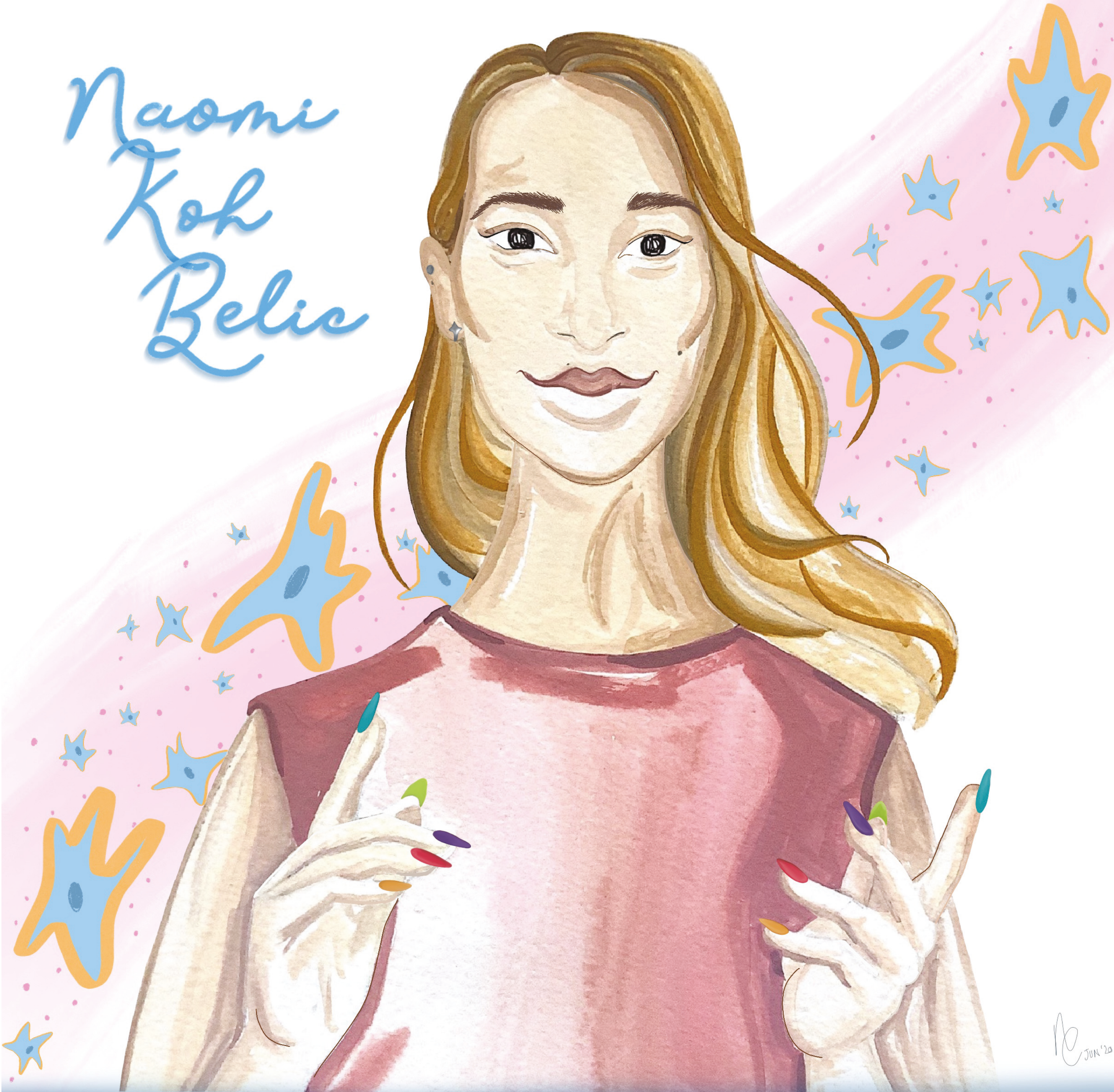
New Stemsation:

A COMPREHENSIVE ANALYSIS OF ADIPOSE STEM CELLS

DOCTOR OF PHILOSOPHY FACULTY OF SCIENCE UNIVERSITY OF TECHNOLOGY SYDNEY



I, Naomi Koh Belic, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Science Faculty at the University of Technology Sydney. This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research is supported by an Australian Government Research Training Program.

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Over the last eight years that I've been at the University of Technology Sydney my life has been tumultuous. But there has always been one place that's remained with me through it all. I've always found comfort on a tiny red plastic stool, waiting under the dusty grapes hung up on the roof, littered with Christmas decorations that have never been taken down, the giant deer rug staring into my soul… sometimes my mind wanders, and I remember the round aquarium on the wall that housed a dead goldfish for years. I'm never left with my thoughts for long before my wobbly table is piled high with steamed egg and chive dumplings, hand sliced noodles with fragrant spicy sauce and special braised eggplant. The trifecta that I fell in love with when I had first moved to Sydney at the big age of 17. Chinese Noodle House has seen me through my highest highs while I sloppily scoffed dumplings with a plastic cup full of wine in hand surrounded by friends, and in my absolute lowest of lows when it offered a place where I could come on my own and find joy in the food in front of me. Thank you for offering stability amongst your chaos and for always keeping my belly and heart full.

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Abstract

Stem cells are defined by their ability to self-renew and differentiate into multiple cell types. Because of this, they have potential to repair or replace damaged tissue and are of great interest for regenerative medicine which is advancing at an astonishing rate. However, patient hope is also being exploited by predatory clinics offering unproven stem cell treatments. These treatments have little to no scientific evidence of safety, let alone efficacy and are detrimental to scientific progression. The growth of unproven autologous stem cell treatments can be attributed to the realisation that liposuction can be used to extract stem cells. As adipose stem cells are being used in both proven and unproven treatments it is vital to understand how they function. This project successfully characterised the proteome of adipose stem cells through the analysis of the whole cell lysate, membrane bound fraction, extracellular vesicles and select secreted cytokines. Isolation and cryopreservation techniques were also investigated as there is a lack of standardisation in these areas. When comparing traditional and xeno free media for the isolation and expansion of adipose stem cells, there was a distinct shift in the proteome, and this is largely associated with proteins involved in metabolism. Furthermore, the cytokine profiles were wildly different, and the absence of vital stem cell cytokines suggest that traditional media may be preferential, however substantially more research is required to validate this. It was revealed in this study that cryopreservation also causes a shift in the adipose stem cell proteome, albeit not as distinct as the one observed from different media. This proteomic shift also manifests in the metabolome with evidence of particular pathways being altered after cryopreservation, but again this requires further validation. Metabolites involved in the kynurenine pathway were further investigated as this pathway accounts for the metabolism of more than 95% of tryptophan. This is the first study to confidently demonstrate that the kynurenine pathway of tryptophan metabolism is activated by IFN-γ in human adipose stem cells. Lastly, because it is the responsibility of scientists to ensure that science is accessible for everyone, the use of digital media for scientific education was explored. The knowledge from this project can be adapted to produce better scientific educational video content, and should be employed to educate the broader community about stem cell therapies, as it is known that patients use digital media to access unproven treatments.

Abbreviations

ASC adipose stem cell

BMSC bone marrow stem cell

DMEM Dulbecco's modified eagle medium

ESC embryonic stem cell

FBS fetal bovine serum

FGF fibroblast growth factor

FSC fetal stem cell

G-CSF granulocyte colony-stimulating factor

GM-CSF granulocyte macrophage colony-stimulating factor

HSC hematopoietic stem cell

IDO 1 indoleamine 2,3-dioxygenase

IFN interferon

IL interleukin

IP interferon gamma-induced protein

KMO kynurenine 3-monooxygenase

LC-MS/MS liquid chromatography coupled with tandem mass spectrometry

MCP monocyte chemotactic protein

MIP macrophage inflammatory protein

NSC neural stem cell

PGDF platelet-derived growth factor

RANTES regulation on activation normal T cell expressed and secreted

TDO tryptophan 2,3-dioxygenase

TNF tumour necrosis factor

VEGF vascular endothelial growth factor

Chapter One

The hope and hype of stem cells

As our technological tools advance, so do scientific discoveries. The knowledge of the complex human body is constantly increasing. Scientists and medical practitioners are contributing to new breakthroughs, and one of the areas of interest is stem cells. Stem cell therapy is still a relatively immature field but is often portrayed as the next revolution in healthcare. This is due to stem cells ability to self-renew and to differentiate into multiple cell types. Because of this, they have the potential to repair or even replace damaged tissue, and there has been an understandable amount of excitement around this prospect [1].

