HIV viral mRNA from the nucleus to the cytoplasm along with XPO1. As a result, this contributes to increased translation of viral mRNA in the cytoplasm and subsequent viral replication, which could explain why female fetuses are more susceptible to HIV

infection from the mother than the male fetuses (odds ratio 2.14; 95% CI: 1.56 to 2.95)[99]. For other engagement in immunity, RANBP3 modulates the nuclear export of mitogen-activated protein kinase (MEK) and p53, regulating the life cycles of cells. It has been shown that it boosts the proliferation of melanoma cells via the MAPK/ERK pathway[145]. Lastly, importin subunit alpha-1 (KPNA2), is a protein principally committed to the import of proteins from the cytoplasm into the nucleus[146]. The cellular function of KPNA2 is complicated and broad. High expression of KPNA2 in cancer is often positively correlated with poor prognosis, as it participates in the translocation of various proteins which are in charge of DNA repair and the genes of which operate as tumour suppressor genes in normal cell activities but mutate in the process of carcinogenesis. These proteins include checkpoint kinase 2 (CHK2), breast cancer type 1 susceptibility protein (BRCA1), and nibrin (NBS1). In viral infections, KPNA2 imports viral materials to the nucleus, assisting their replication. So far, HIV, Epstein-Barr virus, polyomavirus and HPV have been found to benefit from this mechanism[147]. In SARS coronavirus, KPNA2 is entrapped in the endoplasmic reticulum (ER) and Golgi membrane, restrained from importing STAT1, an important transcription factor to promote the immune responses against viruses, into the nucleus. Except for STAT1, KPNA2 also mediates the translocation of several other immune related proteins, such as interferon regulatory factor 1 (IRF1), interleukin-2-inducible T-cell kinase (ITK), paired box 5 (PAX5) and recombination activating gene 1 (RAG1). IRF1 and ITK are more orientated towards immune responses, while PAX5 is engaged in the differentiation of B cells and RAG1 involved in the V(D)J recombination[147].

Another identified protein, double-stranded RNA-specific adenosine deaminase (ADAR) is a protein responsible for RNA editing, mainly dsRNAs in the cytoplasm. dsRNAs generated from all RNA viruses and even dsDNA viruses are recognised by PRRs. In the cytoplasm, dsRNAs are mostly detected by one kind of PRRs - the RIG-I-like receptors[148]. RIG-I signals initiate the pathways that lead to the activation of NF-κB and IRF3, which consequently sparks the production of type I and III interferons and antiviral gene products. This cascade plays a critical role in the defense against viral infections[149]. Editing of dsRNAs by ADAR in the cytoplasm precludes them from being sensed, imposing an inhibitory effect on the antiviral responses. Though host has evolved several mechanisms to overtake this blocking impact in viral infections, in the pathogenesis of autoimmune diseases, it still has a crucial role in strengthening self-tolerance, avoiding autoimmunity. Considering that females are much more likely to suffer autoimmune diseases compared to males, higher expression of ADAR in female HBCs might be one of the established systems in females to lessen the possibility of developing cross-reactive antibodies, which are normally induced by preceding viral infections and

eventually attack the tissues of host himself. Editing by ADAR is also required in preventing the apoptosis of certain cell types in the late phase of infections[148].

H/ACA ribonucleoprotein complex subunit 4 (DKC1), ATP-dependent RNA helicase DDX50 (DDX50), 5-3 exoribonuclease 2 (XRN2) and Treacle protein (TCOF1) are all proteins engaged with the processing of rRNAs. Mutations of DKC1 are the underlying reason for Hoyeraal-Hreidarsson (HH) syndrome, a serious multisystem condition affecting males with symptoms of microcephaly, growth restriction, aplastic anaemia and immunodeficiency[150]. Different from other proteins identified differentially regulated in male HBCs and female HBCs related to immunity, the gene coding this protein is located on X chromosome, so the higher expression in female HBCs can be a result of the escape from X inactivation as described in the section of sexual dimorphism, especially the part `sexual dimorphism in general'. DDX50, a kind of RNA helicase, supervising the modifications of RNA secondary structures including the assembly of rRNAs[151], has been shown to have suppressing effects on the replication of dengue virus by enhancing the production of IFN-β via an additive effect on the promotors of IFN-β genes[152]. Other contributions by DDX50 in immunity are unrevealed. Thirdly, XRN2, a director in pre-rRNA cleavage and mature rRNA generation[153], also induces the termination of transcription to repair DNA double strand breaks, maintaining the stability of genomes, which is a key factor in preventing the occurrence of malignancy[154]. Lastly, TCOF1, which regulates the transcription of ribosomal DNA, also has an important role in fixing DNA damage, preserving the integrity of genome[155].

CD34, though broadly regarded as a common marker for haematopoietic cells is, in fact, expressed on a diversity of cell lines, such as mesenchymal stromal cells, muscle satellite cells, interstitial cells, epithelial cells and vascular endothelial cells. Its functions are associated with cell adhesion and the modulation of cell differentiation and proliferation, also immune regulation[156]. In transplantation, adopting purified $CD34^+$ cells from the donor displays inhibitory effects on the formation of cytotoxic T lymphocytes (CTLs) in the host. It has been demonstrated that this is achieved by cell contact[157].

Finally, Enolase2 (ENO2) works cooperatively with PFKL in the process of glycolysis. Please refer to the part about PFKL for the significance of glycolysis in immunity.

As a summary for all proteins related to immunity, proteins up-regulated in male HBCs function appeared more focused on the interactions between proteins and substances, while proteins up-regulated in female HBCs appeared more concentrated on the processing of DNAs and RNAs.

Proteins related to mitochondrial functions

For proteins involved in functions linked with mitochondria, 6 proteins were significantly up-regulated in male HBCs compared to females'. NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 (NDUFA6) and Cytochrome B-C1 complex subunit 9 (UQCR10) are both proteins substantially engaged in the electron transport chain (ETC). Located within the inner membrane of mitochondria, the ETC is composed of four multi-protein complexes (I-IV) and is the area generating most ATP inside the cell (during which process the non-enzymatic superoxide can also be shaped). Following the products of NADH and $FADH_2$ from the TCA cycle are oxidised in Complex I and II, creating electrons which are passed to ubiquinol, which further transfers electrons to Complex III. Complex III carries electrons to Cytochrome C, which eventually transports electrons to Complex IV. Receiving electrons and subsequently being charged, Complex IV pumps out H^+ from mitochondrial matrix to the inter-membrane space, creating the final proton gradient in the ETC. Complex IV gives electrons to the last electron receiver - the oxygen, which is split into two oxygen ions and then combines with H^+ respectively, generating H_2O. ATP synthase utilises the proton gradients formed in Complex I, III and IV so far, converting ADP to ATP[158]. Individually, NDUFA6 is required in Complex I and UQCR10 in Complex III. Malfunction of NDUFA6 leads to the inactivation of Complex I, the largest and most elaborate enzyme union in the ETC. This will cause the apoptosis of cells, which is an underlying reason for the death of T-cells induced by HIV[159][160].

Protein Mpv17 (MPV17) is a mitochondrial inner membrane protein which contributes to the metabolism of reactive oxygen species[161] and the maintenance of mtDNA[162]. Mutations of MPV17 trigger mtDNA depletion syndromes (MDS), which are characterised by decreased mtDNA copy number and the impairment of oxidative phosphorylation. The clinical symptoms include neurological abnormalities, hypoglycaemia, early onset progressive liver failure and enhanced level of blood lactate[162]. On the other hand, aspartate-tRNA ligase, mitochondrial (DARS2) is a synthetase producing L-aspartyl-tRNA from L-aspartate and tRNA[163]. This process is necessary for the translation of mitochondrial mRNA into usable protein. There are 19 mitochondrial amino-acyl tRNA synthetases, which are encoded in the nucleus and translated in the cytosol, then transported to mitochondria. Mutations of DARS2 cause leukoencephalopathy with brainstem and spinal cord involvement (LBSL), in which slowly progressive weakening of motor skills can be observed in childhood or adolescence. In some cases, the cognitive functions are also affected[164].

Alpha-methylacyl-CoA racemase (AMACR) is an enzyme committed to the degradation of branched-chain fatty acids and the conversion of cholesterol to primary bile acids. Deficiency of AMACR leads to the build-up of pristanic acid and

intermediates of C-27 bile acid, which influences the activities of the brain and liver respectively. Mutations of AMACR have been found in patients with sensory neuropathy and certain single nucleotide polymorphisms (SNPs) of AMACR have been confirmed in male patients with schizophrenia[165]. Moreover, the overexpression of AMACR has been detected in 80% of prostate cancers diagnosed by biopsies[166], though the mechanism of how AMACR is involved in the pathogenesis of prostate cancer is unclear. In contrast, succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial (OXCT1) is a central enzyme in the metabolism of ketone bodies. It is in charge of the first and rate-limiting step in the reactions of ketolysis, promoting the change of ketone bodies to acetoacetyl CoA, which will be eventually transformed to acetyl-CoA and supplied to the TCA cycle for oxidation and ATP generation[167][168]. OXCT1 exists in mitochondrial matrix of all tissues except for the liver[169], which is the main site for ketogenesis. However, in hepatocarcinoma, where there is a stress caused by the shortage of energy, liver cancer cells can express the enzymes involved in ketolysis including OXCT1 to utilise ketone bodies for the creation of ATP to sustain the growth of cancer cells. Mutations of OXCT1 have been identified in various cancers and it has been demonstrated that OXCT1 prevents the apoptosis and antophagy of cancer cells, while it facilitates their development and migration[168]. In diabetes, the activity of OXCT1 has been found to be significantly decreased by 92% in pancreatic islets of diabetic patients compared to non-diabetic controls, although the reason for this remains undiscovered[170].

Three proteins associated with mitochondrial functions were significantly up-regulated in female HBCs compared to male HBCs. Cytochrome b-c1 complex subunit 6, mitochondrial (UQCRH) is a protein involved in Complex III in the ETC. Due to Warburg effect, the activity level of Complex III is repressed in invasive breast cancers, nonetheless, the expression of UQCRH is often highly regulated in breast cancers, which can be observed in many other types of malignancy[171].

39S ribosomal protein L12, mitochondrial (MRPL12) and Treacle protein (TCOF1) are both proteins for ribosomes. Although the genes for mitochondrial ribosomes are encoded in the nucleus, they permit mitochondrial mRNAs encoded by mtDNAs to be translated[172]. While an absolute majority of proteins engaged in the ETC are encoded by DNAs in the nucleus, 13 proteins essential for ETC are encoded by mtDNAs. It has been detected that the mutations of MRPL12 change the embedding with the large ribosomal subunit of mitochondrial ribosome, giving rise to the defect of mitochondrial translations and causing growth delay and neurological impairment[173]. Finally, TCOF1 is a protein contributing to both immune and mitochondria related functions. Though TCOF1 is a protein mainly involved in modulating the transcription of ribosomal DNAs in the nucleus[155], it is also vital in

protecting cells, especially neuroepithelial cells and progenitor neural crest cells from the stress caused by reactive oxygen species (ROS), which is generally released by mitochondria in the process of development. Mutations of TCOF1 induce the occurrence of Treacher Collins syndrome, which is marked by the deformity of craniofacial structures including conductive hearing loss and brain defects[174].

Proteins related to vesicular functions

For proteins involved in vesicular functions, 6 proteins were significantly up-regulated in male HBCs compared to female HBCs. ADP-ribosylation factor 4 (ARF4), is one kind of protein of the ARF families involved in the regulation of vesicular trafficking. ARF GTPases modulate the recruitment of coat proteins, which are important in creating transport vesicles, adjust the construction of the actin cytoskeleton and change membrane lipid composition through the activation of phospholipase D (PLD). It has been verified that the interactions between ARF4 and the C terminus of rhodopsin are crucial for the post-Golgi trafficking of rhodopsin, an essential protein in visual photo-transduction. ARF4 particularly detects the sorting signal released from the C-terminal of rhodopsin, facilitating its merge with rhodopsin transport carriers (RTCs) for its transport to its functional destinations - photoreceptor rod outer segments (ROS). Mutations in rhodopsin are the fundamental reasons for many retinal conditions including retinitis pigmentosa (RP), a genetic disorder and a major cause for early onset blindness. It has become clear that critical autosomal dominant form of RP is induced by point mutations occurring within the C-terminal of rhodopsin, which could affect the integrity of the C terminus of rhodopsin impairing its molecular communication with other proteins, such as ARF4[175].

Coatomer subunit delta (ARCN1), Protein transport protein Sec24A (SEC24A), SEC23-interacting protein (SEC23IP), Trafficking protein particle complex subunit 3 (TRAPPC3) and Vesicle-fusing ATPase (NSF) are all proteins involved in vesicle mediated protein transport between the ER and Golgi apparatus. ARCN1 is a subunit of coat protein I (COPI), which are types of proteins coating the surfaces of vesicles, responsible for the transport from the Golgi apparatus to ER (retrograde transport). Recent studies have revealed that COPI not only contributes to the retrograde transport but is also engaged in the transport from the ER to Golgi apparatus, the anterograde transport. Mutations in ARCN1 cause ARCN1-related syndrome, which is characterised by facial dysmorphisms, microcephalic dwarfism, micrognathia and intellectual retardation. The mechanism of the pathogenesis of this condition is considered as the results of ER stress, which is triggered by the over piling of proteins at the ER site from the disturbance of intracellular trafficking. Decrease of ARCN1 may influence the intracellular transport of collagen as well,

which is another underlying reason for ARCN1-related syndrome[176]. SEC24A is an element of coat protein II (COPII), which is one of a family of proteins specialised in the coating of vesicles for the anterograde transport. SEC24A modulates the secretion of proprotein convertase subtilisin kexin 9 (PCSK9), the binding of which, with the low-density lipoprotein (LDL) receptors, induces the degradation of LDL receptors in the lysosomes, resulting in high levels of LDL in the blood stream. It has been demonstrated that SEC24A deficiency in mice caused reduced secretion of PCSK9, which in turn led to lessened levels of LDL in the plasma. This finding suggests the potential role of SEC24A in the metabolism of cholesterol via the regulation of PCSK9[177]. SEC23IP works closely with SEC23, another component protein of COPII. SEC23IP has been proved to be indispensable for spermatogenesis in mice[178]. TRAPPC3, a protein functioning at the interface between the vesicle and Golgi apparatus, facilitates the tethering of COPII vesicle with the membrane of Golgi apparatus. NSF is also a protein committed to membrane fusion. It disassociates the complexes of SNAREs (Soluble NSF Attachment protein REceptors) formed by SNAREs on the vesicles and those on the target membranes for the docking of vesicles with the target protein[179]. NSF is important for the interaction between the sperm and the oocyte, particularly for the acrosome reaction[180] during which various fusions of the outer acrosomal membrane with the sperm plasma membrane happen to release the inside materials of the acrosome to the outside of the sperm cell. These secreted materials will form a hole in the oocyte, enabling the fusion between the sperm and the oocyte[181].

The only protein which was significantly up-regulated in female HBCs compared to males relating to vesicular functions was Gamma-soluble NSF attachment protein (NAPG). It cooperates with NSF, mediating the fusion of vesicles with the target proteins[182].

Proteins related to transcription functions

For proteins involved in transcription functions, 5 proteins were significantly up-regulated in male HBCs compared to females'. Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform, also called serine/threonine-protein phosphatase (PPP3CA) and WD repeat-containing protein 82 (WDR82) are both proteins engaged in the regulation of transcription. PPP3CA encodes the alpha isoform of a subunit of calcineurin[183]. Calcineurin up-regulates the transcription of multiple cytokines and co-stimulatory molecules, which are necessary for the activation of T cells[184]. In the brain, calcineurin has a pivotal role in synaptic transmission through modulating the signalling responses to calcium ions. De novo mutations in PPP3CA are associated with the occurrence of neurodevelopmental conditions with seizures[183]. WDR82 is a C-terminal domain-

binding protein promoting the initiation of transcription by recruiting the Setd1A histone H3-Lys4 methyltransferase complex to the start sites of transcription[185]. In colorectal cancer, low expression levels of WDR82 are related with poor prognosis[186].

The other three proteins which were up-regulated in males HBCs were all involved in the metabolism of sugars. Alpha-galactosidase A (GLA) hydrolyses the half terminal α-galactose from glycoconjugates such as glycolipids and glycoproteins. Mutations of GLA are the reasons for Fabry disease, which is a X-linked genetic condition affecting various organs as a result of the accumulation of globotriaosylceramide, a type of glycosphingolipid requiring GLA for its degradation[187]. Plasma alpha-L-fucosidase (FUCA2) is an enzyme to generate L-fucose from α-L-fucoside. In the infections of *Helicobacter pylori*, fucose is utilised as an additional energy source for the bacteria and FUCA2 has been shown to be necessary for *H.Pylori* adhesion, especially for the strains specifically causing duodenal ulcers and gastric cancers. Furthermore, FUCA2 also increases the expression of Lewis x antigens in *H.pylori*. Lewis x antigens are known to be crucial for bacterial cell adhesion and the escape from host surveillance[188]. In general, males have a higher incidence of developing gastric cancers compared to females[189]. Lastly, Di-N acetylchitobiase (CTBS) is a glycosidase required for the degradation of asparagine-linked oligosaccharides on glycoproteins[190]. For these three proteins, which were all involved in the metabolism of sugars, their direct contributions to transcription related functions are unknown.

Five proteins associated with transcription functions were significantly up-regulated in female HBCs compared to males'. Protein dpy-30 homolog (DPY30) is a subunit of the SET1-family (SET1A, SET1B, MLL1, MLL2, MLL3 and MLL4 in humans)[191] which constitutes the SET1/MLL complex, a main histone H3-Lys4 methyltransferase in mammals. DPY30 is not only essential for regulating gene transcription, but also vital for sustaining the functions of haematopoietic stem cells in adults by modulating the gene expressions of multiple haematopoietic development-related genes[192]. Paired amphipathic helix protein Sin3a (SIN3A) is another transcription regulator, controlling cell cycle through development. It is particularly critical for embryonic development during the period of implantation. Mouse embryos without SIN3A endured unsolved DNA damage and acute p53-independent apoptosis[193]. For genes associated with immune responses, Sin3a suppresses the transcription of STAT3. On the other hand, it up-regulates the transcription of interferon stimulated gene factor 3 (ISGF3) to produce interferons in response to viral infections such as influenza A and hepatitis C[194]. Transcription activator BRG1 (SMARCA4), also a transcription regulator, is often linked with the growth of cancer cells. High expression of SMARCA4 has to be proved to lead to

poor prognosis in cancers[195], while loss of SMARCA4 in certain cancers, for example in small cell carcinoma of ovary, indicates the existence of cancers with high sensitivity and specificity[196].

Cyclin-dependent kinase 6 (CDK6) plays important parts in the regulation of cell proliferation. It aids the cells progress into the DNA synthetic (S) phase of the cell division cycle. The inhibitors of CDK6 have been therapeutic targets for the treatments of cancers[197]. Finally, protein O-GlcNAcase (MGEA5) is a protein involved in the post-translational modification conducted by O-linked N-acetylglucosamine (O-GlcNAc). The cellular level of O-GlcNAc is adjusted by O-GlcNAc transferase (OGT) and MGEA5 which is also called OGA. Adding the modification by OGT and erasing the modification by MGEA5 maintain the balance of O-GlcNAc, which is important for gene transcription, cell cycle progression, and stress responses. Disruptions of this homeostasis are reasons for the pathogenesis of cancers, diabetes and Alzheimer's disease[198].

In summary, the proteomics results revealed significant differences in protein expression in a number of molecules involved in four major pathways. The validation experiments on PSME3, PSMB2, TCOF1, SEC24A and SMARCA4, which corroborated the proteomics differences, suggest that the data is valid.

Results Chapter 3

Investigating sexual differences in HBCs using next-generation sequencing

1 Sample preparation

RNAs were extracted from the cells of same samples that were subjected to proteomics in the previous chapter. Please refer to appendix 3 for the methodology of RNA isolation. Concentrations of RNAs were measured by nanodrop spectrophotometer to ensure that there was a minimal concentration of 20ng/µl for each sample. To assess the purity of RNA, a 260/280 ratio was also measured and ~2.0 was regarded as appropriate for each sample. As there was a female HBCs sample which did not meet the requirements stated above, 6 male HBCs samples and 5 female HBCs samples were sent to Novogene Co. Ltd. (Hong Kong) for analysis (20µl at 20ng/µl for each sample). Illumina NovaSeq platforms were applied to perform sequencing.

2 Results

To analyse the sequencing data, DESeq2 was utilised with the help from Dr. Thomas Brown (Big Data Institute, University of Oxford).

Figure 1 shows the read assignment by category for each sample. The numbers of reads for genes are shown in pink colour, with other numbers of reads for other constitutional factors including external RNA control consortium (ERCC), fragment length, chimera, mapping quality and unmapped shown in other different five colours.

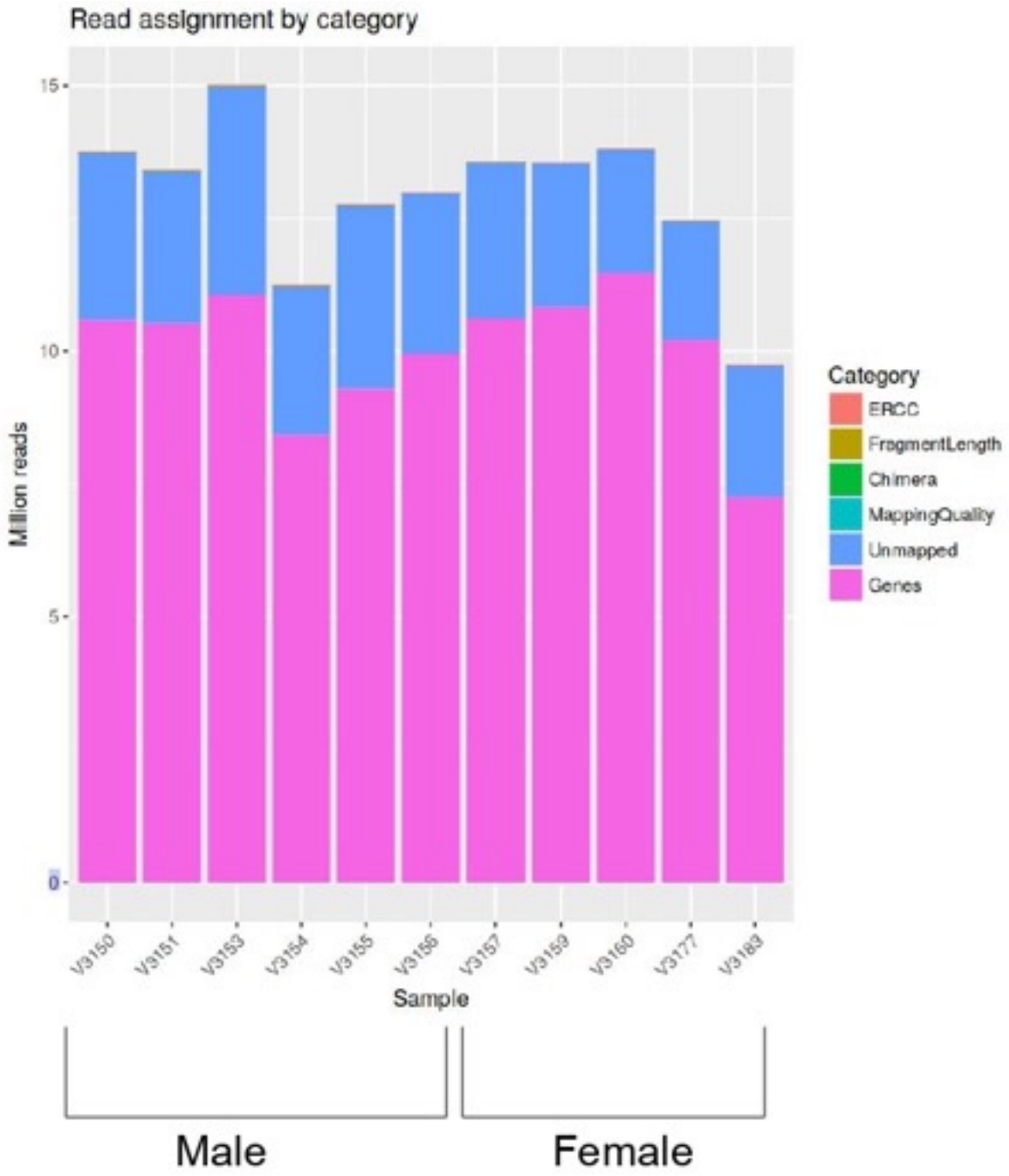


Figure1: An absolute majority of reads for each sample were genes compared to a small part of reads assigned for external RNA control consortium (ERCC), fragment length, chimera, mapping quality and unmapped.

Figure 2 shows the read biotype counts, which include the counts for protein coding RNAs and other types of RNAs for each sample.

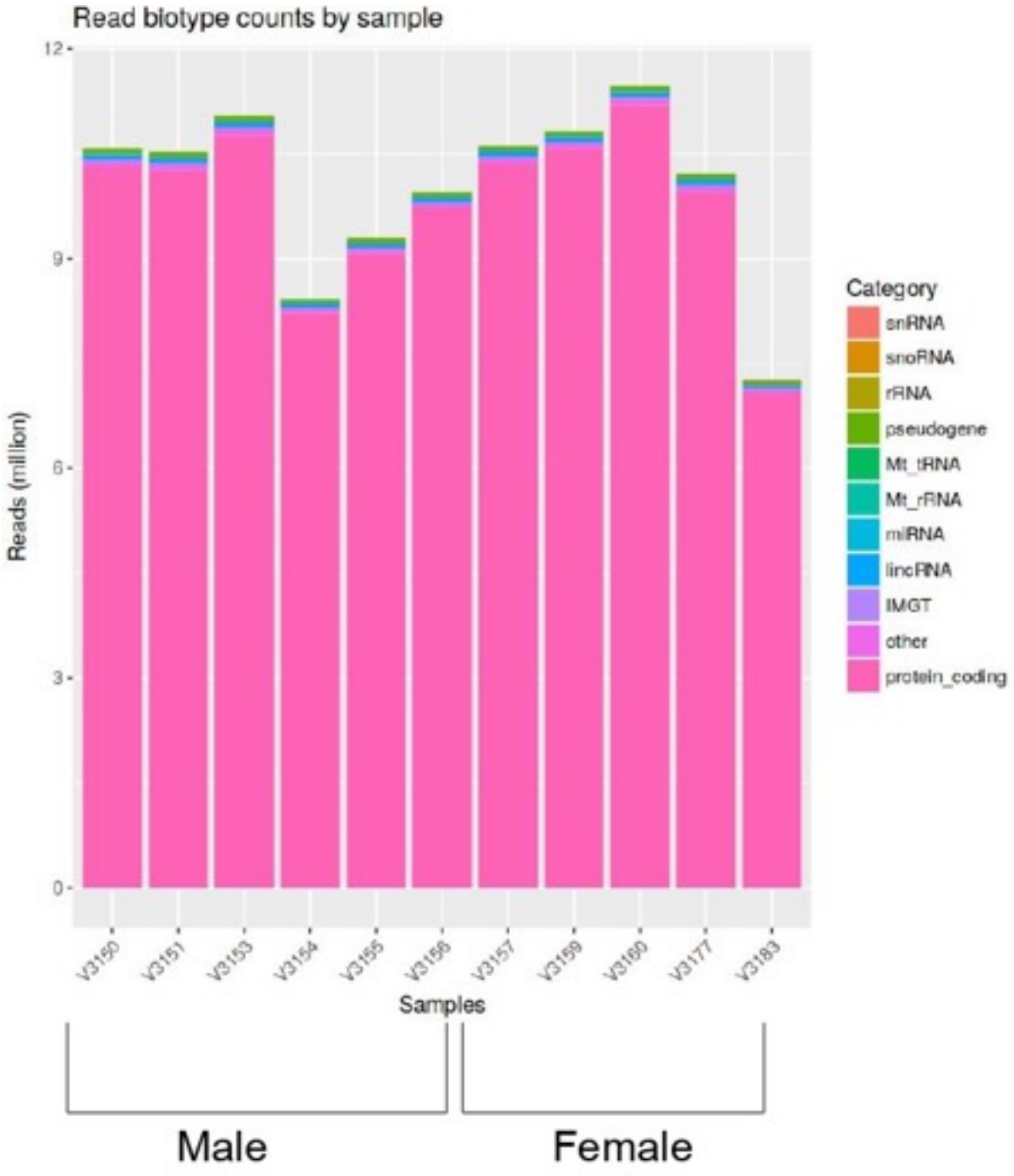


Figure2: Most of the reads for each sample were protein coding genes, while small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), ribosomal RNAs (rRNAs), pseudogenes, mitochondrial transfer RNAs (Mt_tRNAs), mitochondrial ribosomal RNAs (Mt_rRNAs), micro RNAs (miRNAs), long intervening noncoding RNAs (lincRNAs), ImMunoGeneTics (IMGTs), and others were also recorded at an extremely low level.

Figure 3 shows distance-based hierarchical clustering.

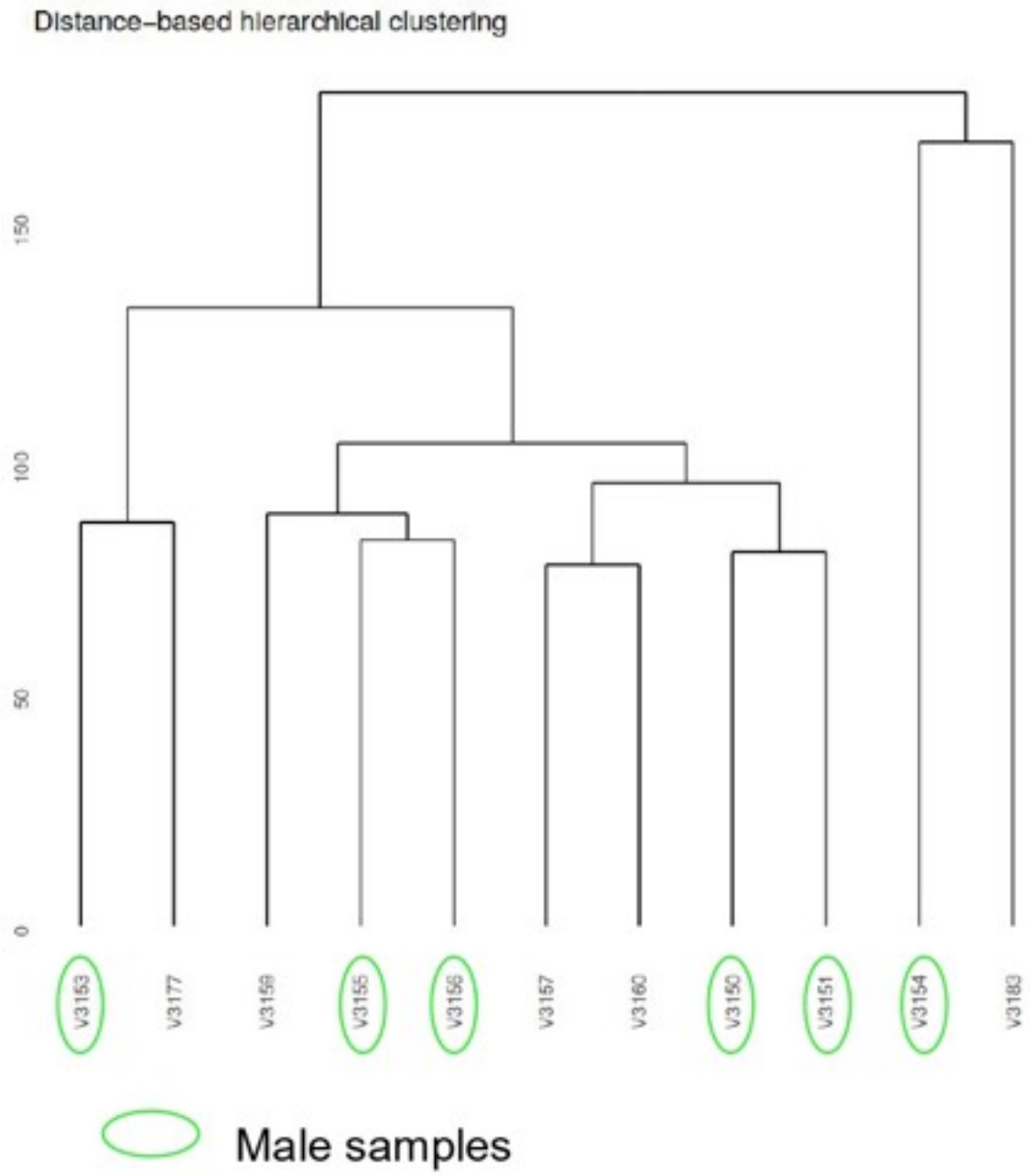


Figure3: From the distance-based hierarchical clustering, a male sample (V3154) and a female sample (V3183) were distantly distinct from other samples.

Figure 4 shows Spearman's rank-based hierarchical clustering.