Stem cells can be broadly classified by their differentiation ability, being either multipotent or pluripotent. The best known stem cell are embryonic stem cells (ESCs) which were discovered in 1998 [2]. ESCs originate from a pre-implantation blastocyst and can differentiate into every somatic tissue, while also having the capacity to self-renew and proliferate significantly [1,3]. However, well documented ethical concerns, regulations, and potential immune rejection hinder the use of ESCs in regenerative medicine [4]. Another source of pluripotent stem cells is induced pluripotent stem cells (iPSCs) these are reprogrammed somatic cells that exhibit the same essential characteristics as ESCs [5]. Initially iPSCs were reprogrammed in a way that would leave residual transgene sequences that raised concern as the effect of these transgenes on phenotype were unpredictable [6,7]. This has been somewhat overcome as new methods have been developed such as the use of viral vectors that no longer leave residual sequences in the genome [7].

Most other stem cells are multipotent and can be defined by their source, whether they are adult or non-adult. Non-adult multipotent stem cells include fetal stem cells (FSCs), which are isolated from fetal blood or umbilical cord blood [8]. While FSCs have shown greater multipotency and are more immune privileged than adult stem cells, there are safety concerns and significant ethical constraints surrounding their use [9,10]. There are many sources of adult stem cells, which can be isolated from blood, bone, and even adipose tissue just to name a few [11]. Whilst adult stem cells have restricted growth potential when compared to

ESCs, they are not clouded by the same ethical concerns and are not subject to immune rejection if returned to the host they were isolated from [8,10].

Adult stem cells are often called 'mesenchymal stem cells' however this is a problematic term as there are significant inconsistencies and ambiguity with its use [12]. Ideally the term would no longer be used as it is poorly defined, yet its use is rampant with more than 32,000 published articles using the term, many of which don't indicate the tissue the cells were even sourced from [13]. The US Food and Drug Administration attempted to characterise mesenchymal stem cell-based products and found them to be diverse with almost no consistent molecular characterisation [14]. Another study determined that it wasn't just significant differences in gene expression, but there were also substantial differences in differentiation capacity [15]. There is a strong push in the scientific community to change the name, illustrated in a poignant example from Arnold Caplan who first coined the term, who released a publication in 2017 titled "Mesenchymal Stem Cells: Time to Change the Name!" [16]. However, this could cause greater confusion, and in this thesis the term 'mesenchymal stem cell' will not be used. Rather, the specific source of the stem cell will be used, and if that isn't available then they will simply be referred to as adult stem cells.

The most widely utilised sources for adult stem cells are from bone marrow and adipose tissue [17]. These multipotent stem cells are able to differentiate along mesodermal lineages (stromal cells, cartilage cells, fat cells and bone cells) and to transdifferentiate along ectodermal (neuronal cell and epithelial cells) and endodermal lineages (muscle cells, gut cells and lung cells) *in vitro* [17-23]. When sourced from bone marrow, they are termed bone marrow stem cells (BMSCs) while those sourced from adipose tissue are termed adipose stem cells (ASCs) [24]. The differences in morphology and phenotype of ASCs and BMSCs is not significant [25], however ASCs have been shown to have higher proliferation rates [26]. BMSCs are harvested from the central region of bone marrow; this process is exceptionally painful and poses a significant risk of morbidity [27,28]. In contrast, adipose tissue is obtained from liposuction, which is minimally invasive, and is a relatively safe procedure [20,29]. This was highlighted through an analysis of over 65,000 liposuction procedures which discovered that only 0.68% of patients had a serious adverse event post-surgery [30]. Furthermore, the quantity of stem cells obtained from adipose tissue is comparatively higher than that obtained from bone

marrow [26,31], which is advantageous for regenerative medicine as clinically applicable quantities are available without further expansion in culture [32].

Stem cells collectively have two broad uses for which they are best known, either as a research model or as a therapeutic treatment. Stem cells are the bedrock of regenerative medicine, a multidisciplinary branch of translational research involving tissue engineering and cellular therapy [8,33]. Tissue engineering focuses on the interaction of cells with biologically-active molecules and biomaterials, while cellular therapy requires donor tissue from which cells can be isolated, proliferated and then differentiated into a desired cell type for transplantation [8,33]. The field of regenerative medicine is advancing at an extraordinary rate and with real potential [34]. While regenerative medicine was initially established through treatments such as human stem cell therapy, it has since expanded to encompass many cell sources and there are tens of thousands of patients receiving cell therapies worldwide [35]. However, stem cell therapies are often viewed with extreme optimism and this is largely due to exploitation of patient hope. There is considerable investment into both preclinical and clinical trials, however this has resulted in only minimal successful treatments [36]. Adult stem cells are the most common stem cell being used in clinical trials, however both ESCs and iPSCs are also being used [36]. Pluripotent stem cells are typically used in situations where adult stem cells are difficult to isolate or expand [36].

To date there has been success with stem cell treatments for cancers such as leukaemia and multiple myeloma. However, apart from hematopoietic stem cells, all other stem cell therapies are still in the research, pre-clinical or clinical trial phases [37]. Stem cells are used to treat cancer only after an attempt to kill all existing cancer cells, and this is typically achieved through high doses of chemo, occasionally coupled with radiation therapy. This process not only kills cancer cells but also kills stem cells in the bone marrow. This is why stem cells are used in cancer treatment, to replenish the body's stem cells. This process is called engraftment and has been performed for more than 50 years [38]. Stem cells are also used to treat blood disorders such as sickle cell disease and thalassemia. Sickle cell disease is an umbrella term for inherited blood cell disorders. The only cure is currently blood and bone marrow transplants, and only very few people with the disease are allowed to have these transplants [39].

Another stem cell therapy with success is skin replacement. In order for humans to survive their skin needs to be able to repair itself after injury. The process of wound healing is complex as it relies on a synchronised response involving immune cells, haematopoietic cells and skin cells. This applies to chronic wounds and severe burn injuries. In both cases stem cells are used to replace skin [40,41]. Other stem cells that have shown some early success are neural stem cells (NSC) which appear to have potential for regenerative repair. They are primarily being utilised in central nervous system repair [36]. In addition, limbal stem cells have also been successfully used for corneal regeneration and it has been demonstrated that after transplantation allogenic limbal cells do not persist for long [42,43].

Standard medical therapies follow a linear model: basic research, applied research and development, and production and diffusion [44]. While this is not always the case with stem cell therapies, there are still many clinical trials that involve stem cells. Australia has a phase II trial for multiple sclerosis using autologous HSC transplantation [45]. Other adult stem cells are similarly being investigated as immunosuppressive treatments such as the use of autologous BMSCs after renal transplant [46,47]. Autologous BMSCs have also been used to treat progressive forms of multiple sclerosis, demonstrating they were safe with some minor indication of improvement [48-50]. A randomised double-blind placebo-controlled crossover phase II study using BMSCs to treat relapsing-remitting multiple sclerosis also successfully demonstrated safety and suggested that BMSCs do have immunomodulatory properties [51]. Adult stem cells have also been used to treat severe graft-versus-host disease in a phase II clinical trial. It was found that they may be an effective therapy for steroid-resistant acute graft-versus-host disease irrespective of the donor of the stem cells [52]. Another pilot study suggested that adult stem cells are a suitable treatment for osteoarthritis although further research is required to confirm this [53]. However, these clinical trials have not always resulted in success. Autologous adult stem cells were transplanted to treat liver cirrhosis as part of a phase II trial and no significant difference was noted for clinical efficacy [54]. Adult stem cells have been used in a variety of trials to treat amyotrophic lateral sclerosis and have demonstrated safety but minimal to no clinical benefit [55-57].

Interestingly most adult stem cell clinical trials involve allogenic adult stem cells, despite the fact that one of the large benefits of adult stem cells is that they can sourced from the patient

themselves as they have immunosuppressive functions [36,58]. Allogenic BMSCs were utilised in a double-blind randomized placebo controlled phase I/II study for critical limb ischemia and, while serious adverse events occurred, they were determined to not be related to the stem cells and consequent safety parameters were determined with minimal evidence of positive trends in regards to ankle pressure [59]. There is also growing interest in using iPSCs for allogenic transplants or as partially compatible human leukocyte antigen haplotyped derivatives [60].

Non-adult stem cells are also being used in the clinic. Idiopathic pulmonary fibrosis was treated with intravenous injection of placental stem cells is a non-randomised phase I clinical trial, resulting in acute adverse events but no signs of improving the condition [61]. There is a phase I trial that has been conducted using human fetal spinal cord NSCs for amyotrophic lateral sclerosis. This involved intra spinal injections of up to 1 million cells and resulted in some small improvement in survival and no adverse events [62-64]. Children with neuronal ceroid lipofuscinosis have been treated with doses of 1 billion human fetal-derived NSC progenitors into their brains and no adverse events were detected [65]. Furthermore, these cells had long term survival and were detected 2.5 years after treatment, 1.5 years of which were without immunosuppression [65]. Fetal-derived NSC progenitors were also transplanted directly into the brain of 4 young boys who had severe, fatal form of Pelizaeus-Merzbacher disease in which oligodendrocytes cannot myelinate axons [66]. The results of which suggest some improvement in neurological function and indication of myelination at the site of transplant [66].