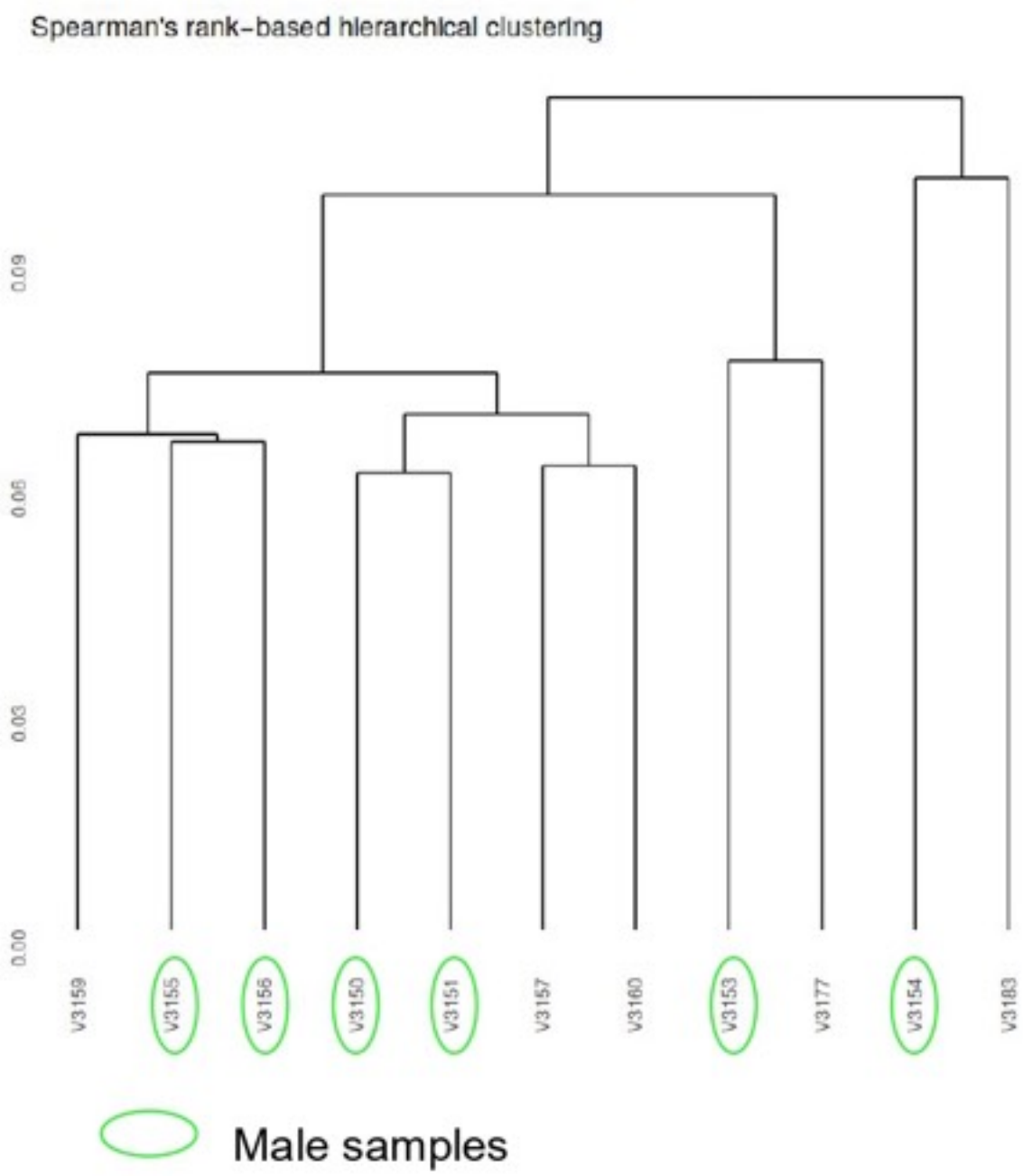
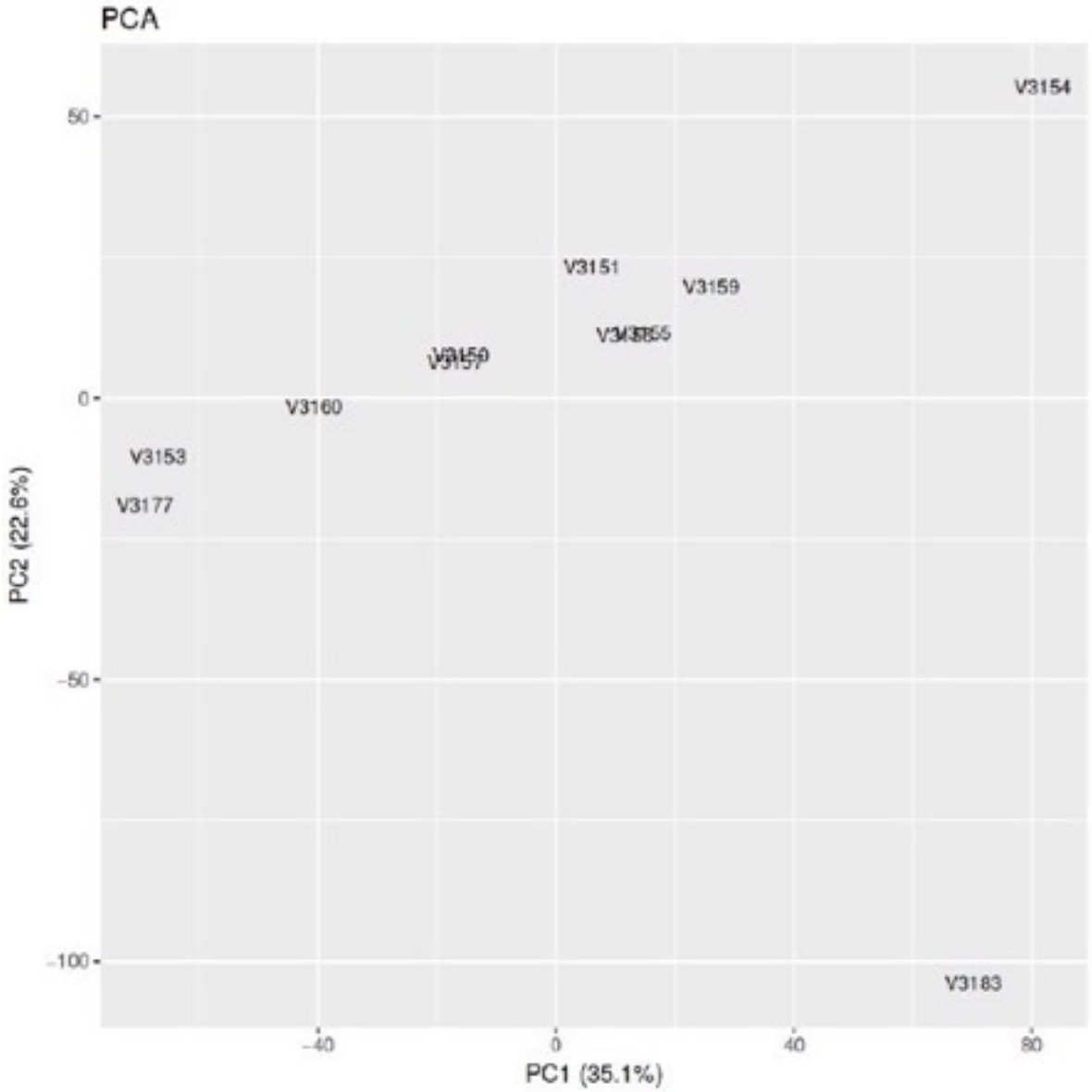


Figure4: Spearman's rank-based hierarchical clustering showed the same clustering trend with the distance-based hierarchical clustering.

Figure 5 shows the variability between samples using principal component analysis (PCA).

(A)


PCA
V3154
50
V3151
V3159
0
V3160
V3153
V3177
PC2 (22.6%)
-50
-100
V3183
-40
0
40
80
PC1 (35.1%)

(B)

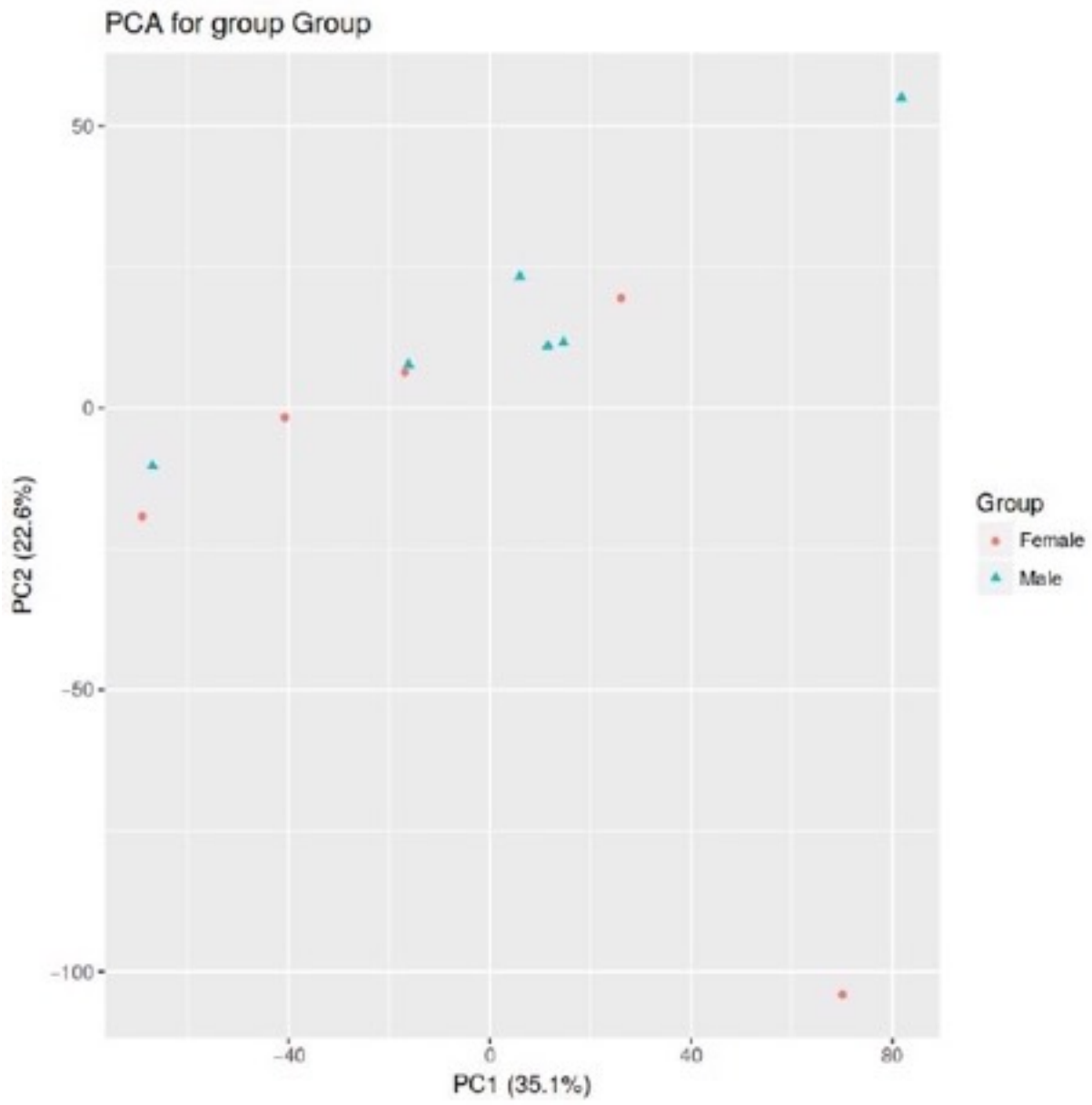


Figure5: (A) and (B) both show the variability between samples using principal component analysis. A female sample (V3183) clustered away from others.

Analysis was run and 14 transcripts were found to have a false discovery rate (FDR=adjusted p value)<0.05.

Figure 6 shows the summary of the result of this analysis.

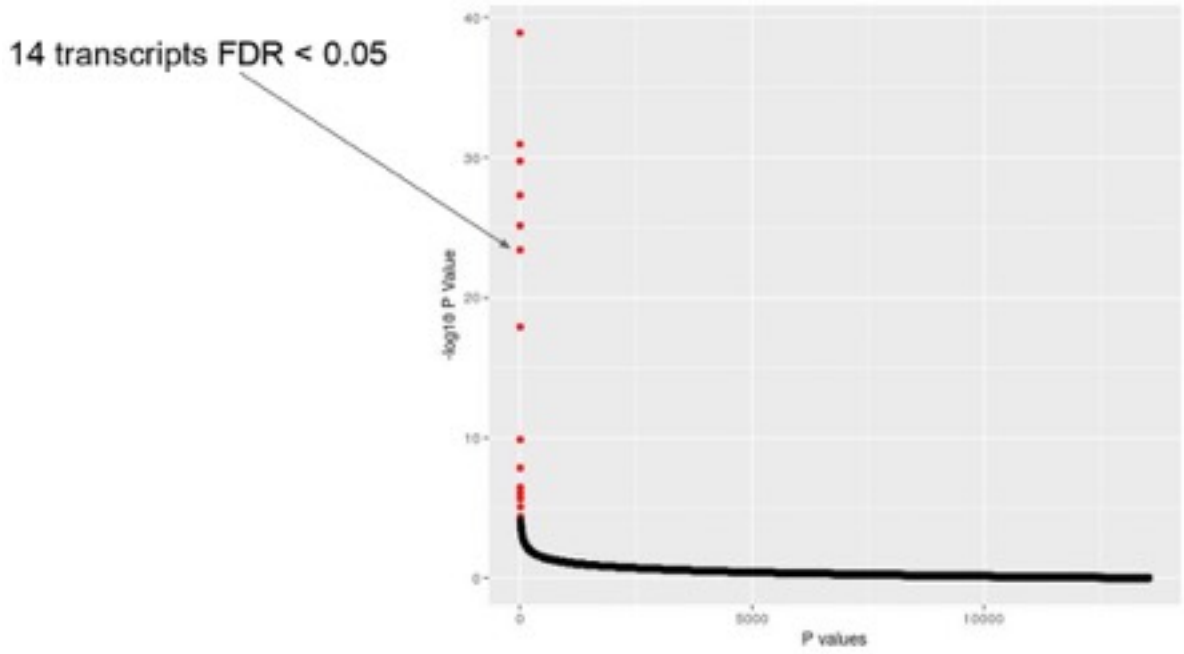


Figure6: 14 transcripts had an adjusted p value<0.05 in the first attempt of analysis.

As one female sample (V3183) had lower read depth and clustered away from other samples, this sample was excluded from the input samples list and the analysis was re-run.

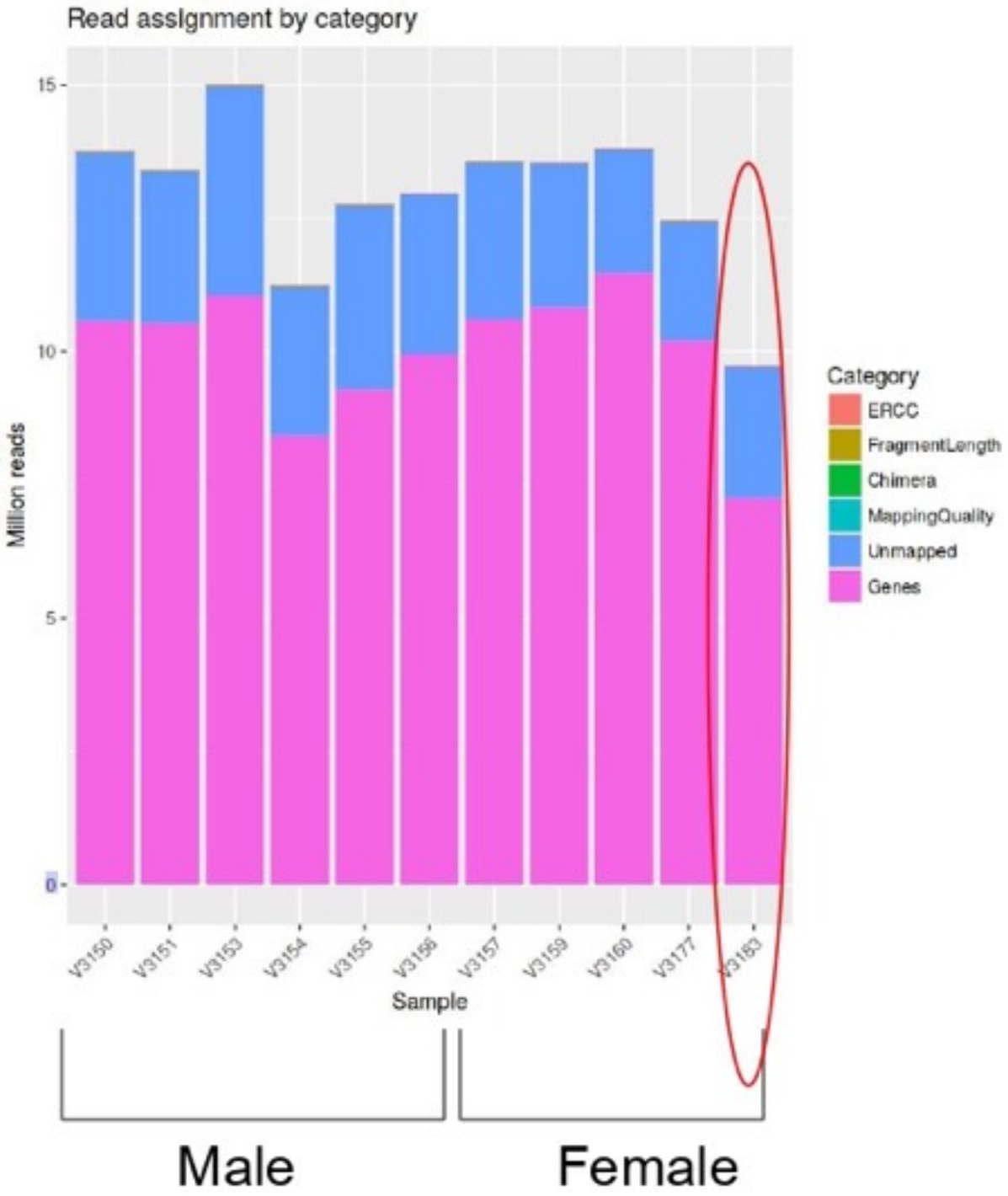
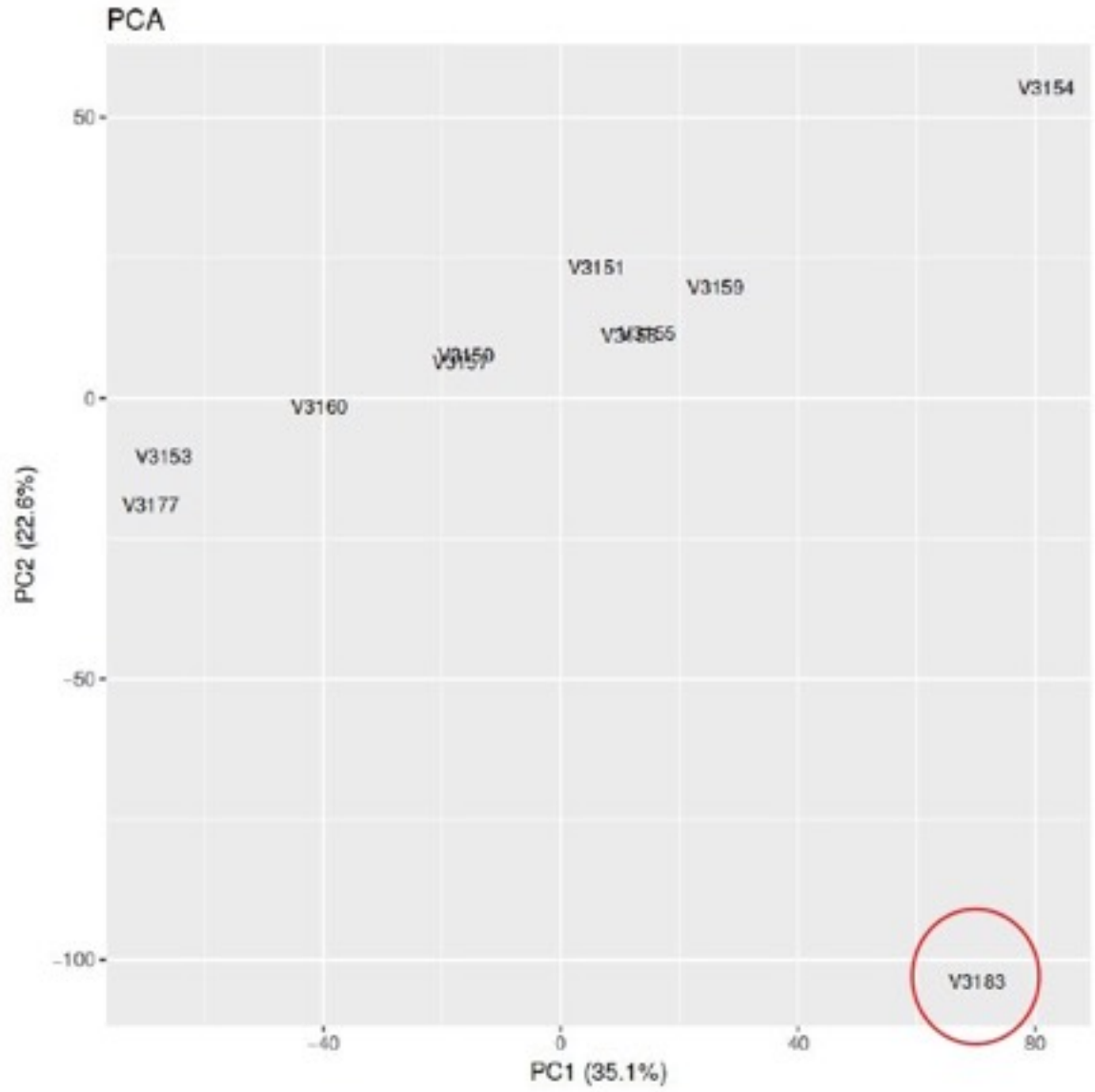


Figure7: One female sample (V3183) was excluded from the input samples list as it had lower read depth compared to others.

(A)


PCA
V3154
V3151
V3159
V3160
V3153
V3177
V3183
50
0
-50
-100
-40
0
40
80
PC2 (22.6%)
PC1 (35.1%)

(B)

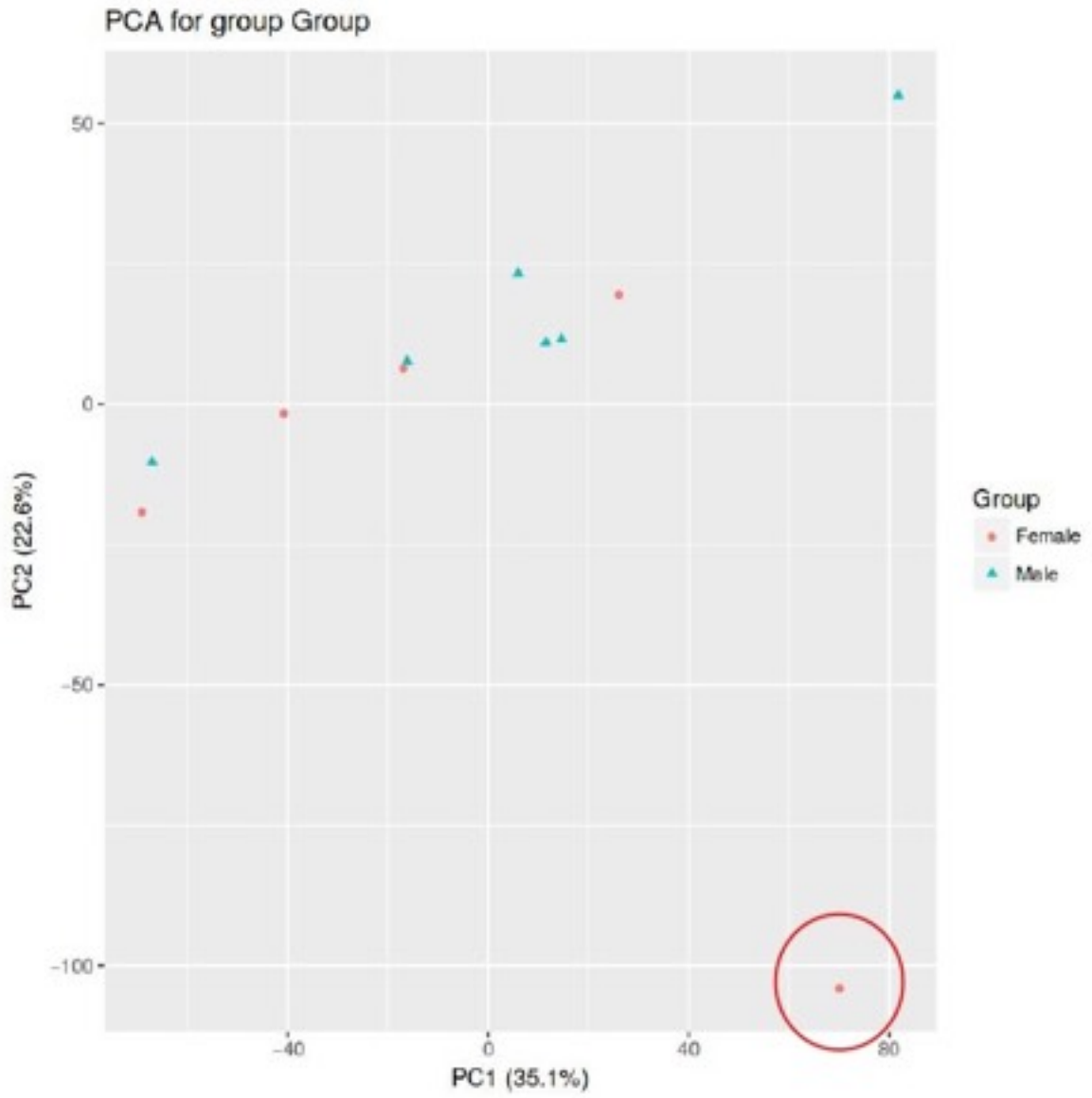


Figure8: (A) and (B) both show that one female sample (V3183) was excluded from the input samples list as it clustered away from other samples.

According to the analysis re-run, 49 transcripts were found to have an adjusted p value<0.05.

Figure 9 shows the summary of the result of the re-run analysis.

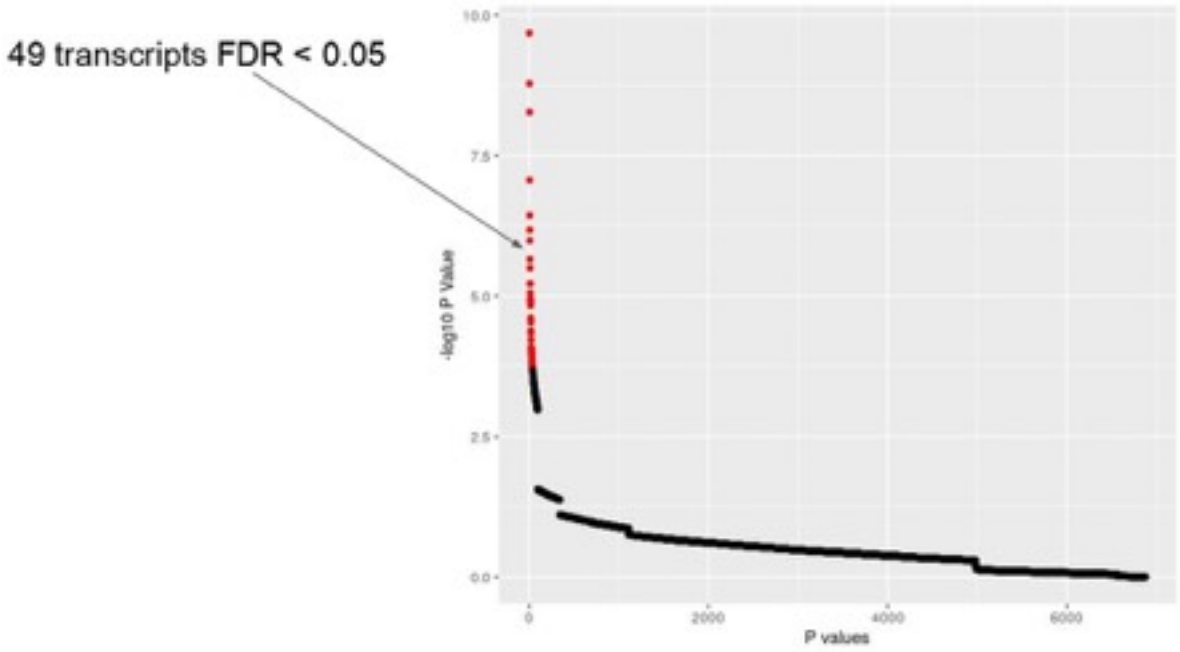


Figure9: 49 transcripts had an adjusted p value<0.05 in the re-run analysis.

Of 6844 included transcripts, 26 transcripts were significantly up-regulated in male HBCs samples and 23 transcripts were significantly up-regulated in female HBCs samples (adjusted p value<0.05).

Figure 10 shows the heat map which presents the expression pattern of these 49 differentially regulated genes identified between male and female HBCs in individual sample.

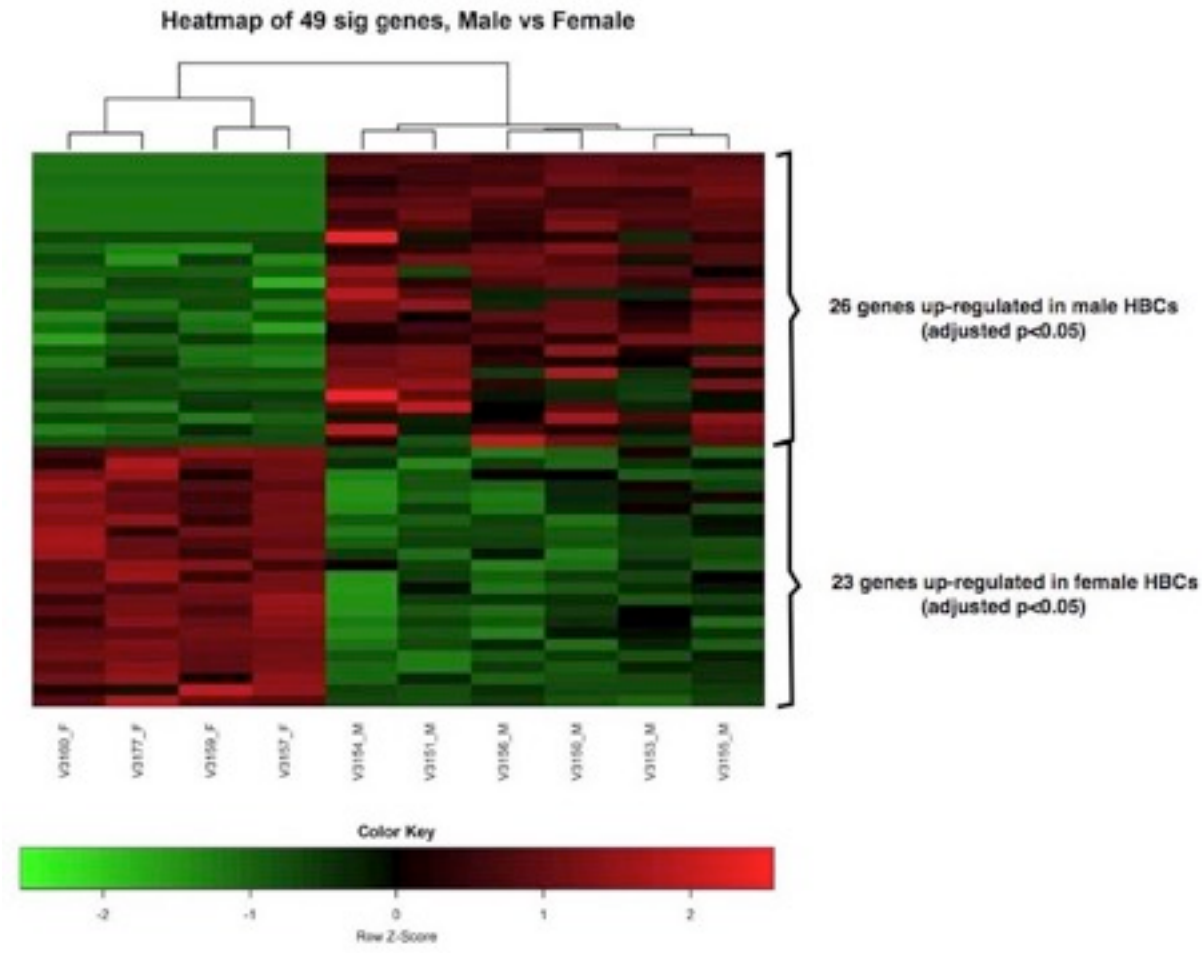


Figure10: Of 49 genes identified, 26 genes were significantly up-regulated in male HBCs, while 23 genes were significantly up-regulated in female HBCs.

Table 1 shows the names of genes and the fold changes of genes which were significantly up-regulated in male HBCs. Orders are corresponding to their adjusted p value (from the smallest). For some genes, which are located on Y chromosomes, the information is provided.

Gene Name	Fold Change	Chromosome
KDM5D	5148.73	Y
EIF1AY	2086.69	Y
UTY	1927.35	Y
TTTY15	1393.99	Y
USP9Y	794.66	Y
TXLNGY	633.85	Y
TTTY14	366.08	Y

Gene Name	Fold Change	Chromosome
TMSB4Y	167.38	Y
COX20	1.90	
CTA-445C9.15	1.96	
RP11-424G14.1	62.58	Y
HEBP2	1.37	
LINC00278	64.97	Y
NICN1	1.96	
RING1	1.24	
EID2	1.32	
EBPL	1.35	
AHSP	10.37	
SNORD3A	1.87	
ACCS	2.56	
S100A1	4.05	
WNK2	7.68	
SCUBE2	1.90	
RP11-324I22.4	4.51	
CD99P1	2.46	
RP-11-350D17.3	5.90	

Table 2 shows the names of genes and the fold changes of genes which were significantly up-regulated in female HBCs. Orders are corresponding to their adjusted p value (from the smallest).