While this may collectively sound promising, it is important to know that there are many clinical trials utilising stem cells that have failed or been cancelled, raising concern that these clinical trials are being poorly designed and are ultimately slowing the progression of successful clinical translation [36]. There are also stem cell treatments on offer that do not follow conventional clinical trial models, many of these being unproven stem cell treatments. The term ‘unproven stem cell treatment’ unsurprisingly carries negative connotations, however this term also encompasses some legitimate in-progress research that is yet to be sufficiently proven [67]. The definition of an unproven cell therapy is “unclear scientific rationale to suggest potential efficacy, lack of understanding on the mechanism of action and/or the biological function to support clinical use, insufficient data from in vitro assays,

animal models and clinical studies regarding the safety profile to support the use in patients, lack of a standardized approach to confirm product quality and ensure consistency in cell manufacturing, inadequate information disclosed to patients to enable proper informed consent, use within non-standardized or non-validated administration methods or uncontrolled experimental procedures in humans" [67]. It is incredibly difficult to determine the number of patients who have received unproven stem cell treatments, but it is suggested to be in the tens of thousands or even higher [68]. It is important to remember that stem cell treatments are invasive, and their potential is often misrepresented without sufficient supporting scientific evidence [69]. Unproven stem cell treatments are often perceived as being offered in countries with more relaxed medical legislation.

Medical tourism is when patients seek treatments abroad either because they not available in their home country, or because they are more affordable [70]. Medical tourism is growing rapidly and has implications for the health systems of both countries involved [71]. Considering the popularity of stem cell treatments, it should come as no surprise that stem cell tourism is on the rise. One significant downside of stem cell tourism is that it is drawing people away from clinical trials, ultimately slowing down research progress. It is an internet-driven industry that relies on patients travelling to receive unproven stem cell treatments [68] and it portrays the success of therapies in an overly optimistic manner [72]. Patients who embark on stem cell tourism collectively appear to have conducted ample research on the topic, but despite this appear to know little about the cells they were receiving such as where the cells were sourced from. It appears most of these types of stem cell treatments are administered through intramuscular or intravenous injections, and often by a lumbar puncture [73]. In China, fetal brain tissue was transplanted into more than 400 patients with spinal cord injury and the safety and efficacy of these treatments was only reported anecdotally. There were significant complications including meningitis and perioperative death, and there was no evidence of improvement of sensory, motor, or autonomic functions, nor any improvement of disability [74]. Researchers in China have made it clear that they are firmly against the over availability of stem cell treatments, however these clinics continue to practice as there is a lack of regulation [75]. It is important to recognise that it is not only consenting adults who receive these treatments. Parents or guardians of children have the right to make health care decisions on

behalf of their children, and consequently minors are exposed to many unproven stem cell treatments.

One of the first popular stem cell clinics was X-Cell centre in Germany, a clinic practicing due to a regulatory loophole. The clinic practiced from 2007-2011 treating thousands of people [76]. Their website claimed to treat almost every disease including diabetes, stroke, cardiovascular disease and multiple sclerosis. The clinics closure came after several adverse events including the death of an 18-month-old child and injection of stem cells into the brain of a 10-year-old boy. An investigation by German regulators established that these intra-cranial injections produced more harm than benefit. Once this centre was closed, the owner went on to open a new clinic in Lebanon operating under similar regulatory loopholes [76]. To reiterate, there is little to no evidence that these treatments are safe, let alone that they actually work. In fact, when it comes to stem cell treatment for stroke there is no substantial evidence to indicate stem cell treatments can replace damaged cells, reconstruct neural circuits or improve loss of function [77-79]. Not to mention these treatments come with a hefty price tag, costing between $10,000 - $60,000 per treatment [73].

Without regulation it is difficult to know if the origin of cells, or if the cells are even being administered. In India, partially differentiated ESCs were intravenously and intramuscularly injected into 91 children with cerebral palsy, some as young as 30 days old. This was part of a clinical trial, despite the risks of transplanting undifferentiated pluripotent cells that could have formed teratomas. Upon evaluation of the methods used, it is likely that most cells used in the treatment were dead [36]. This has occurred in other studies that have not clarified if the treatment was provided locally or abroad, such as a boy with ataxia telangiectasia who was given intrathecal injection of human fetal neural stem cells. Four years later a brain tumour was detected, and it was determined that it was derived from the transplanted neural cells [80]. In another case, a 46-year-old female patient who had lupus nephritis was treated with direct renal injection of autologous HSCs, developed masses at the site of injection, hematuria and angiomyeloproliferative lesions [81]. There is also case of a 21-year-old woman who had olfactory mucosal cell implantation in her spine after complete spinal cord injury who, 8 years later, had a multicystic mass detected in her spine. These are just a small handful of the reported cases available, and countless more unproven stem cell treatments are going

undetected, many of which won't have follow ups and won't be investigated by a scientific research team. These findings reinforce the importance and desperate need of long, thorough monitoring for all patients [82].

Unproven stem cell treatments are also administered outside the context of stem cell tourism. The growth of unproven autologous stem cell treatments can be attributed to the use of liposuction to extract stem cells [83]. In Australia, autologous ASCs are being offered outside of clinical trials for osteoarthritis, cartilage repair, stroke, multiple sclerosis, retinal neuropathy, spinal cord injury, amyotrophic lateral sclerosis and autism [83]. The number of private clinics offering autologous stem cell therapies is continuing to grow in Australia [84]. It is extremely unethical to be offering unproven stem cell treatments outside of clinical trials [85]. However, the unfortunate reality is that predatory clinics are continuing to exploit vulnerable patient populations due to a lack of regulation and oversight [86]. Despite regulatory efforts to reduce their commercial promotion, unproven stem cell treatments are still a growing global problem [87]. Australia has the 5th highest total number of stem cell clinics and the 5th highest number of clinics per capita in the world [88].

In Australia, stem cells are being marketed as treatments for neurodegenerative diseases, stroke, spinal cord injury, autism, osteoarthritis, asthma, headaches and even as anti-aging and facial rejuvenation treatments [85]. For vulnerable populations, hope is a crucial part of their self-identity and agency to have the potential to control their future, but these stem cell therapies come with extremely high risks [73]. They also come with a huge financial burden for patients, in Australia the cost of autologous stem cell treatments ranges between $750 and $2500 for cosmetic purposes and this number rises steeply for medical treatments, typically costing at least $9000 for a single intra-articular injection alone, with several Australian clinics offering payment plans for between $60,000 and $70,000 [85]. In 2016, an Australian woman underwent fat stem cell isolation at a Sydney clinic, and died from complications. Her death was entirely avoidable, and it highlights the predatory nature of these clinics that exploit regulatory loopholes to provide unnecessary and unjustified treatments at a profit [89]. Australian predatory clinics are operated by medical professionals such as general practitioners, cosmetic surgeons, sports medicine physicians and orthopaedic surgeons.

However, specialists of the diseases being treated are typically not involved [85]. These vulnerable populations are having their hope blatantly exploited by medical professionals.

The reality is that stem cell treatments do have real potential, but widespread unproven stem cell treatments and the constant depiction of stem cell treatments as the golden cure in the media will ultimately leave patients both disappointed and disillusioned [90]. In Australia, the US and Canada, stem cells are perceived to have far greater benefits than risks [91]. The combination of traditional and online digital media has allowed for patients to generate their own perception of significant and successful treatments [92]. However, before delving into the role that traditional and digital media play in shaping the perception of stem cell treatments, it vital to recognise that many researchers themselves contribute to this excessive hype by overstating their findings to publish with greater impact and to secure grants to further their research.

Unproven stem cell therapies use direct-to-consumer online marketing [93]. This marketing strategy for stem cell therapies is extensively used in countries such as Australia, the United States, Germany and Ireland [88]. This online marketing involves aggressive promotion of these therapies on websites that make broad unproven claims and often lack details on the procedures themselves [88]. While direct-to-consumer marketing is employed across many other fields of advertising, it is important to recognise that the consumer market niche in this case is predominantly patients whose hope can be exploited [73]. These websites target patients who have exhausted conventional treatment options by providing reassurance of safety and efficacy of the treatments they provide in the form of reports from independent sources such as news coverage, and through patient testimonials [94]. Narratives are employed in this advertising style, to showcase the optimistic perspective of patients and doctors offering these treatments [85]. Communities of hope are created and maintained online where they continue to depict stem cell treatments in a positive light, despite the lack of scientific evidence [92]. Sharing health experiences online gives individuals a sense they are participating in the creation of health information, and reading these first-person accounts effect other individual’s health predominantly through the memorability of a story and building an online community [95]. There are literally hundreds of US clinics offering unproven stem cell treatments through direct-to-consumer advertising. Between 2014-2016, there was average

of 90-100 new stem cell business websites each year [96]. This direct-to-consumer marketing has drastically shifted the relationship patients have with actual experts in the field [73]. It is clear that patients use digital media to access these unproven treatments [92]. There is a lack of comprehensive information and reliable resources available to combat the existing predatory online market [68]. What is needed is more engagement and resources for patients, and for organisations and industry with leverage to push for this outreach [97].

While the International Society for Stem Cell Research (ISSCR) released guidelines for the clinical translation of stem cells in 2008, it is the responsibility of individual governments to enforce legislation that reflects these recommendations [98]. These guidelines included recommendations for unproven stem cell treatments stating that medical innovation is allowed when there are exceptional circumstances involving small numbers of severely ill patients, that the procedure would require independent peer review that interrogates scientific rationale, institutional accountability, informed consent, patient monitoring, transparency, reporting of adverse events, and a commitment to proceed with formal clinical trials [99]. The guidelines explicitly state "the ISSCR condemns the administration of unproven uses of stem cells or their direct derivatives to a large series of patients outside of a clinical trial, particularly when patients are charged for such services. Scientists and clinicians should not participate in such activities as a matter of professional ethics." [98]. ISSCR has also released additional statements that provide further clarification, such as the one in 2013 that reinforced that even autologous stem cells still should adhere to their guidelines, as well as encouraging the entire medical community to discourage unproven autologous stem cell treatments outside of clinical trials. In 2016, ISSCR launched their guidelines for stem cell research and clinical translation and this revised edition emphasises the need for accurate public communication, highlighting the need for more thorough regulation at all stages of research and translation. They also reiterated the importance that researchers play in science communication [100].

In Australia, autologous stem cell therapies do not need to comply with TGA regulations [101]. Establishing legislation around stem cell therapies is not only the responsibility of government, but also research scientists, medical professionals and ethicists [102]. The current model most commonly adopted for legislation of stem cell therapies is the rational actor

model, which assumes that when an individual is provided with information, they will rationally decide which is the optimal decision. However this model does not account for the exploitation of hope [73]. The complexity of hope needs to be understood and that understanding applied to the regulation of stem cell therapies [73]. To reiterate, there is insufficient evidence that stem cell therapies will provide more benefits than they do harm [103,104]. Until there is sufficient evidence, governments should be restricting availability of stem cell treatments [102]. Patient demand should not mean that stem cell treatments are provided in a commercial context outside of these existing norms [83]. Greater regulatory oversight and action are needed to protect vulnerable patients as unproven stem cell therapies offered outside of clinical trials are completely unethical [85]. The future of stem cell therapies is hindered by the lack of rigorous clinical trials. However, there is hope on the horizon if legislation is created that protects patients, if the scientific community broadly communicates that clinical translation takes time, and greater online educational resources are made available to protect patients from exploitation.

While stem cell therapies still hold genuine potential in the clinic, they are also exceptionally useful as research models. Animals are typically used for research models, however there are significant differences between animals and humans. To improve reliability of predictive tests and methods for research, it would be beneficial to have intraspecies models rather than interspecies models [105]. Human stem cell models offer an alternate to animal models [106]. iPSCs are the most popular stem cell used in research models, because they are pluripotent and are an immortalised cell line. This is not to say that other sources of stem cells can't be used, simply that most research utilises iPSCs. Stem cell research models can focus on specific diseases and provide insight on the phenotypes of the disease and to generate potential therapies [106]. Disease models can also be derived from autologous patient cells. Stem cell disease models provide insight on disease pathophysiology, identification of novel drug targets, and increasing clinical success of new drugs [107]. Stem cell disease modelling typically starts with the development of cell lines that have the molecular defect of interest, allowing for a robust disease phenotype [108]. Stem cell models could contribute to the development of personalised or precision medicine by allowing drugs to be tested *in vitro* [109]. The human body is complex, comprised of different systems, organs, and tissues that involve simple tissues

containing cells, controlled by subcellular components. Recapitulating the complexity of the human body is the greatest challenge for research models.

The simplest stem cell model is a monolayer that contains a single cell type. This is advantageous as it is relatively easy to maintain, easy to interrogate and cost effective in comparison to more complex models. These models allow for information from the donors to be obtained and have the potential to provide insight into the pathological mechanism and progression of the disease. Furthermore these models can also be used for drug screening [109]. For example, iPSCs have been used to model Parkinson's [110], Huntington's disease [111] and Amyotrophic lateral sclerosis [112]. Amyotrophic lateral sclerosis has been modelled by differentiating motor neurons from patient derived iPSCs [112], which has resulted in a wealth of knowledge on the disease such as the observation of dysregulation of neuronal synaptic activity in these motor neurons [113], neurofilament aggregation [114], insight on TAR DNA-binding protein 43 [115-117], expression and underlying contributors to C9ORF72 repeat expansion [118,119] and the role that astrocytes have in this disease [120,121]. Other models have differentiated neurons from schizophrenia patient iPSCs and found key cellular and molecular phenotypes were ameliorated after treatment with the antipsychotic loxapine [122]. There have been many Alzheimer's disease models created using iPSCs which have provided disease insight such as gene expression patterns, increased p-tau production, accumulation of intracellular Aβ oligomers, demonstrated difference in $A\beta_{42}/A\beta_{40}$ ratios [123-126].

Monolayer models are reliant on homogenous cell cultures as single cell analysis is still difficult to do and is very expensive. The limitations of these models are the reduced cell-cell interactions when compared to more complex 3D models. There are understandable variations between 2D and 3D cellular models such as morphology, proliferation and differentiation capabilities [127]. 3D stem cell models allow for more complex cellular interaction [109]. Scaffolds can be used to generate 3D cellular models that are made up of multiple layers of cells. However seeding cells onto a scaffold can make them more difficult to analyse for research purposes as they are likely harder to retrieve and they are also more challenging to image [109]. 3D stem cell models can also be achieved through creating aggregates, also known as spheroids [128]. Even more complex 3D models can be created that resemble whole organs, termed organoids [129]. Organoids garner significant research interest

as they recapitulate spatial organization of cells, cellular interactions and particular physiological functions, consequently providing insight on the stem cell niche [130]. A large variety of organoids have been developed, often demonstrating only a single or partial element of the desired tissue [130]. Many organoids have been created including those from pluripotent stem cells such as brain [131], kidney [132], intestine [133], liver [134], and those from multipotent stem cells such as prostate [135], pancreas [136], intestine [137] and stomach [138]. Organoids have been successfully created from both pluripotent and multipotent stem cells and can be used to model genetic disorders by using patient-derived stem cells or by initiating mutations specific to that disease [129]. This makes organoids a useful candidate for drug testing, as they will likely be more accurate representations of the human body as opposed to animal models and will consequently reduce the need for loss of animal life [129]. While organoid models are considerably more complex than other stem cell models, this means that standardization is going to be significantly harder to achieve. This will be a continual challenge for the field as this lack of standardisation will make it difficult to compare different studies [130].

Organoids have had success modelling diseases such as Parkinson's disease through the generation of neural and intestinal organoids from patients with familial Parkinson's disease [139], or the generation of midbrain-specific organoids with spatially organised dopaminergic neurons [140]. Organoids have significantly contributed to forming a better understanding of Parkinson's, even though these organoids do not have microglia which have a substantial role in disease progression [141]. Success with organoids has also been observed with intestinal organoids. Intestinal organoids allow not only for the study of intestinal diseases, but importantly they allow for the study of intestinal development [133]. Intestinal organoids contain all the same major cell types that are found in intestinal epithelium including enterocytes, entero-endocrine cells goblet cells and Paneth cells [137]. iPSCs from a patient with cystic fibrosis were used to develop intestinal organoids, these successfully recapitulated the aberrant swelling in response to forskolin [142]. Gastric organoids have also been created from human pluripotent stem cells and have been used to model gastric diseases through infection with *Helicobacter pylori* [143].

There are several approaches being employed to reduce the difference between *in vitro* organoids and *in vivo* systems. One of these approaches is bioengineering, this includes controlling the microenvironment, or directly modifying the cells themselves [130]. *In vivo* systems are vascularised, understandably there is significant research into vascularising *in vitro* organoids. This has been achieved through seeding endothelial cells that go on to form blood vessels into an organoid, or alternatively by using of engineered scaffolds [130]. Organoids have even been created through bio-printing which allows for co-culturing of cells in specific arrangements [144]. Human blood microvessel, liver and muscle organoids have all successfully been bio-printed [144]. Bio-printing organoids is becoming more common, but still has limitation that are being improved. Like all the aforementioned stem cell models, organoids could also be used beyond research and could be translated into clinical practice. For example, there is potential in the future for organoids to be used for transplants, as these autologous tissues would not be rejected by the immune system [129].