Gene Name	Fold Change	
EIF1AX	1.39	
HLADRB5	9.09	
MBTPS2	1.72	

Gene Name	Fold Change	
EIF2S3	1.25	
TMEM248	1.18	
RASA3	1.20	
JPX	1.56	
PSMD5	1.33	
POLR1B	1.43	
AAR2	1.25	
CCDC71	1.35	
BRIX1	1.32	
VMA21	1.35	
NA	1.44	
BNIP3P17	4.76	
PRUNE	1.28	
RIOK2	1.25	
CEP44	1.25	
IFIT5	1.45	
GUF1	1.25	
PTGIR	1.25	
NKAP	1.33	
NAPEPLD	1.32	

To identify the correlations between RNA-sequencing data and the proteomics data, genes which were significantly differentiated between male HBCs and female HBCs (adjusted $p<0.05$) were analysed for the expressions in proteomics.

Table 3 shows the comparison of the expressions of genes with significant differences and the corresponding proteins. This excludes the genes where no corresponding proteins were detected. Fold changes are rates of male expression to female's. NaN suggests no information was available.

Gene Name	Fold Change in RNA Seq	Fold Change in Proteomics
EIF1AX	0.72	0.92
HLADRB5	0.11	0.91
EIF2S3	0.80	1.17
COX20	1.90	NaN
HEBP2	1.37	1.21
PSMD5	0.75	0.84
POLR1B	0.70	1.08
RING1	1.24	0.92
AAR2	0.80	NaN
AHSP	10.37	NaN
BRIX1	0.76	0.70
S100A1	4.05	1.93
WNK2	7.68	NaN
CEP44	0.80	NaN
IFIT5	0.69	0.68
GUF1	0.80	NaN

Table3: Of 49 genes with adjusted $p<0.05$, corresponding proteins were detected for 16 genes. Of these, 2 genes and their corresponding proteins were both up-regulated in male HBCs (font in red), while 5 genes and their corresponding proteins were both down-regulated in male HBCs (font in blue). None of these corresponding proteins were identified as significant ($p<0.05$) in the proteomics data.

To identify the correlations between the proteomics data and RNA-sequencing data, proteins which were significantly differentiated between male HBCs and female HBCs ($p<0.05$) were analysed for the expressions in RNA-sequencing.

Table 4 shows the comparison of the expressions of proteins identified as significant in the proteomics data and their corresponding genes. This excludes the proteins where no corresponding genes were detected. Fold changes are rates of male expression to female's.

Gene Name	Fold Change in Proteomics	Fold Change in RNA Seq
ALDH1A1	6.18	4.17
GSTM1	2.56	0.77
FUCA2	2.49	1.11
AMACR	2.29	1.02
GLA	2.23	1.19
ARF4	2.08	1.03
NNT	2.00	0.93
DPYSL3	1.98	1.32
IQGAP2	1.93	1.43
XPO7	1.91	0.98
PPP3CA	1.89	1.14
SERPINB9	1.67	0.99
MPV17	1.67	1.14
SAR1B	1.65	0.86
SEC24A	1.51	0.95
GBE1	1.50	1.01
AP3M1	1.46	0.92
PSME3	1.43	0.92
PSMB2	1.36	0.96
TRAPPC3	1.34	1.11
NDUFA6	1.31	1.03
RAP1GDS1	1.31	0.86
NSF	1.30	0.94
ARCN1	1.29	0.94
TANC1	1.12	0.89
XRN2	0.84	0.93

Gene Name	Fold Change in Proteomics	Fold Change in RNA Seq
SRSF3	0.75	1.05
ADAR	0.75	0.92
PDXDC1	0.75	0.88
SON	0.73	1.02
IK	0.71	1.00
HNRNPAB	0.71	1.09
SF3B4	0.70	0.92
DKC1	0.69	0.82
HIST1H2BC	0.68	1.30
DPY30	0.68	1.05
MGEA5	0.66	0.99
UQCRH	0.65	1.13
MRPL12	0.63	0.88
TCOF1	0.63	0.98
DDX50	0.61	0.85
ENPEP	0.60	0.83
H1FX	0.60	1.11
GPX8	0.58	0.81
PTMS	0.55	1.12
GGCX	0.53	0.94
ENO2	0.53	0.79
CREB1	0.50	0.88
SHROOM2	0.50	0.90
HIST1H2AJ	0.47	0.91
RANBP3	0.44	0.98
CA1	0.40	1.94

Table4: Of 86 proteins which were significantly differentially expressed between male HBCs and female HBCs, corresponding genes were detected for 52 proteins. Of these, 12 proteins and their corresponding genes were both up-regulated in male HBCs (font in red), while 17 proteins and their corresponding genes were both down-regulated in male HBCs (font in blue). None of these corresponding genes were identified as significant (adjusted p<0.05) in the RNA-sequencing data.

To identify the signalling pathways between differentially expressed genes, results from RNA-sequencing data were applied to KEGG (Kyoto Encyclopedia of Genes and Genomics) by comparing all significant genes (adjusted p value<0.05) to all the genes obtained in the analysis and pathways overrepresented were identified (the signalling pathways analysis was done with the kind help from Dr. Toluwalase Awoyemi).

Amongst the pathways obtained, "cytokine-cytokine receptor interaction pathway" was most related to the functions of Hofbauer cells as immune cells and had the smallest p value (0.001) and adjusted p value (0.073).

Figure 11 shows the entire cytokine-cytokine receptor interaction pathway.

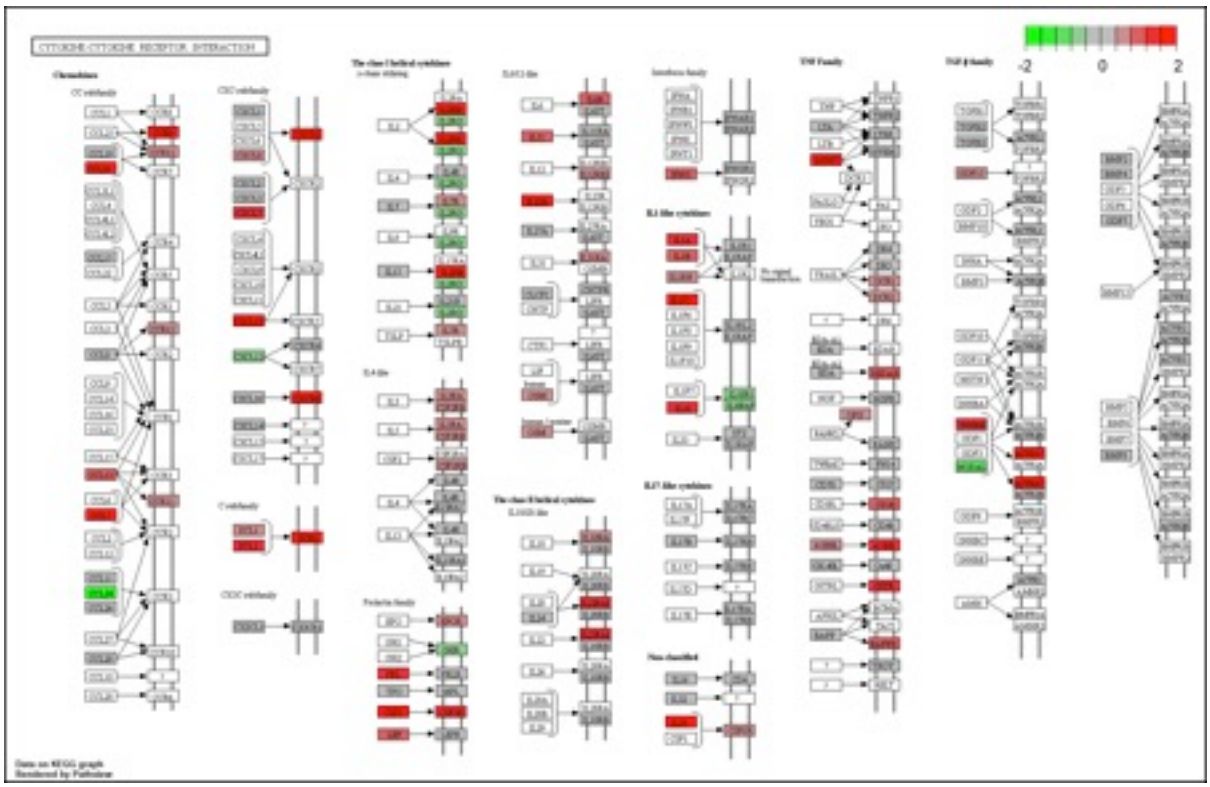


Figure11: The entire cytokine-cytokine receptor interaction pathway. Scale indicating the fold change is at the right top corner (male expression rates versus female's).

Cytokine-cytokine receptor interaction pathway consists of 8 parts from 265 genes. The eight main components are chemokines, the class I helical cytokines, the class II helical cytokines, IL-1 like cytokines, IL-17 like cytokines, non-classified, TNF family, and TGF-β family.

To show the pathway in a clearer way, these components are shown individually.

Figure 12 shows the part of chemokines.

Chemokines

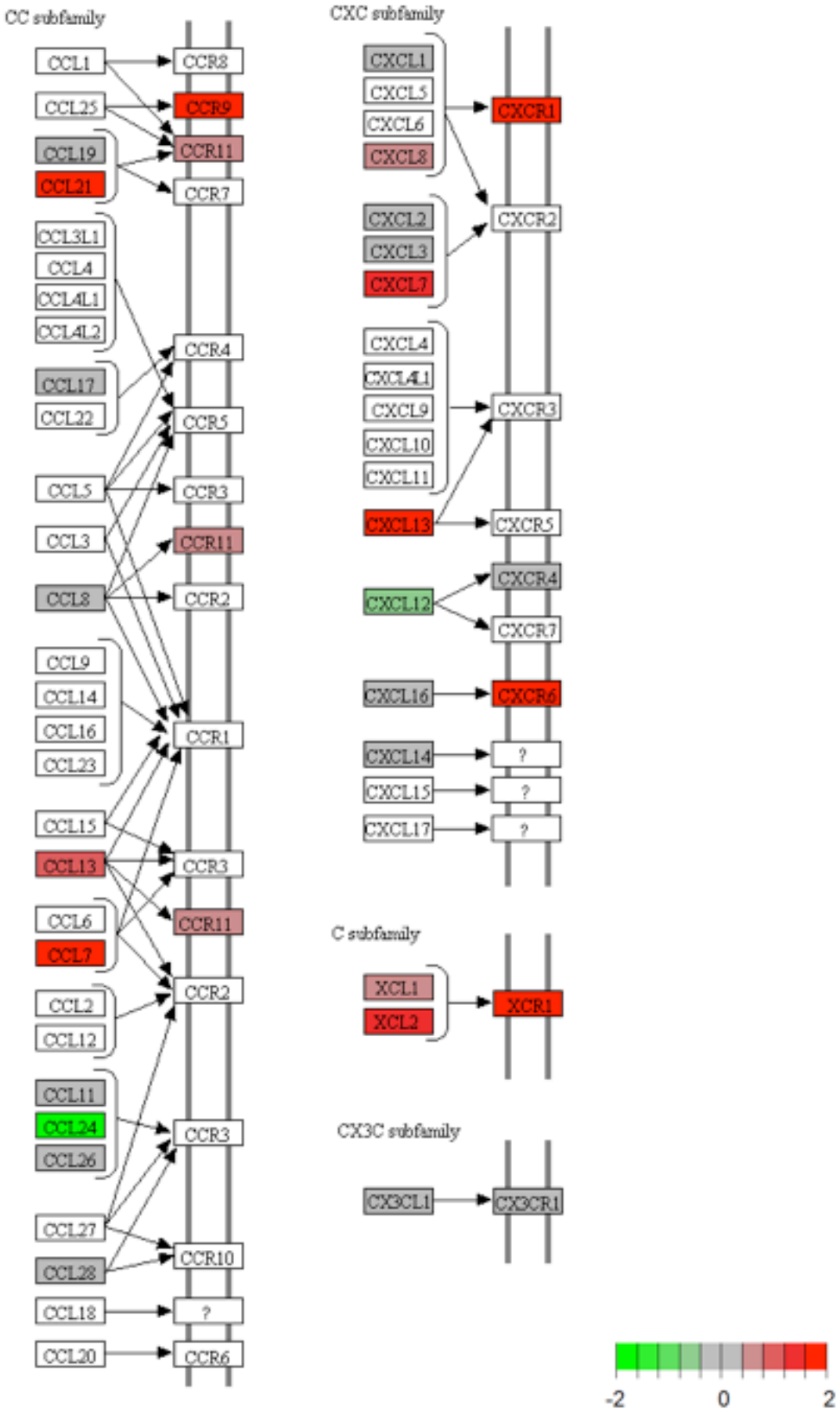

CC subfamily
CCL1
CCL25
CCL19
CCL21
CCL3L1
CCL4
CCL4L1
CCL4L2
CCL17
CCL22
CCL5
CCL3
CCL8
CCL9
CCL14
CCL16
CCL23
CCL15
CCL13
CCL6
CCL7
CCL2
CCL12
CCL11
CCL24
CCL26
CCL27
CCL28
CCL18
CCL20
CCR8
CCR9
CCR11
CCR7
CCR4
CCR5
CCR3
CCR11
CCR2
CCR1
CCR3
CCR11
CCR2
CCR3
CCR10
?
CCR6
CXC subfamily
CXCL1
CXCL5
CXCL6
CXCL8
CXCL2
CXCL3
CXCL7
CXCL4
CXCL4L1
CXCL9
CXCL10
CXCL11
CXCL13
CXCL12
CXCL16
CXCL14
CXCL15
CXCL17
CXCR1
CXCR2
CXCR3
CXCR5
CXCR4
CXCR7
CXCR6
?
?
?
C subfamily
XCL1
XCL2
XCR1
CX3C subfamily
CX3CL1
CX3CR1
-2
0
2

Figure12: Chemokines in the cytokine-cytokine receptor interaction pathway. There were more chemokines which were up-regulated in male HBCs compared to female HBCs, especially in the C subfamily.

Figure 13 shows the part of class I helical cytokines.

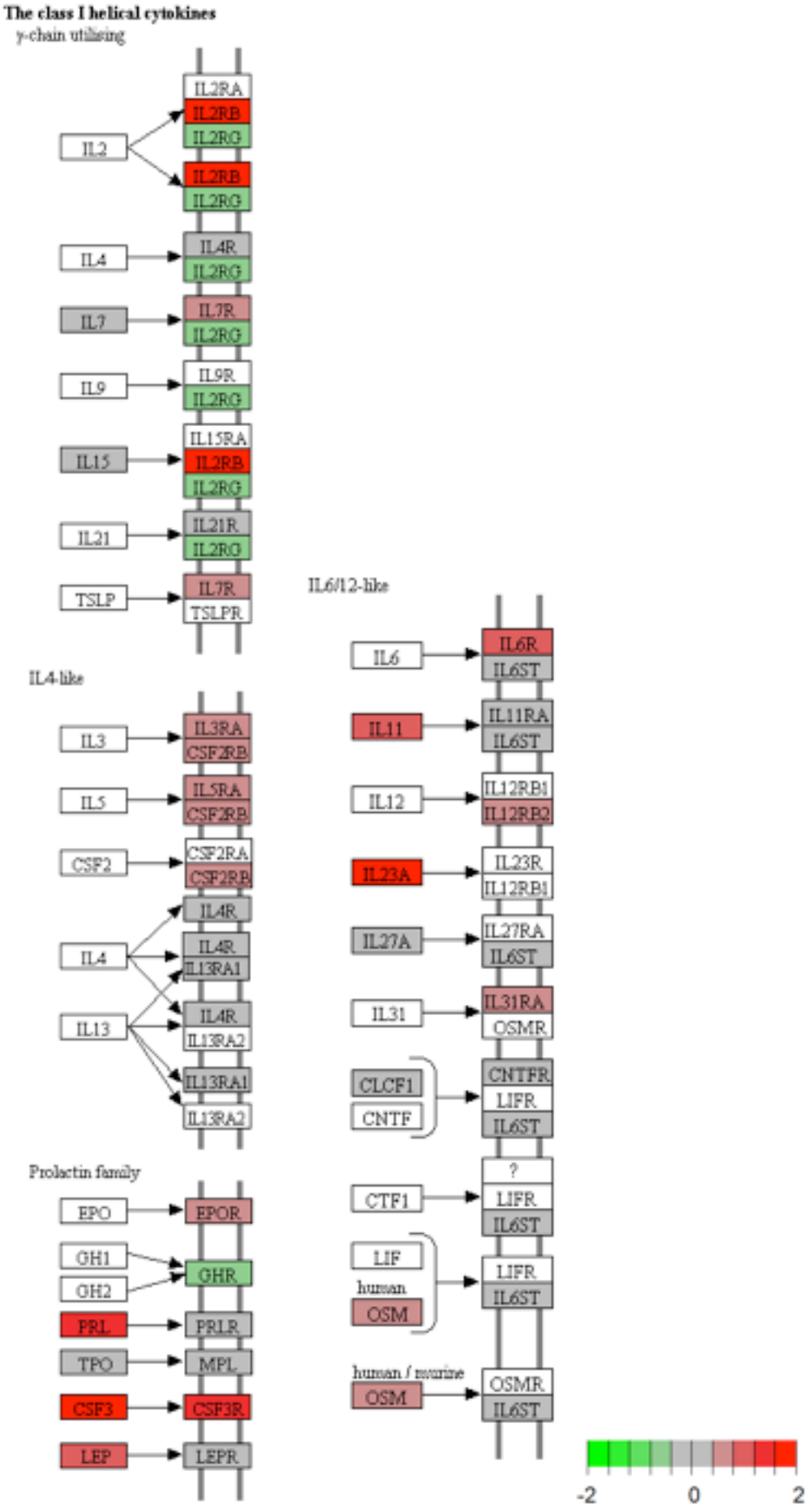

The class I helical cytokines
γ-chain utilising
IL2
IL2RA
IL2RB
IL2RG
IL2RB
IL2RG
IL4
IL4R
IL2RG
IL7
IL7R
IL2RG
IL9
IL9R
IL2RG
IL15
IL15RA
IL2RB
IL2RG
IL21
IL21R
IL2RG
TSLP
IL7R
TSLPR
IL4-like
IL3
IL3RA
CSF2RB
IL5
IL5RA
CSF2RB
CSF2
CSF2RA
CSF2RB
IL4
IL4
IL4R
IL13RA1
IL13
IL4R
IL13RA2
IL13RA1
IL13RA2
Prolactin family
EPO
EPOR
GH1
GH2
GHR
PRL
PRLR
TPO
MPL
CSF3
CSF3R
LEP
LEPR
IL6/12-like
IL6
IL6R
IL6ST
IL11
IL11RA
IL6ST
IL12
IL12RB1
IL12RB2
IL23A
IL23R
IL12RB1
IL27A
IL27RA
IL6ST
IL31
IL31RA
OSMR
CLCF1
CNTF
CNTFR
LIFR
IL6ST
CTF1
?
LIFR
IL6ST
LIF
human
OSM
LIFR
IL6ST
human / murine
OSM
OSMR
IL6ST
-2
0
2