Stem cells can even be used in organ-on-a-chip systems. Organ-on-a-chips are microfluidic cell culture devices that have perfused chambers lined with cells that recapitulate tissue or organ physiology [145]. These complex devices produce superior high-quality tissue or organ functionality compared to most other modelling systems [145]. Organ-on-a-chips will be particularly useful for drug discovery and development as they allow for thorough interrogation of the molecular mechanism of action, toxicology testing and identification of potential biomarkers [145]. Intestine, lung, liver, heart, kidney, cornea, fat, bone marrow, skin and even blood-brain barrier have been made on chips [145]. Dopaminergic neurons have been differentiated from neuroepithelial stem cells in 3D microfluidic culture to study Parkinson's [146]. One of the benefits of organ-on-a-chip over an organoid is that it is much easier to track cell activity [145]. It should also be particularly useful for drug discovery through the study of molecular mechanisms of action, prioritization of the best drug candidates, testing of toxicity and identification of biomarkers [145].

Stem cells have immense potential as a research model. However, there is a broad lack of standardisation across all stem cell research models, such as how the stem cells are sourced (isolation, sorting, reprogramming etc), how they are grown and how they are analysed [145]. ESCs were initially used for models, however once iPSCs emerged they became the preferred

choice [147]. A hindrance to the use of iPSC disease models is the genetic instability that arises from the reprogramming of iPSCs from diseased patient samples [148]. Long-term iPSC culture frequently results in mutations and chromosomal abnormalities that could also skew the disease model [149]. In addition, iPSCs are notoriously time consuming to maintain and require a significant amount of optimization [149,150]. iPSCs are utilised for disease modelling as they are a primary cell line, they are capable of self-renewal**,** and they can differentiate into a variety of cell lineages [151]. All these positive characteristics are shared with adult stem cells, and in some cases adult stem cells can even be preferable for disease modelling. For example, they do not need to be reprogrammed and therefore are more genetically stable, and maintenance of adult stem cells is relatively simple when compared to iPSCs. There are also concerns over genetic and epigenetic variations in iPSCs and the possibility of epigenetic memory which may hinder their use as a research model [152-154]. While there are benefits of using iPSCs, such as their apparent ability to be passaged indefinitely, adult stem cells are a viable alternative for disease modelling.

While this thesis cannot address all the issues present in the stem cell industry, it aims to cover a few of these issues in depth. This project will on the stem cell used most commonly in unproven stem cell treatments: ASCs. This thesis will establish a comprehensive understanding of ASCs that can act as a baseline for future research models, will investigate the use of ASCs in the clinic and will also address the need for better science communication. While ASC treatments are available around the globe, these cells are still poorly understood. Clarity will be provided through the proteomic characterisation of these cells and the data generated from this will act as a baseline for future ASC research models. ASCs will also be investigated as a model for a specific pathway, and metabolites will be measured. The conditions under which ASCs are isolated, expanded and stored in will be investigated, as unfortunately there is minimal research in this despite their regular application in the clinic. And finally, the desperate need for better online educational resources and science communication will be addressed.

Chapter Two

Chewing the fat: proteomic analysis of human adipose stem cells

1. Introduction

1.1 Background

Stem cells are defined by their ability to self-renew and to differentiate into multiple cell types [1]. In adults, stem cells can be sourced from all over the body; from blood, bone and, specifically important to this work, adipose tissue [11]. Whilst adult stem cells have restricted growth potential when compared to embryonic stem cells (ESCs), they are not clouded by the same ethical concerns and can be used in an autologous nature [10]. The term mesenchymal stem cell is used to describe many adult stem cells but is actually problematic due to the significant inconsistencies and ambiguity of the term's use [12]. Ideally mesenchymal stem cell as a term would no longer be used as it is poorly defined, however its use is still rampant with many researchers publishing their findings without indicating which tissue the mesenchymal stem cells were even sourced from [13]. Currently, the most widely utilised source of adult stem cells are bone marrow and adipose tissue [17]. While adult stem cells possess limited proliferative capacity in comparison to ESCs, they still have the ability to differentiate along a mesodermal lineage and are able to transdifferentiate into ectodermal and endodermal cells lineages *in vitro* [17-23]. When sourced from bone marrow, they are termed bone marrow stem cells (BMSCs) while those sourced from adipose tissue are called adipose stem cells (ASCs) [24]. The differences in morphology and phenotype of ASCs and BMSCs is not significant [25], however ASCs have been shown to have higher proliferation rates [26]. BMSCs are harvested from the central region of bone marrow, a process that is exceptionally painful and poses a significant risk of morbidity [27,28]. In contrast, adipose tissue is obtained from liposuction, which is minimally invasive and a relatively safe procedure [20,29]. This was highlighted through an analysis of over 65,000 liposuction procedures which discovered that only 0.68% of patients had a serious adverse event post-surgery [30]. Furthermore, the quantity of stem cells

obtained from adipose tissue is comparatively higher than that obtained from bone marrow [26,31], which is advantageous for regenerative medicine as clinically applicable quantities are available without further expansion in culture [32].

Adipose tissue is a vascularised connective tissue that functions as an endocrine organ [155,156]. Within the body adipose tissue has many functions, such as protecting or cushioning with its mechanical properties, providing insulation and helping to streamline aquatic mammals, regulation of energy balance, and nutritional homeostasis [155]. The functional cells within adipose tissue are the adipocytes accounting for 90% of adipose tissue volume [157]. Adipocytes crosstalk with immune cells and neighbouring cells and tissues [155] and have also been shown to interact with many stromal cells other than immune cells such as vascular and neuronal cells. Enzymatic digestion of adipose tissue yields a population of cells termed the stromal vascular fraction, which contains endothelial cells, pericytes, fibroblasts and ASCs [19,20,158-160]. ASCs are can differentiate into different cell types as seen in Figure 1. They are capable of differentiating along mesodermal lineage (shown in blue) and can transdifferentiate along endodermal (shown in pink) and ectodermal (shown in orange) lineages.

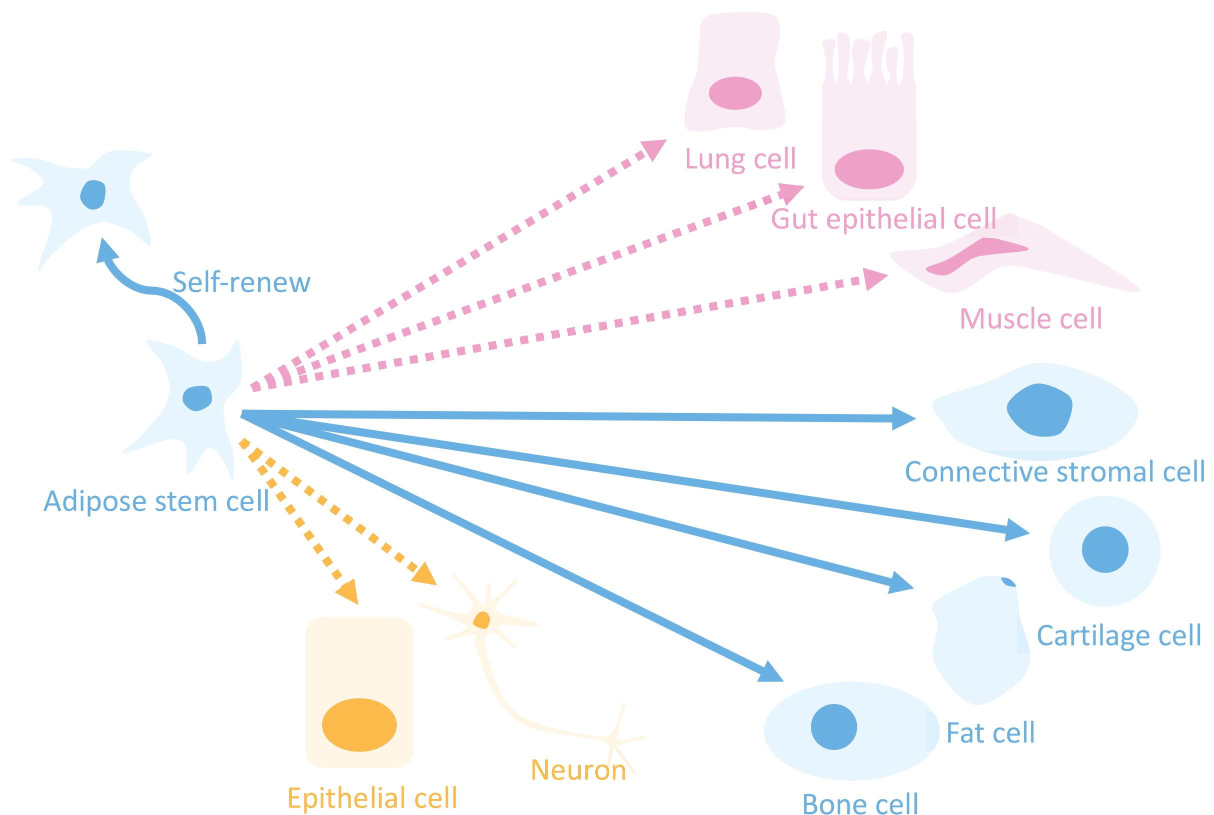


Figure 1: ASC differentiation capacity.

With regard to the development of therapies for regenerative medicine, the stromal vascular fraction can be used independently, or ASCs can be further isolated for use. It has been proposed that the presence of endothelial progenitor cells within the stromal vascular fraction may be advantageous for treatments of some diseases, however isolating and culturing ASCs provides significantly more cells which is preferable for treatment [161]. ASCs have been demonstrated to be immune privileged both *in vivo* and *in vitro* as they regulate both an inflammatory and immune response due to their ability to suppress T cells and activate antigen-specific regulatory T cells [162-164]. They also have immunosuppressive abilities such as suppressing lymphocyte reactivity, inhibiting production of inflammatory cytokines and the secretion of proteins which assists in immunomodulation and tissue regeneration [165-167]. The ability of ASCs to modify host tissue responses and overall immunobiological properties suggests minimal immune rejection [10,159]. To further support the use of ASCs in regenerative medicine they have also been shown to accumulate at sites of injury, often in response to an inflammatory reaction, [168,169]. ASCs have also been shown to improve formation of the extracellular matrix through remodelling [170]. However, ASCs like other stem cell treatments still face challenges such as immune mediated rejection, problems with cell

survival, loss of function and potential to transform into malignant cells. Stem cells have been isolated from human lipoma tissue [171] and stromal cells that are similar to BMSCs have been isolated from primary osteosarcoma [172], and this evidence suggests that stem cells are involved in tumour development. BMSCs have been shown to promote tumour growth through expression of growth factors, increasing vessel formation within tumours and creating a tumour stem cell niche [173].

To date there has been success with stem cell treatments for cancers such as leukaemia and multiple myeloma. However, with the exception of hematopoietic stem cells, all other stem cell therapies are still in the research, pre-clinical or clinical phases [37]. The field of regenerative medicine is advancing at an extraordinary rate and with real potential [34]. Overall there appears to be considerable investment in both preclinical and clinical trials, yet this is resulting in only minimal success [36]. This often isn't communicated to the broader public, and they tend to view stem cell treatments through a lens of false optimism. Stem cell treatments are ripe for patient exploitation and predatory stem cell clinics have been abusing patient hope to sell unproven stem cell treatments around the globe. Stem cells are being sold as treatments for everything from anti-ageing facial treatments that lack clinical evidence [174] through to intra-articular injections of ASC extracts for osteoarthritis, and intravenous injection of crude stem cell extracts for stroke, spinal cord injury, amyotrophic lateral sclerosis, autism and multiple sclerosis to just name a few [83]. All of the aforementioned treatments are available outside of clinical trials. ASCs are being employed in these unproven stem cell treatments due their ease of extraction, and this detracts from their potential use as an actual, viable, tested, treatment option. While it is still being debated, there is hope that conditioned media from ASCs could be utilised in place of the cells themselves as these contain cytokines. There is interest in extracellular vesicles released by ASCs into the media as there are reported to be more stable than the cells themselves [175]. There are many studies that have investigated the regenerative potential of stem cell extracellular vesicles and these have shown some promising results [176-178]. They also pose lower therapeutic risk and could be used as an alternate therapeutic, obviously after undergoing much needed rigorous testing so they don't fall into the same trap as existing predatory ASC therapies. However just as there as stem cell treatments that have tumorigenic potential, so do the extracellular vesicles themselves, with BMSC derived exosomes having been shown to promote tumour growth [179].

When examining the stem cell treatments that have undergone clinical trials, it is adult stem cells that are used more commonly. Pluripotent cells are also being studied clinically, but to a lesser extent that adult stem cells [36].

Stem cells also have use outside of the clinic, namely as a research model. Unlike clinical trials, pluripotent stem cells are used more than their multipotent counterparts in research models. Stem cell research models are dominated by iPSCs which are reprogrammed somatic cells that exhibit the same essential characteristics as ESCs [5]. It is logical for research models to favour these stem cells over adult stem cells. Firstly, they are pluripotent rather than multipotent, meaning they have greater differentiation capacity, and secondly, they are immortalised cell lines, meaning they can be expanded in culture indefinitely. Adult stem cells are seldom used because they must be extracted directly from patients, are a primary cell line that can only be utilised for a certain number of passages and have a limited multipotent differentiation capacity. It is for these reasons that there are limited research models that utilise ASCs and the cells remain poorly understood, with no single marker nor panel of markers that can sufficiently identify ASCs, for example [180]. It is imperative that a thorough understanding of ASCs is developed, particularly because of their rampant use in unproven stem cell therapies, but also to further their use as a research model.

1.2 Chapter proposal

This project aims to provide the most comprehensive catalogue of the ASC proteome to date. As stated, ASCs are poorly characterised, and their proteome has garnered little research. For example, undifferentiated human ASC lysates have been analysed by 2D gel electrophoresis and tandem mass spectrometry which identified 170 proteins [181]. While useful, this only provides a very small glimpse of the ASC proteome and is not nearly comprehensive enough. As these stem cells are being used widely in both clinical trials and in unproven stem cell treatments, it is important that their proteome is thoroughly characterised to understand if any adverse problems may result from their use as a therapeutic and through their expansion before use. Furthermore, ASCs have potential as a research model and establishing a thorough understanding of their baseline proteome is critical for further models to be

developed. This will also help bridge the current gaps in ASC knowledge, for example the lack of sufficient surface markers for the identification of ASCs can be addressed through proteomic analysis. Characterisation of the proteins present on the surface of the cells could provide insight into alternative markers capable of distinguishing ASCs.

The determination of what proteins are present, or identified, on and in a cell and the measurement of the abundance of those proteins is achieved through the combination of numerous disciplines [182]. Characterisation of proteins is crucial for developing a sound understanding of the complexity of biological samples as they are a dominant feature of cellular phenotype [183]. Analysis of the proteome provides more information on the functional complexity of a cell than the analysis of the genome alone, because proteomes differ spatially and temporally within cells and tissues, making proteomics a useful tool for studying biological phenotypes [183]. Proteomics also allows for the analysis of functional changes that can't be detected by genomics due to post-translational modifications and processing into mature proteoforms [183]. Biological samples are extremely complex, and identification of proteins within a sample is possible through the coupling of liquid chromatography (LC) with tandem mass spectrometry (MS/MS) [183]. Mass spectrometry-based proteomics allows for identification, quantification and characterisation of proteins from complex samples [182,184]. The most commonly utilised method for the identification and quantification of proteins is bottom-up proteomics. This essentially involves the analysis of a complex mixture of proteins that have been digested into peptides, which are then fractionated by reversed phase chromatography, ionised, and analysed by MS/MS [183]. The abundance of proteins, or more specifically the products of open reading frames, is inferred through the abundance of peptides that map to the sequence of the protein.

In order to identify as much of the ASC proteome as possible, multiple fractions will be investigated. Analysis of the whole cell lysate will provide a broad catalogue of the ASC proteome. As ASCs are encased in a cellular membrane, the membrane bound from ASCs will be examined to provide some insight into potential surface markers that warrant further investigation. The method utilised to extract the membrane bound fraction will extract all cellular membranes, and this will provide more depth to the analysis by allowing detection of membrane bound proteins that may not be identified in the proteomic analysis of the ASC

whole cell lysate. Secreted proteins packaged into extracellular vesicles will also be analysed, as ~10% of the human genome encodes for secreted proteins [185]. Secreted proteins are vital for cellular communication such as cell signalling, cell adhesion, binding, differentiation, invasion, metastasis, angiogenesis and apoptosis [186]. Proteins are secreted out of the cell constitutively or in a regulated manner.

The classical endoplasmic reticulum-golgi secretory pathway is the conventional way that proteins are secreted. This is initiated by ribosomes directing the proteins that contain an endoplasmic reticulum signal sequence to the rough endoplasmic reticulum. Here the polypeptide chain is translocated to the lumen where post-translational processing occurs. Glycosylation of correctly folded proteins occurs in the golgi complex, along with protein sialyation, tyrosine sulphation and proteolytic cleavage if necessary, before the proteins are released by exocytosis [187]. Proteins which lack an endoplasmic reticulum signal sequence are likely secreted by being translocated across membranes or packaged into membrane or protein coated vesicles [188]. Regulatory proteins stored within secretory vesicles can be released at the appropriate time after a specific signal and [189]. Stem cells are known to secrete a large quantity of vesicles [190] and these extracellular vesicles are capable of transferring proteins, mRNA and miRNA to other cells, making them important for intercellular communication [191]. Studying extracellular vesicles allows for a more comprehensive analysis of the cellular proteome as extracellular vesicles often carry low abundance and membrane bound proteins that may not otherwise be detected. Analysis of the three fractions stated allows for a more comprehensive picture of the ASC proteome, not only through the identification of more unique proteins, but also because these fractions increase the number of detected peptides for each individual protein, allowing for more reliable and robust quantification of proteins.