Figure13: Of class I helical cytokines, there were more cytokines and cytokine receptors which were up-regulated in male HBCs, while IL2RG, which is located on X chromosome, was highly regulated in female HBCs.

Figure 14 shows the part of class II helical cytokines.

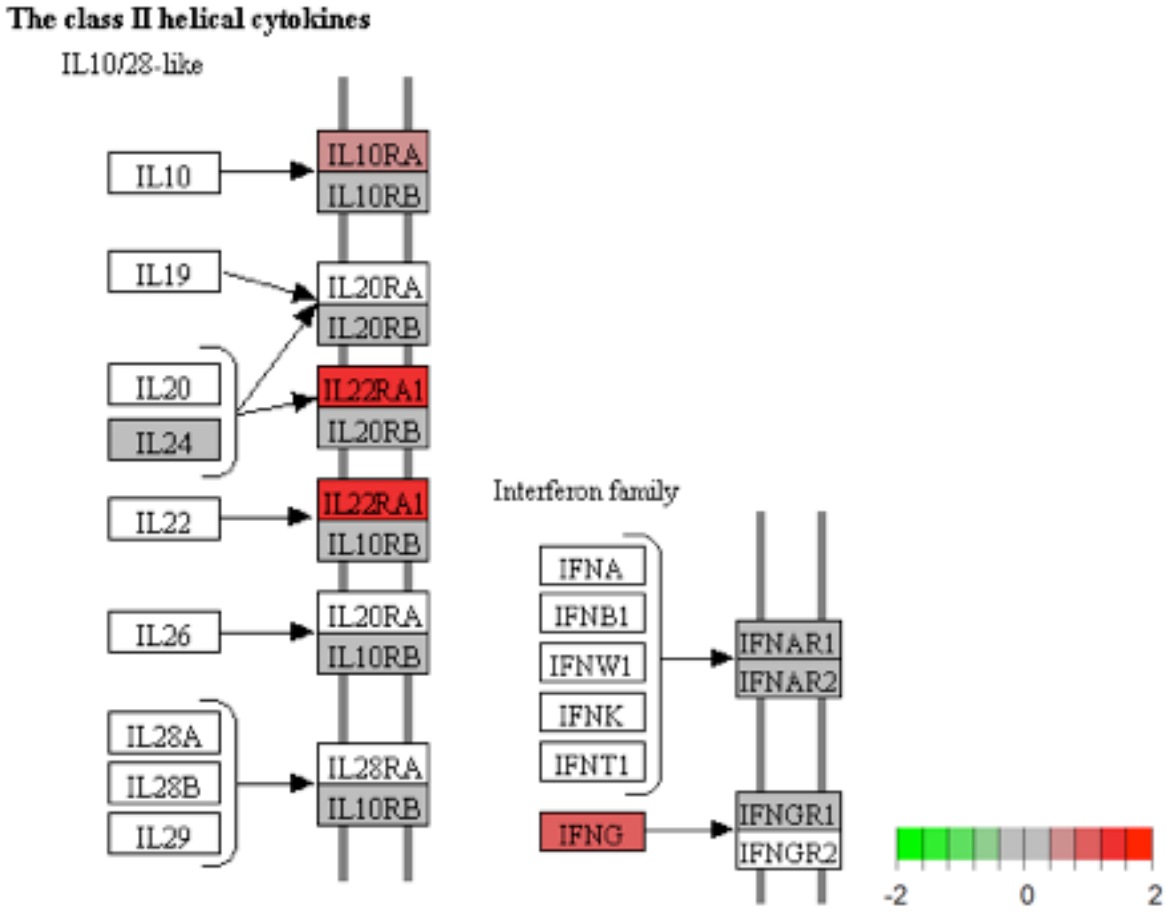


Figure14: Of class II helical cytokines, differentially regulated cytokines and cytokine receptors were all up-regulated in male HBCs.

Figure 15 shows the part of IL-1 like cytokines, IL-17 like cytokines, and non-classified.

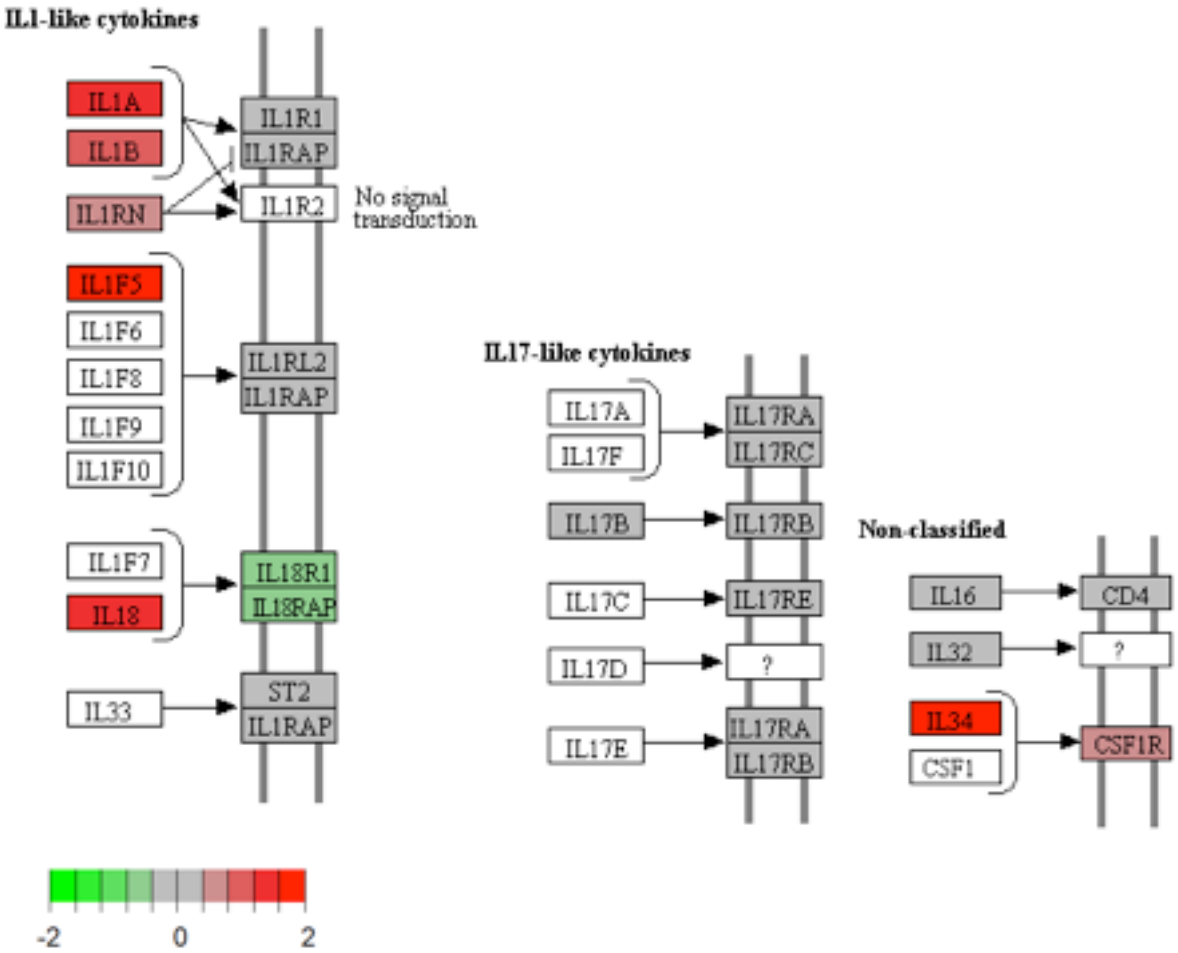


Figure15: IL-1 like cytokines, IL-17 like cytokines, and non-classified in the cytokine-cytokine receptor interaction pathway. No differentially regulated genes were identified in IL-17 like cytokines.

Figure 16 shows the part of TNF family.

TNF Family

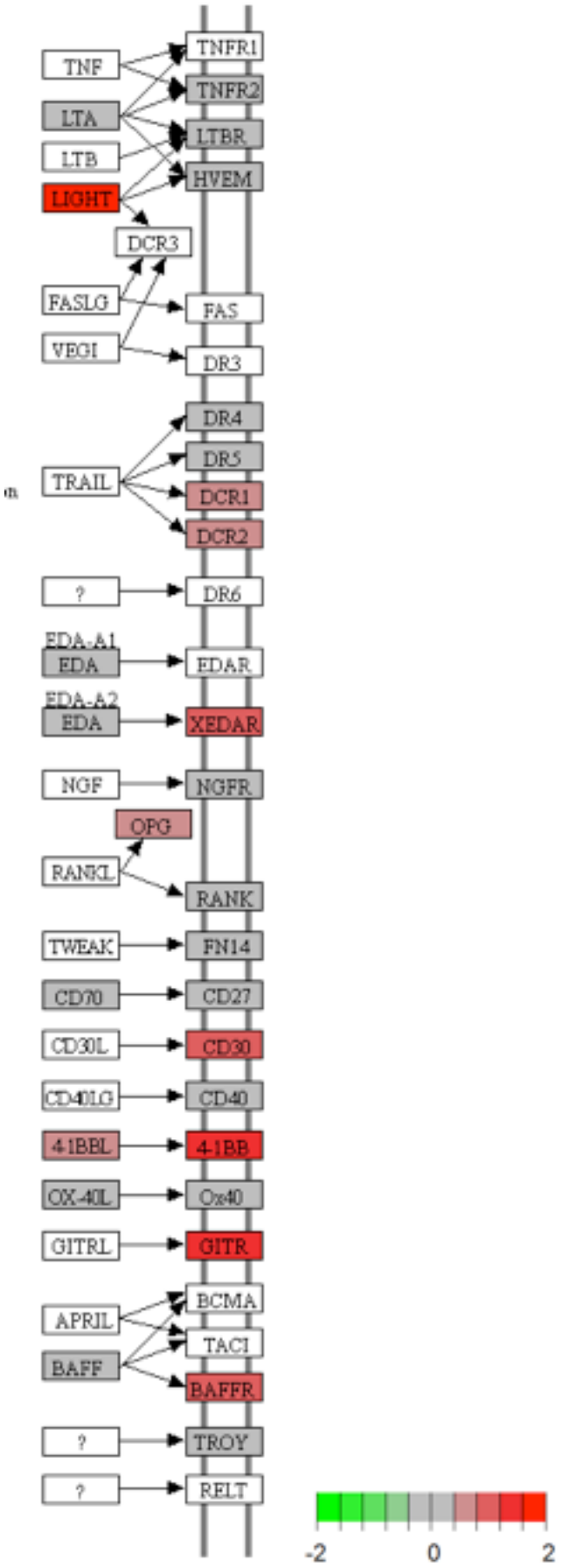

TNF
LTA
LTB
LIGHT
FASLG
VEGI
TRAIL
?
EDA-A1
EDA
EDA-A2
EDA
NGF
RANKL
TWEAK
CD70
CD30L
CD40LG
4-1BBL
OX-40L
GITRL
APRIL
BAFF
?
?
TNFR1
TNFR2
LTBR
HVEM
DCR3
FAS
DR3
DR4
DR5
DCR1
DCR2
DR6
EDAR
XEDAR
NGFR
OPG
RANK
FN14
CD27
CD30
CD40
4-1BB
Ox40
GITR
BCMA
TACI
BAFFR
TROY
RELT
-2
0
2

Figure16: In TNF family, the differentially regulated cytokines and cytokine receptors were all up-regulated in male HBCs.

Figure 17 shows the part of TGF-β family.

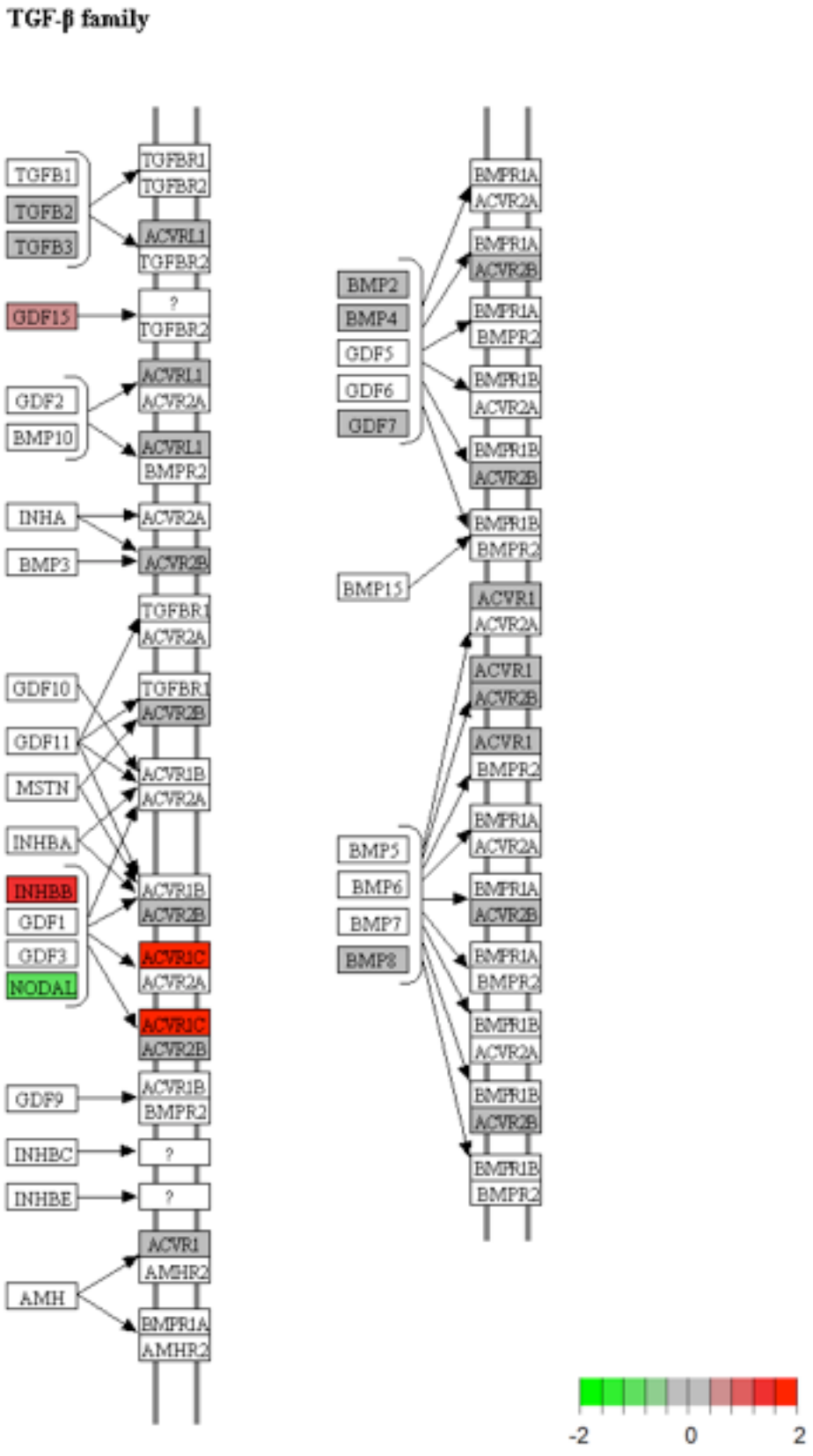


Figure 17: TGF-β family in the cytokine-cytokine receptor interaction pathway.