Detecting cytokines using a mass spectrometer is difficult due to their low abundance and to overcome this technical hurdle a multiplex immunoassay was utilised. Cytokines are present in relatively low abundance, making them difficult to detect by electrophoretic or mass spectrometry based proteomic methods. However, cytokines can be quantified by the Bio-plex multiplex assay (Bio-Rad) which provides a robust and reproducible assay system that allows for accurate quantification of this niche of the proteome. The Bio-Plex uses a liquid

suspension array and unique hexapeptide-antibody library of complementary colour coded magnetic beads specific for a panel of human cytokines which are quantified by being drawn through a flow cell, fluorescently excited with lasers, and measured by a dual-laser based reader, allowing for the consequent identification and quantification of the secreted cytokines [192]. The Bio-Plex Pro Human Cytokine 27-Plex Immunoassay that will be utilised quantifies the following panel of proteins: fibroblast growth factor (FGF) basic, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, interferon gamma-induced protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulation on activation normal T-cell expressed and secreted (RANTES), tumour necrosis factor (TNF)-α and vascular endothelial growth factor (VEGF).

Through quantifying proteins in the whole cell lysate, membrane bound fraction, extracellular vesicles and select secreted cytokines, a more complete view of the ASC proteome will be developed. This proteome is desperately needed as ASCs are already being used in the clinic, both in controlled clinical trials and in unproven stem cell treatments. The catalogue of proteins from this project are vital for a comprehensive understanding of ASCs and can shape their use in the clinic. Furthermore, this will serve as a thorough baseline library of proteins that can be used to support the use of ASCs as a research model.

2. Methods

The work presented in this thesis has biosafety approval from University of Technology Sydney (2013000437) and human ethics approval from the University of Technology Sydney (UTSHREC 2015000118).

2.1 Stem cell isolation and expansion

A total of 8 humans volunteered to donate their subcutaneous lipoaspirates for this PhD, these 8 patient samples are utilised across Chapter Two, Chapter Three and Chapter Four of this thesis. It is important to recognise that n=8 is a reasonably small sample number. These subcutaneous lipoaspirates were obtained through the Matraville Medical Centre from a collaboration with the Schwartz Foundation. Lipoaspirates were washed with phosphate buffered saline and digested with collagenase type 1 (Gibco) for 40 minutes at 37°C to disrupt connective material. The samples were centrifuged at 1600 x g for 10 minutes, separating the stromal vascular fraction containing the cells of interest [18]. The supernatant was carefully decanted, and the remaining pellet was gently resuspended in pre-warmed basal growth medium, DMEM Glutamax/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco). The suspension was aliquoted into tissue culture flasks (Falcon) in an appropriate volume for the flask size (T25 3mL, T75 9mL and T175 21mL) and incubated at 37°C in a 5% CO_2 atmosphere, allowing the cells to adhere to the flask. After 48 hours the basal growth media was replaced, removing any non-adherent cells which were discarded. The basal growth media was replaced every 84 hours thereafter. When the cells reached 80% confluence, the cells were rinsed with phosphate buffered saline and TrypLE (Gibco) was utilised to detach the ASC with volumes appropriate for the size of the flask (T25 0.5mL, T75 1.5mL and T175 3mL). ASCs were isolated from the stromal vascular fraction by expanding their numbers in tissue culture to eliminate non-adherent cells [193]. ASCs were grown to passage 5, and all analysis in this thesis was conducted at passage 5. For all experiments a T175 was seeded with 100,000 cells which were harvested after 84 hours for subsequent analysis.

2.2 Extraction of cellular fractions

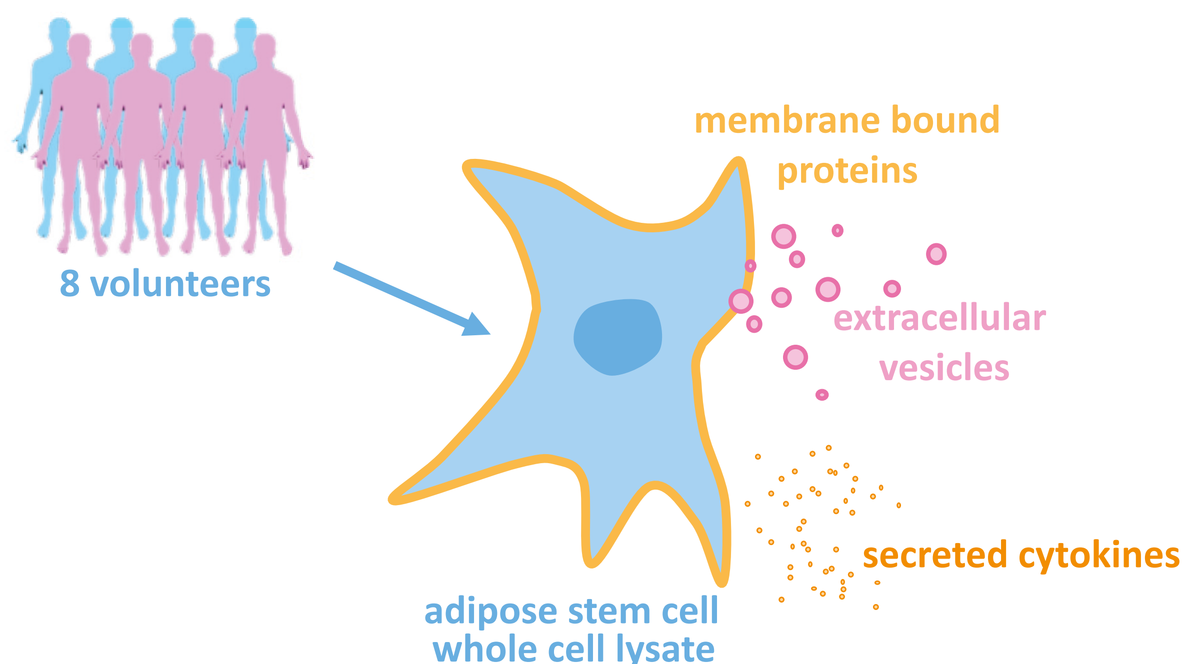


Figure 2: ASC proteomic analysis workflow.

In order to gain a comprehensive understanding of ASCs, the proteins able to be extracted from the whole cell lysate, membrane bound fraction, extracellular vesicles and secreted cytokines were analysed as shown in Figure 2. When harvesting ASCs, the cells were detached using the aforementioned method and the cell resuspension from a T175 (~3 mL) was split across three microtubes. These were centrifuged 1200 x g for 10 minutes and the supernatant was discarded. Of these 3 aliquots, 1 was utilised for the extraction of the whole cell lysate, 1 for the membrane bound fraction, and the other stored for future use at -80°C.

Stem cell extracellular vesicles are typically isolated by differential centrifugation [186]. It is typically recommended to expand stem cells in the absence of FBS prior to extracellular vesicle extraction as highly abundant serum proteins such as albumin will make it mask low abundance proteins [186]. However, serum starvation causes stem cells to respond differently to how they normally would, such as membrane blebbing and growth arrest [194,195]. Serum

starvation has been shown to affect the transcription of over 100 genes in ASCs [196] which will be investigated in the next chapter. For this reason, extracellular vesicles were not serum starved and were isolated through differential centrifugation. This involved collecting media from ASCs, centrifuging them at 300–400 × g for 10 min to remove cells, followed by 2000 x g to remove cell debris, then 10,000 × g to remove the aggregates of biopolymers, apoptotic bodies, and the other structures with the buoyant density above those of extracellular vesicles. Extracellular vesicles contained in the resulting supernatant are sedimented by ultracentrifugation at 100,000 × g for 2 hours [197,198]. There are additional steps that could be taken to purify extracellular vesicles, but these also reduce the quantity of extracellular vesicles [199]. For this reason, no further purification was conducted, as each LC-MS/MS injection already required extracellular vesicles extracted from 3 T175s. Extracellular vesicles were stored at -80°C for future protein extraction using the single-pot, solid-phase-enhanced sample-preparation method described above. The media from ASCs was also collected and frozen at -80°C for future analysis of secreted cytokines using a multiplex immunoassay explained bellow.