Discussion for results chapter 3

To analyse the RNA-sequencing of HBCs, next-generation sequencing was applied. The innovation in the field of genomics traces its history back to the breakthrough achieved by Sanger sequencing, which was considered by F. Sanger et al in 1977[199]. Sanger sequencing, also called the traditional sequencing, detects the DNA sequence by randomly incorporating the dideoxy terminators to stop the elongation of DNA chains, resulting in the formation of diversely sized DNA fragments. The inserted dideoxy terminators are usually radioactively or fluorescently labelled to be detected by equipped machines. Sanger sequencing, and other sequencers which were based on Sanger sequencing and improved from it are generally regarded as the first-generation DNA sequencing machines. They generate reads sightly less than one kilobase (Kb) in length. To analyse longer strands, the overlapping parts of DNA fragments had to be duplicated and sequenced separately, then combined into one sequence[200]. It is not hard to imagine how difficult it would be to analyse the whole human genome solely relying on the technical power provided by Sanger sequencing. Human Genome Project (HGP), which was founded in 1990, aimed to sequence and interpret the 3.2 billion nucleotide base pairs constituting the human genome in order to discover their potential involvements in various medical conditions. The cost for this project was estimated to be massive until the introduction of next-generation sequencing (NGS) was made in the early 2000's[201]. NGS conducted by illumina® consists of four steps - sample preparation, cluster generation, sequencing, and data analysis. Sample preparation starts from extracting genetic materials from interested samples. In the case of RNA sequencing, unstable RNAs are usually converted to complementary DNAs (cDNAs) with the aid of reverse transcriptase and DNA polymerase. Subsequently, sequencing libraries are constructed by generating numerous DNA fragments and attaching the 'adaptors' to the both ends of these fragments. Adaptors possess not only primer binding sites and capture sequences, but also sample specific tags. Once the sequencing libraries are completed, cluster generation will be performed on flow cells. The flow cell is a glass slide with lanes of small fluidic channels. Each channel is coated with multiple oligonucleotides of two types. Hybridisation is made possible as capture sequences in the adaptors will bind with the complementary oligonucleotides attached on the flow cell. Single strand will be complimentarily replicated by primer into double strands. The double strand molecule will then be denatured and the original strand will be washed away. The strand will fold over to make a bridge so the adaptor region will complimentarily hybridise to the second type of oligonucleotides on the flow cell. Primer will create the complimentary strand, forming a double strand bridge. This bridge will be

denatured, generating two single strand copies of the molecule that are bound to the flow cell. This process will be repeated over and over, resulting in the generation of millions of amplifications for all DNA fragments. The reverse strands will be cleaved and removed, leaving only the forward strands on the flow cell. Subsequently, sequencing is executed by adding one fluorescently labelled nucleotide at a time based on the sequence of the template. And the fluorescent signals will be captured. This way of sequencing is termed sequencing by synthesis. The length of read will be determined by the number of cycles. All identical strands are read at the same time in a cluster. Immense clusters will be sequenced in a paralleled manner. Amplifications by double strand bridge will be repeated as occurs in the cluster generation. The reverse strand will be left this time instead of the forward strand. Sequencing process will be continued until the desired length is achieved. Finally, for data analysis, sequences obtained from all fragments will be separated based on the specific indices tagged in the sample preparation. Strands with similar weights will be clustered and forward reads will be paired with reverse reads. Contiguous sequences generated in this way will be lined up to create a reference genome. As a result, multiple ways of analysis are enabled in the end[202].

For the comparison of expressions of mRNAs and corresponding proteins and vice versa, the results did not show an exact correlated relationship between them, which should not be received as surprising. Though there might be a strong expectation that coding genes of mRNAs should be translated into proteins accordingly as the central dogma suggests, in the real biological environment, the protein concentrations often do not follow a strong correlation with the abundances of their corresponding mRNAs - normally only ~40% of the variation in protein concentrations can be described from the known abundances of their corresponding mRNAs[203]. To understand the variation in the remaining 60%, several processes after mRNA is made should be considered. Firstly, mRNAs are much less stable and have much shorter half-lives compared with proteins. As a result, the concentrations of mRNAs in cells fluctuate much more than those of the corresponding proteins which have longer lives. Secondly, regarding transcriptional or post-translational regulation, gene-specific translate rates have to be taken into account[203][204]. It has been discovered that proteins which are engaged in transcriptional regulation and chromatin organisation are more likely to be produced and degraded speedily, while structural proteins like housekeeping proteins are more durable. From these facts, it is logical to consider that the functions of proteins and individual biological environment surrounding proteins are extremely important in the regulation of the concentrations of proteins. Furthermore, microRNAs (miRNAs) and RNA-binding proteins also play essential roles in transcriptional and post-translational regulation[203]. Lastly, though there has been significant

improvement in the technique regarding proteomics and transcriptomics, there are still certain error and noise in both protein and mRNA related experiments that prevent us from getting a clearer image[205]. In general, protein abundances are more evolutionarily conserved than the levels of their corresponding mRNAs[203]. As a conclusion, although the levels of proteins should be credited with more importance in elucidating the underlying mechanisms, the information from mRNA and protein levels are complementary and both are indispensable to obtain an integrated appreciation of the functions of the cells. There might not be any significant correlations between them, however, the fact itself is revealing and valuable[205].

To analyse the data from RNA-sequencing, data was further analysed in R with a package of Cluster Profiler and Pathview. Cluster profiler performs a gene set enrichment analysis utilising KEGG pathways. There are many enrichment factors including the number of genes detected, the expression pattern of the genes and probably the most important element - the sample size, which influence the adjusted p value in a pathway. Considering that cytokine-cytokine receptor interaction pathway consists of 8 parts (chemokines, the class I helical cytokines, the class II helical cytokines, IL-1 like cytokines, IL-17 like cytokines, non-classified, TNF family and TGF-β family) from 265 genes, p value of 0.001 in the pathway we obtained before multiple comparison testing is still highly scientifically suggestive. From the cytokine-cytokine receptor interaction pathway acquired, it is noteworthy that several cytokines and cytokine receptors that are involved in inflammation and the pathogenesis of pregnancy-related medical conditions such as preeclampsia (PE) are elevated in male HBCs. These include IL-11, IL23A, IL-1 (IL1A, IL1B, IL1RN and IL1F5), IL22RA1, and IL34. IL-11, originally from the bone marrow stromal cell line, is known as a haematopoietic growth factor with versatile functions. It stimulates myelopoiesis, megakaryopoiesis and erythropoiesis[206]. It regulates cell cycle, invasion and migration in various cell types, playing a crucial role in the process of placentation[207]. Although IL-11 is indispensable in decidualization[206][207], in vitro it prevents the invasion of human extravillous trophoblasts (EVT) and is enhanced in the decidua in women who suffer PE. Further research has found that the level of IL-11 in serum increases not only in women who suffer PE, but also in those who are prior to the onset of PE, indicating the predictive role of IL-11 in PE. Administration of IL-11 to mice caused impaired trophoblast invasion, spiral artery remodelling, and placentation, all of these having resulted in elevated systolic blood pressure, proteinuria and intrauterine growth restriction, the features of PE. The mechanism that IL-11 is involved in the pathogenesis of PE is mediated by pappalysin-2 (PAPPA2), which is up-regulated by IL-11 and impedes the outgrowth of human EVT[207], and STAT3, the inhibitor of which lessens the inhibiting effects caused by IL-11 on EVT[208]. IL-23, which is produced by

macrophages and dendritic cells, is a key element in inflammatory responses. Along with TGF-β, it promotes the differentiation of $CD4^+$ T cells into IL-17-producing Th17 cells[209]. Both IL-23 and IL-17 play important parts in the pathogenesis of many autoimmune conditions, such as psoriatic arthritis, inflammatory bowel diseases, and spondyloarthritis[210]. In pregnancy, IL-23 enhances the concentration of matrix metallopeptidase 9 (MMP-9) locally and facilitates the process of angiogenesis. These functions have made IL-23 an extremely important factor in proper implantation and placentation. However, clinical studies have disclosed that the levels of expressions of IL-23 are increased in patients with recurrent pregnancy loss. Recent studies support this fact by having revealed that IL-23 is the key pro-inflammatory cytokine released by dendritic cells and dendritic cells present the antigens in inflammatory processes through the regulation of expressions of IL-23, which eventually leads to the secretion of pro-inflammatory IL-17. This is especially critical in early pregnancy when dendritic cells present antigens of paternal origin promoting or inhibiting the growth of relevant T cell subpopulations, which gives a fundamental impact on the tolerance or rejection of syncytiotrophoblast cells[209].

The IL-1 cytokine family are influential members of the innate immune system, linking the innate and adaptive immune responses. In pregnancy, IL-1A and IL-1B are the most-investigated pro-inflammatory cytokines and it has been confirmed that the level of IL-1B is increased in the plasma of women who have preeclampsia. Most IL-1 cytokine members activate the receptors belonging to the toll-interleukin receptor (TIR) family, inducing the activation of nuclear factor kappa B (NF-κB) and pro-inflammatory effectors[211]. These can be observed in the inflammations triggered by non-infectious conditions including preeclampsia. IL-22, a cytokine produced by diversified immune cells including Th17 cells, plays pivotal roles in tissue repair and host defence at mucosal surfaces. It functions via the IL-22 receptor, a heterodimer composed of unique alpha subunit - IL-22RA1, and a common IL-10 beta subunit - IL10RB. IL-22 works towards epithelial and parenchymal cells at different barrier sites and organs such as lung, liver, intestine and skin. It stimulates the development of epithelial cells, which in the placenta are trophoblast cells originated from the fetus. Trophoblast cells are proved to be responsive to the stimuli released by IL-22 in their proliferation and reduced expressions of IL-22 are found to be involved in the events of spontaneous miscarriage[212]. In pregnancy, IL-22 is not only essential for the regeneration of trophoblast cells and wound repair, it also has crucial roles regarding the defence against pathogens at the maternal-fetal interface. In mice, it has been found that IL-22 simultaneously increases its expression in response to LPS stimulation and contributes to the prevention of the apoptosis of placental cells, which has a protecting role in reducing the preterm delivery induced by LPS[212]. In preeclamptic

pregnancies, the level of IL-22 is increased in mothers as well as in the cord blood of newborns, suggesting the underlying compensational system responding to the dysfunction in the placenta and all other organs caused by preeclampsia[213]. Lastly, IL-34, a cytokine engaged in the regulation of multiple tissue-resident macrophages such as microglia, osteoclasts and Langerhans cells, is a substantial macrophage-regulating agent along with macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are important in polarising macrophages to a M2 type and M1 type respectively. In pregnancy, it has been demonstrated that IL-34 facilitates the polarising of decidual macrophages to a M2-like regulatory phenotype, presenting immunosuppressive traits including the suppression of the proliferation of T cells. This function of IL-34 is extremely important in the acquirement of tolerance at the maternal-fetal interface during early pregnancy[214]. The expression of IL-34 is enhanced in patients with obesity and positively correlated with insulin resistance. In patients with gestational diabetes mellitus (GDM), both the mRNA and protein expression of IL-34 are elevated in placental tissues, cord blood and serum. Upregulation of IL-34 not only impairs the secretion of insulin by pancreatic β cells but also leads to the apoptosis of pancreatic β cells, which expand the sizes of themselves in pregnancy to compensate for the insulin resistance seen in pregnancy. The apoptosis is considered to be induced via the IL-34/CSF-1R/ STAT3 pathway[215]. Taking into account the fact that male fetus is more likely to suffer GDM compared to female fetus[98], it is tempting to consider that the overexpression of IL-34 and its receptor CSF-1R in male HBCs could be an underlying reason for the mechanism.

Other striking discoveries from the pathway are for example from the chemokine pathway, many chemokines and their related receptors were up-regulated in male HBCs. Considering the diversified functions of chemokines in pregnancy including the enrichment of immune cells, promotion of the invasion by trophoblast cells, and neoangiogenesis[216], the upregulation of the expressions of various chemokines and corresponding receptors in male HBCs may suggest the fact that it is a part of the endeavour that male placenta manages to create a supportive milieu during pregnancy.

In the pathway regarding TNF family, LIGHT, a type II transmembrane protein, is a relatively new member of the TNF family and recently been found to be involved in the pathogenesis of preeclampsia. Being an immune signalling component, LIGHT acts on two specific cellular receptors - lymphotoxin β receptor (LTβR) and herpes virus entry mediator (HVEM). In preeclamptic patients, the expression of LIGHT is increased and enhanced LIGHT signalling leads to the secretion of vasoconstrictive factors including endothelin-1 (ET-1) and antiangiogenic agents such as sFlt-1.

These subsequently trigger the dysfunction of the placenta and maternal organs, resulting in the occurrence of maternal symptoms and IUGR[217]. 41BB and glucocorticoid-induce tumour necrosis factor-related receptor (GITR) are both co-stimulatory molecules for T cells. 41BB tends to break the immunotolerance by enforcing the immune responses via $CD8^+$ T cells[218], while GITR alleviates immunotolerance on the other hand[219].

Finally, in TGF-β pathway, ACVR1C also known as ALK-7, was highly up-regulated in male HBCs. TGF-β has an inhibitory impact on the proliferation of trophoblast cells functioning via ALK-7 and the overexpression of ALK-7 has been observed in preeclamptic patients[220]. Other functions of ALK-7 in pregnancy still needs to be explored.

All of the components identified in cytokine-cytokine receptor interaction pathway could not be identified in the results from proteomics. The reason for this is considered to be that the technique of proteomics is not sensitive enough to capture and compare the expressions of chemokines and cytokines yet.

Summarily, though there are some genes which are related to immune functions and are located on X chromosomes, such as IL2RG, male HBCs manage their immunocompetence by regulating the expressions of chemokines, chemokine receptors and several cytokines, cytokine receptors including IL-11, IL23A, IL-1 (IL1A, IL1B, IL1RN and IL1F5), IL22RA1, IL34 and IL2RB, as well as members from the TNF and TGF-β family. Some of them are heavily involved in the pathogenesis of pregnancy related conditions represented by preeclampsia and GDM. To understand the mechanism of how these cytokines function in a more detailed way could be a logic and scientific approach to answering the questions why male fetuses have more serious preeclampsia compared to female fetuses[96] and why male fetuses have more GDM cases compared to female fetuses[98].

Final discussion

This project started from the establishment of the methodology of isolating HBCs from the whole placenta with high yield and purity, which was a new and difficult process. After the methodology of isolating pure HBCs was established, analysis of HBCs utilising proteomics was performed. Proteins that were identified as differentially regulated between male HBCs and female HBCs were found to be involved in mainly four pathways - immune functions, mitochondrial functions, vesicular functions and transcriptional regulation functions. Validation of the results from proteomics have been conducted by application of western blotting. Subsequently, sexual differences of HBCs at RNA levels have been investigated using next-generation sequencing. Comparisons between the results of deep sequencing and proteomics have been made, also comparisons have been performed the other way around between the results of proteomics and deep sequencing. To determine pathways between differentially regulated genes between male HBCs and female HBCs, data was further analysed on R and applied for KEGG pathways, and amongst multiple pathways acquired, cytokine-cytokine receptor interaction pathway was the most significantly different with the smallest p value as well as with adjusted p value. Considering that HBCs are immune cells, this pathway is also regarded as the most important pathway concerning the functions of HBCs from the prospects of mRNAs. From cytokine-cytokine receptor interaction pathway obtained, male HBCs had significantly higher expression of most chemokines as well as in interleukins and cytokines. Amongst these discoveries, several interleukins and interleukin receptors which were significantly up-regulated in male HBCs including IL-11, IL23A, IL-1 (IL1A, IL1B, IL1RN and IL1F5), IL22RA1, and IL34, all having essential roles in the maintenance of pregnancy with some of them engaged in the pathogenesis of pregnancy-related conditions represented by preeclampsia and GDM. Summarily, while there are specific genes committed to immune functions which are located on X chromosomes and have higher expressions in female HBCs probably due to the escape from X chromosome inactivation such as IL2RG, male HBCs regulate their expressions in multiple chemokines, interleukins, and cytokines to sustain their immunocompetence. Some of them are heavily involved in the pathogenesis of pregnancy related conditions such as preeclampsia and GDM. To elucidate the underlying mechanisms associated with the discoveries clarified in proteomics and RNA-sequencing is a novel and critical approach to resolving the clinical questions such as why female fetuses may suffer more infective complications e.g. HIV infection[99] and more mild preeclampsia cases compared to male fetuses, and why male fetuses have more GDM[98] and more serious preeclampsia cases compared to female fetuses[96].

For the limitations for this project, firstly, it might be considered that isolating HBCs from placentae of preeclampsia and GDM would be more suitable to demonstrate the differences between HBCs in pregnancy related conditions including preeclampsia and GDM and in normal states (controls). However, the experiments performed in this thesis permitted more fundamental differences in sexual dimorphism to be identified. It would be important to consider the role of sexual dimorphic differences in studies looking at preeclampsia or GDM. Secondly, for the validations of the results concerning chemokines, interleukins and cytokines, culturing HBCs and collecting the plasma for further analysis could be suggested as one approach. Having said so, HBCs are fully differentiated cells, they do not divide and are less suitable for culturing. In fact, several attempts of culturing HBCs have been made and the results were not ideal. If there had not been COVID-19, I would have done further functional analysis regarding PSME3 and PSMB2 as identified in the immune function pathway in proteomics, as we consider these two proteins carry substantial roles in antigen processing and presentation via MHC class I, which are extremely important in defence against intracellular pathogens including HIV virus. Also validations of gene variations by quantitative PCR (qPCR) were also planned but could not be performed due to COVID-19.

For sexual dimorphism regarding general medical conditions, in general, males are more likely to suffer major chronic conditions, which can endanger their lives. These include coronary heart disease, cerebrovascular disease, cancer, atherosclerosis, emphysema, cirrhosis, and kidney disease, while females are more susceptible to chronic conditions which compromise their quality of life such as thyroid and gall bladder conditions, anaemia, arthritis, migraines, eczema and colitis[221)]. Clinical trials often reject female participants with the intention of protecting them and their unborn children from negative adverse effects that can be possibly caused. As a result, data acquired from male participants often have to be applied to females to predict likely outcomes, except for the reproductive period[221)]. However, various factors related to pharmacokinetics ranging from the differences in body surface (females have lower body surface area), in kidney function, in processes of drug resorption to metabolic processing by hepatic enzymes and excretion have been demonstrated to be significantly varied between females and males. Additionally, differences in elements associated with pharmacodynamics have also become clear. For example, ion channels in heart tissue and kidney are different between females and males[222)]. As a conclusion, it is more important than ever to clarify the impacts of sexual dimorphism and deliver medical care in a sex-specific manner. Also, for areas where detailed data are missing, more investigations are required. For instance, more research may be required on males analysing depression and osteoporosis, whilst more investigations on females concerning cardiovascular conditions[222)].

Although this thesis has been mainly focused on the biological impacts of sexual dimorphism, especially in the placenta, the differences in lifestyle between males and females, differences in economic status which can affect their access to health care, also differences in life experiences which can guide their choices of medical care are some other big factors that should be taken into account when investigating the possible influence of sexual dimorphism on the outcomes of healthcare[221)222)].

For the future work, although there are a few studies which tried to identify the differences between different sexes in other cells types in the placental villi including syncytiotrophoblast and trophoblasts[223)224)], the results remain limited and it is tempting to identify the traits further regarding sexual dimorphism in these cells and compare the results with we have obtained from Hofbauer cells.

Also, further investigations of proteins which were identified highly differentially regulated between male and female Hofbauer cells such as ALDH1A1 would be another novel approach to elucidate their functions in the placental tissue. ALDH1A1 is known to be involved in cell differentiation and self-renewal[225)], and also in multiple cell signalling pathways as described in the discussion in chapter2, however the function of ALDH1A1 is very tissue specific and its roles in placental tissues are not well explored yet.

In summary, HBCs exhibit significant differences in proteomic and genetic signature in male and female placentae. It is likely these differences confer functional alterations in immune cell function which may impact a number of critical functions.

APPENDIX

APPENDIX1:

Details of contents for trypsin solution, collagenase D solution

1 Contents for trypsin solution (all materials were obtained from Sigma-Aldrich unless stated):

0.25% trypsin 3ml

0.2% DNase I 60µl

25mM HEPES 750µl

2mM $CaCl_2$ 15µl

0.8mM $MgSO_4$ 12µl

Hank's balanced salt solution (HBSS) 26.163ml

Total volume: 30ml

2 Contents for collagenase D solution (all materials were obtained from Sigma-Aldrich unless stated):

Collagenase D (dissolved in 10mg/ml with RPMI 1640 medium): 3ml

DNase I (dissolved in 0.2mg/ml with RPMI 1640 medium): 6ml

Roswell Park Memorial Institute (RPMI) 1640 Medium: 21ml

Total volume: 30ml

APPENDIX2:

The components of lysis buffer and the detailed procedures for preparing for protein lysates

Lysis Buffer

Components	10mL	500mL
50mM Tris-HCL pH7.4	60.57mg	3g
150mM NaCl	87.66mg	4.4g
1mM EDTA	3.72mg	186mg
0.1% SDS (10% stock)	100µL	5000µL
1% Triton	100µL	5000µL
Protease and phosphatase inhibitor cocktail (100X)	100µL	5000µL

① Place the cell culture dish on ice and wash the cells with ice cold PBS

Aspirate PBS then add ice cold lysis buffer (300µL/well for 6-well plate)

Scrape adherent cells off the dish → transfer cell suspension into pre-cooled centrifuge tube.

If the cells are already stored in individual tubes, add the lysis buffer directly to the tubes.

② Constant agitation for 30mins at 4°C.

③ Centrifuge at 12000rpm for 20 mins

④ Gently remove tubes from centrifuge → place on ice → transfer supernatant to fresh tube → discard pellet

Recipes of the lysis buffer and procedures for preparation of protein lysate were checked by Dr. Eszter Dombi in Poulton Group, Nuffield Department of Women's & Reproductive Health.

APPENDIX3:

Methodology for Western Blotting

Day 1

Preparing for the experimental samples

Materials: samples, Laemmli buffer 4X, ddH_2O.

Apparatus: ice box, Eppendorf tubes, 10µl pipette and tips, heating-block, bench centrifuge, timer.

Sample	Protein concentration	Mass of Protein to add	Volume of the sample to add	Volume of the water to add	Volume of Laemmli buffer 4x	total volume
xxx	known	Known 20µg	Mass of Protein/ Protein concentration	Total volume - Volume of sample - Laemmli b. volume	5µl	20 µl

① Turn on the heating-block to 95°C before starting with sample preparations.

② Take the samples out from the freezer and put them into the ice-box.

③ Label the Eppendorf tubes and start with adding the ddH_2O to the Eppendorf.

④ Resuspend the sample and add the desired amount to the Eppendorf tubes containing the water and then resuspend again.

⑤ Once the water and the samples have been added, move under the hood to add the Laemmli buffer 4x.

⑥ Warm the samples in the Heating box for 10 min.

⑦ Centrifuge the samples in the bench centrifuge for 1 min at 9000rpm.

Running the SDS-PAGE

Materials: samples, ladder, gel, running buffer.

Apparatus: 20µl and 200µl pipette and tips, electrophoresis apparatus and power pack, timer.

① Remove the gel from the packaging carefully.

② Assemble the electrophoresis apparatus.

③ Pour the running buffer into the inner chamber covering the top of the wells

of the gel. Check if there is any leakage to the outer chamber. Then fill the outer chamber with running buffer.

④ Remove air bubbles from the wells by pipetting 100µl of running buffer in and out for each well.

⑤ Load 5µl of ladder into the first well and add the samples to the other wells.

⑥ Run the gel at 150V. Ensure that there are bubbles coming from the bottom to the top when the power pack starts. Check on the gel every ½ hour and run the gel about 1.5 hours to make sure that all the protein bands are well-expanded.

Electroblotting proteins in the gel onto polyvinylidene difluoride (PDVF) membrane

Materials: Whatman filter papers, PVDF membrane, methanol, anode buffer 1, anode buffer 2, cathode buffer.

Apparatus: Bench rocker, transfer apparatus and power pack, 4 plastic containers, timer.

① Cut out the Whatman filter papers using the existing model. 9 filter papers are needed.

② Cut out the PDVF membrane using the existing model.

15 minutes before stopping the gel:

③ Peel-off the PDVF membrane and put it in a container with methanol for 5 min.

④ Pour the methanol back into its bottle. Then put the membrane in a container with tap water for 10 min.

⑤ Discard the tap water and put the membrane in anode 2 for 2 min.

⑥ Prepare for 3 containers:

- Anode 1: 4 filter papers
- Anode 2: 2 filter papers
- Cathode: 3 filter papers

Place the filter papers into the containers according to the instructions above and pour the buffers (Anode1, Anode2, and Cathode) onto the filter papers so that they are completely saturated by the buffers.

⑦ Open the transfer apparatus and place the anode 1 filter papers firstly and then place the anode 2 filter papers on the top of anode 1 filter papers.

⑧ Stop the power pack when the proteins reach the bottom. Remove the gel cassette, and open it up with the gel cassette opener.

⑨ Put the PDVF membrane on the top of anode 2 filter papers. Subsequently put the gel on the top of PDVF membrane.

⑩ Ensure there are no bubbles on the gel, and the ladder is straight. Cover the gel with the cathode filter papers.

⑪ Roll gently on the sandwich of membrane, gel and filter papers using the roller.

⑫ Seal the transfer apparatus evenly and connect the anode and cathode correctly (black end with black hole, red end with red hole) and run at 25V for 45 min.

⑬ Clean everything up and prepare 5% milk.

Materials: non fat-dry milk, TBS-T.

Apparatus: 80ml bottle or a 50ml tube, pipette boy, weighting scale.

Calculate the total volume that would be needed (e.g. 5g for 100ml). Weight the milk powder on the scale and put it into the bottle or into the tube, then top it up with the corresponding volume of TBS-T. Shake it to make the powder to dissolve. Label the tube/bottle and store it into the fridge up to the use.

Checking the membrane transfer

Materials: Ponceau Red stock solution, TBS-T, membrane.

Apparatus: bench rocker, 1 plastic container, absorbing paper.

① Turn-off the power pack and open the transfer apparatus.

② Pour the Ponceau Red solution into a plastic container.

③ Place the membrane into the Ponceau Red solution and place it on the bench rocker for 2 min.

④ Check for protein transfer: one should be able to see proteins' bands on the membrane.

⑤ Place the membrane in a plastic container with TBS-T.

⑥ Wash the membrane for 2 min (Repeat the washing until the red colour goes off the membrane).

Blocking reaction

Materials: 20ml 5% milk, membrane.

Apparatus: bench rocker, pipette boy, 1 plastic container, timer.

① Prepare a container with 20ml of 5% milk.

② Pick up the membrane by the tweezer and place it into the milk solution.

③ Place the container on the bench rocker for 1 hour.

Clean the transfer apparatus by wiping the cassette gently.

Primary antibody binding

Materials: primary antibodies, membrane, 5% milk

Apparatus: bench rocker, pipette and tips, 50ml flacon tube, scissors.

① Prior to the end of blocking reaction take the primary antibody out of the fridge.

② Add 10ml of milk to a 50ml tube and add the right amount of the primary antibody.

③ Remove the membrane from the bench rocker. Cut the membrane according to the targeted protein sizes.

④ Insert the membranes into the tubes: the proteins face towards the inside of the flacon.

⑤ Place the tubes on a roller at 4°C incubator overnight.

Day 2

Secondary antibody binding

Materials: secondary antibodies, membrane, TBS-T, 5% milk.

Apparatus: bench rocker, pipette and tips, pipette boy, plastic containers, timer.

Antibodies (HRP)	Diluted Concentration	Volume of Ab
Anti-mouse IgG	1/1000	5 µl
Anti-Rabbit IgG	1/1000	5 µl

① Add TBS-T to the plastic containers. Remove the membranes from the primary Ab tubes and place them in the containers with TBS-T.

② Place the container on the bench rocker for 5min. Discard the TBS-T in the sink after 5 min.

③ Repeat step ② x 3 times always using fresh TBS-T.

④ Prepare for another container for the secondary Ab. Add 20ml of milk to the container.

⑤ Add the HRP secondary Ab following the recommended diluted concentrations in the table above.

⑥ Place the containers on the bench rocker for 60 min.

Chemiluminescent Detection

Materials: EZ-ECL kit (fridge), TBS-T, developing reagents.

Apparatus: bench rocker, pipette and tips, cling film, (G-Box), USB stick, developing films, cassette, tape, scissors, timer.

① Discard 5% milk TBS-T from the containers and wash the membrane strips 3 times by adding fresh TBS-T, placing on the bench rocker for 5 min each time.

② Make 1ml of detection solution by mixing 0.5ml of EZ-ECL A and 0.5ml of EZ-ECL B in an Eppendorf tube. Leave to equilibrate for 1 min.

③ Place two sets of cling films on the bench. After the washing is done, lay the membrane on the cling film so that the protein surface is facing up. Pipette the detection solution on the membrane and ensure to spread it evenly using the side of the pipette tip. Wait for 1 min.

④ Place the membrane onto the second cling film so that the protein surface is facing down. Wrap the membrane ensuring there are no bubbles or creases.

G:BOX

① Switch the G:BOX on.

② Bring the membrane to the G:BOX and place it inside. Check the position of the membrane and close the G:BOX door.

③ Select the time for exposure and start the exposure.

④ Check the image acquired and save it.

To quantify the proteins loaded, membranes were stripped and incubated with anti-actin antibodies.

Stripping the membrane

Materials: membrane strips, stripping buffer, TBS-T.

Apparatus: container, timer

① Place the membrane in a container with TBS-T on the bench rocker for 5 min.

② Repeat the washing x3 time using fresh TBS-T.

③ Add the stripping buffer to a container and place the membrane into it after the washing. Leave the container on the bench rocker for 15 min.

④ Repeat step ① and ②.

⑤ Start the protocol from the blocking reaction in day 1.

Components for buffers

Running Buffer

- 50ml of stock solution or 25ml of stock solution
- 950ml of ddH_2O or 475ml of ddH_2O

10xTBS

- Add 87.6g of NaCl and 12.1g of Tris to 700ml of dH_2O
- Dissolve in a bottle with magnet
- Adjust the pH to 8 using the pH metre
- Top up with filtered water to make 1L

TBS-T

- 100ml from the 10xTBS stock
- 900ml of ddH_2O

1M Tris solution

- Add 121.4g Tris to 700ml of dH2O, dissolve it using a magnet
- Bring the pH to 9.4 or 10.4 using the pH metre
- Top up with H_2O to make 1L

Anode 1 buffer

- 150ml of Tris 1M pH 10.4
- 100ml of methanol
- Top up to 500ml with ddH_2O

Anode 2 buffer

- 12.5ml of Tris 1M pH 10.4
- 100ml of methanol
- Top up to 500ml with ddH_2O

Cathode buffer

- 12.5ml of Tris 1M pH 9.4
- 100ml of methanol
- 2.6g of α-aminohexanoic acid (AKA 6-Aminocaproic acid)

Recipes of the buffers and procedures of western blotting and membrane stripping are reedited on the basis of Western Blotting Protocol in team Manu, dictated by visiting student Dr.Daniela Anna Laccarino.

APPENDIX4:

Methodology for RNA Isolation

RNA isolation was performed using QIAGEN's RNeasy Plus Mini Kit.

RNeasy® Plus Mini Handbook has been referred for the description of methodology.

① Pellet the cells (<1x107cells) by centrifuging for 5 min at 300g in a centrifuge tube.

Remove all supernatant by aspiration.

② Disrupt the cells by adding Buffer RLT Plus. Add the appropriate volume of Buffer RLT Plus (<5x106 cells:350 µl, 5x106 cells-1x107cells:600 µl). For pelleted cells, loosen the cell pellet thoroughly by flicking the tube.

③ Homogenize the lysate by using a needle (18G) and syringe (1ml). Pass the lysate at least 6-8 times through the needle.

④ Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 30s at 8000g. Discard the column, and save the flowthrough. Make sure that no liquid remains on the column membrane after centrifugation (If necessary, repeat the centrifugation until all liquid has passed through the membrane).

⑤ Add 1 volume of 70% ethanol to the flowthrough, and mix well by pipetting.

⑥ Transfer up to 700µl of the sample to an RNeasy spin column placed in a 2ml collection tube. Close the lid gently and centrifuge for 15s at 8000g. Discard the flowthrough.

⑦ Reuse the collection tube and add 700µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 8000g to wash the spin column membrane. Discard the flow-through.

⑧ Reuse the collection tube and add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 15s at 8000g to wash the spin column membrane. Discard the flow-through.

⑨ Reuse the collection tube and add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 2min at 8000g to wash the spin column membrane.

⑩ Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000g to elute the RNA.

⑪ To detect the quality of obtained RNA, OD260/280 and OD 260/230 were measured by Nano DropTM. Obtained RNA were then stored at -80°C freezer.

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