2.3 Single-pot solid-phase-enhanced sample-preparation

The whole cell lysate tryptic digest was preapred using the single-pot, solid-phase-enhanced sample-preparation protocol [200]. Cell pellets were resuspended in 50µL of single-pot solid-phase-enhanced sample-preparation reconstitution buffer containing 1% sodium dodecyl sulfate, 1% nonionic polyoxyethylene -40, 1% deoxycholate, 1% glycerol, 50mM NaCl and 50mM HEPES pH 8.0 in ultrapure water and heated at 60°C for 30 minutes in a heating block. The cell suspension was then sonicated in a sonicating water bath for 10 minutes at room temperature, and 1µL of benzonase nuclease was added to samples before incubating at 37°C for 1 hour. Following benzonase treatment, samples were reduced and alkylated by adding tris(2-carboxylethyl) phosphine and acrylamide monomers to final concentrations of 5 mM and 20 mM, respectively, and incubating at room temperature for 1 hour before quenching the reaction with dithiothreitol at a final concentration of 20mM. An aliquot of each sample containing ~1×10^6 cells was transferred to a fresh microtube and diluted with SP3 reconstitution buffer. The 'rule of thumb' is 1ug protein:10ug beads [201]. To each sample, 2µL

of a paramagnetic bead suspension (50 mg/mL) was then added along with 50µL of 100% ethanol. Samples were vortexed for 5 minutes and then incubated on a magnetic rack. The supernatant was then removed, and beads washed three times with 80% ethanol, utilizing the magnetic rack each time to remove wash solution. After the final wash, the supernatant was removed and the beads were resuspended in 100µL of 200mM ammonium bicarbonate with 1µg of trypsin gold, mass spectrometry grade (Promega). Samples were then sonicated in a sonicating water bath for 30 seconds to disaggregate beads and incubated overnight at 37°C to allow for trypsin digestion. Following trypsin digestion, samples were incubated on a magnetic rack, and the supernatant containing the peptides removed and stored in a fresh microtube at -80°C for future LC-MS/MS analysis.

2.3 Membrane bound protein extraction

The membrane bound fraction was extracted using the following method. Samples were lysed in 100mM NH_4HCO_3 and 8M urea by sonication in a sonicating water bath for 10 minutes at room temperature, then 1µL of benzonase nuclease was added to samples before incubating at 37°C for 1 hour. Following benzonase treatment, samples were reduced and alkylated by adding tris(2-carboxylethyl) phosphine and acrylamide monomers to final concentrations of 5 mM and 20 mM, respectively, and incubating at room temperature for 1 hour before quenching the reaction with dithiothreitol at a final concentration of 20mM. This suspension was centrifuged at 20,000 g for 10 minutes and the supernatant was stored in a microtube at -80°C for alternate use. The remaining membrane bound protein pellet was resuspended in sodium dodecyl sulfate loading buffer. The samples were then heated to 95°C for 10 minutes and centrifuged for 5 minutes at 16000rcf. After these samples were loaded into a Criterion TGX gel (BioRad) with tris/glycine/SDS running buffer (BioRad) and were electrophoresed at 160V for 5 minutes or until the sample had just run into the gel and all of the proteins were compressed into a single band. The gel was then stained with Coomassie until the samples were visible. The gel pieces containing the sample protein were excised, cut up and placed into their own microtubes and destained by washing twice with 50% acetonitrile/50mM NH_4HCO_3 pH 9 for 10 minutes with vortexing. After the washes were discarded, the gel slices were dehydrated with 100% acetonitrile for 10 minutes and

rehydrated in 100nM NH_4HCO_3 pH 9 containing 12.5ng/µL of trypsin (Promega). After incubating at 4°C for 30 minutes 100nM NH_4HCO_3 pH 9 was added and the samples incubated overnight at 37°C. The digest solution was removed to a new tube and 50µL of 50% acetonitrile, 2% trifluoroacetic acid added to the gel pieces and incubated for 10 minutes in a sonicating water bath at full power. The solution was removed, added to the other collected digest solution, and stored in a fresh microtube at -80°C for future LC-MS/MS analysis.

2.4 Liquid chromatography–mass spectrometry

Using an Acquity M-class nanoLC system (Waters, USA), 5 µL of the sample was loaded at 15µL/min for 3 minutes onto a nanoEase Symmetry C18 trapping column (180µm x 20mm) before being washed onto a PicoFrit column (75 µmID x 300 mm; New Objective, Woburn, MA) packed with Magic C18AQ resin (3µm, Michrom Bioresources, Auburn, CA). Peptides were eluted from the column and into the source of a Q Exactive Plus mass spectrometer (Thermo Scientific) using the following program: 5-30% MS buffer B (98% Acetonitrile + 0.2% Formic Acid) over 90 minutes, 30-80% MS buffer B over 3 minutes, 80% MS buffer B for 2 minutes, 80-5% for 3 min. The eluting peptides were ionised at 2400V. A data dependant MS/MS (dd-MS^2) experiment was performed, with a survey scan of 350-1500 Da performed at 70,000 resolution for peptides of charge state 2+ or higher with an AGC target of 3e6 and maximum Injection Time of 50ms. The Top 12 peptides were selected fragmented in the HCD cell using an isolation window of 1.4 m/z, an AGC target of 1e5 and maximum injection time of 100ms. Fragments were scanned in the Orbitrap analyser at 17,500 resolution and the product ion fragment masses measured over a mass range of 120-2000 Da. The mass of the precursor peptide was then excluded for 30 seconds.

2.5 Proteomic data analysis

The MS/MS data files were searched using Peaks Studio X+ against the Homo sapiens database and a database of common contaminants with the following parameter settings. Fixed modifications: none. Variable modifications: propionamide, oxidised methionine, deamidated asparagine. Enzyme: semi-trypsin. Number of allowed missed cleavages: 3.

Peptide mass tolerance: 10ppm. MS/MS mass tolerance: 0.05Da. The results of the search were then filtered to include peptides with a $-\log_{10}P$ score that was determined by the false Discovery Rate of <1%, the score being that where decoy database search matches were <1% of the total matches.

Several programs were utilised for further analysis. Label-free quantification was performed in Peaks Studio (v. 8.5) with the following parameter settings: Retention time range between 0 and 140; Protein significance ≥ 20; Protein fold-change ≥ 2; Significance method – ANOVA, with at least 1 unique peptide; Normalization method – total ion count. Pathway enrichment analysis was performed on protein identification lists placed into the PANTHER Classification System which categorised proteins by molecular function, cellular compartment, biological process, protein class and protein pathway [202]. PANTHER is only able to detect proteins that are annotated within its own database. Heatmaps were generated using R (v3.6.2) from protein quantification lists which were normalised between samples to total ion count per individual sample. Normalised intensities were then log2 transformed, Z scaled across all samples and plotted as clustered heatmaps with complexheatmap (v2.2.0). STRING was used to showcase functional protein association networks and to illustrate the interactions of the detected proteins [203]. These interactions are based on direct and indirect associations [204], but are also inferred from computational predictions, knowledge from other organisms, and aggregates from primary databases [205,206]. The main databases used by STRING are genomic context predictions, high-throughput lab experiments, co-expression, automated text mining and previous knowledge in databases [207,208]. STRING contains information on 24,584,628 proteins and will only create networks for proteins that it is able to detect from its own database [208]. Network edges were set to display confidence and structure previews were disabled within nodes. Functional enrichments were chosen manually. The output is a STRING network, the number of nodes, the number of edges and the average local clustering coefficient. This number measures the extent to which nodes cluster together and the closer this value is to 1, the greater the clustering. Conversely a value closer to 0 is indicative of low clustering of these nodes.

2.6 Cytokine detection and analysis

Cytokines were quantified by the Bio-plex multiplex assay (Bio-Rad) which provides a robust and reproducible assay system that allows for better representation of the dynamic range of this niche of the proteome. The Bio-Plex uses a liquid suspension array and unique hexapeptide-antibody library of complementary colour coded magnetic beads specific for the panel of human cytokines which were quantified by being drawn through a flow cell, fluorescently excited with lasers, and measured by a dual-laser based reader, allowing for the consequent identification and quantification of the secreted cytokines [192]. The Bio-Plex Pro Human Cytokine 27-Plex Immunoassay that will be utilised quantifies the following panel of proteins: FGF basic, eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF.

3. Results

ASCs were isolated and expanded to passage 5 where proteomic analysis occurred. The whole cell lysate, membrane bound fraction and extracellular vesicles were analysed on the Q Exactive Orbitrap MS system. After identification and filtering 2290 proteins were found to be conserved across all patients.

Table 1: Proteins detected from ASC whole cell lysate, membrane bound fraction and extracellular vesicles from 8 patient samples.

	Whole cell lysate	Membrane bound	Extracellular vesicles
Average proteins per patient	4379	1583	2582
Proteins conserved in all patients	1854	577	1312

Table 1 demonstrates that the largest quantity of proteins detected was in the whole cell lysate which had an average of 4379 proteins detected per patient. This was followed by extracellular vesicles with an average of 2582 proteins per patient and the membrane bound fraction with an average of 1583 proteins per patient. This number is reduced significantly when filtering for proteins that were conserved across all patients, with the whole cell lysate proteins decreasing by 57.67%, membrane bound by 63.55%, and extracellular vesicles by 49.19%.

Proteins that were conserved across all patients are the most accurate representation of ASC proteins, as there is greater confidence that these proteins are expressed in the broader population if they are detected in all patients. It is these proteins that will be the focus for the rest of this chapter.

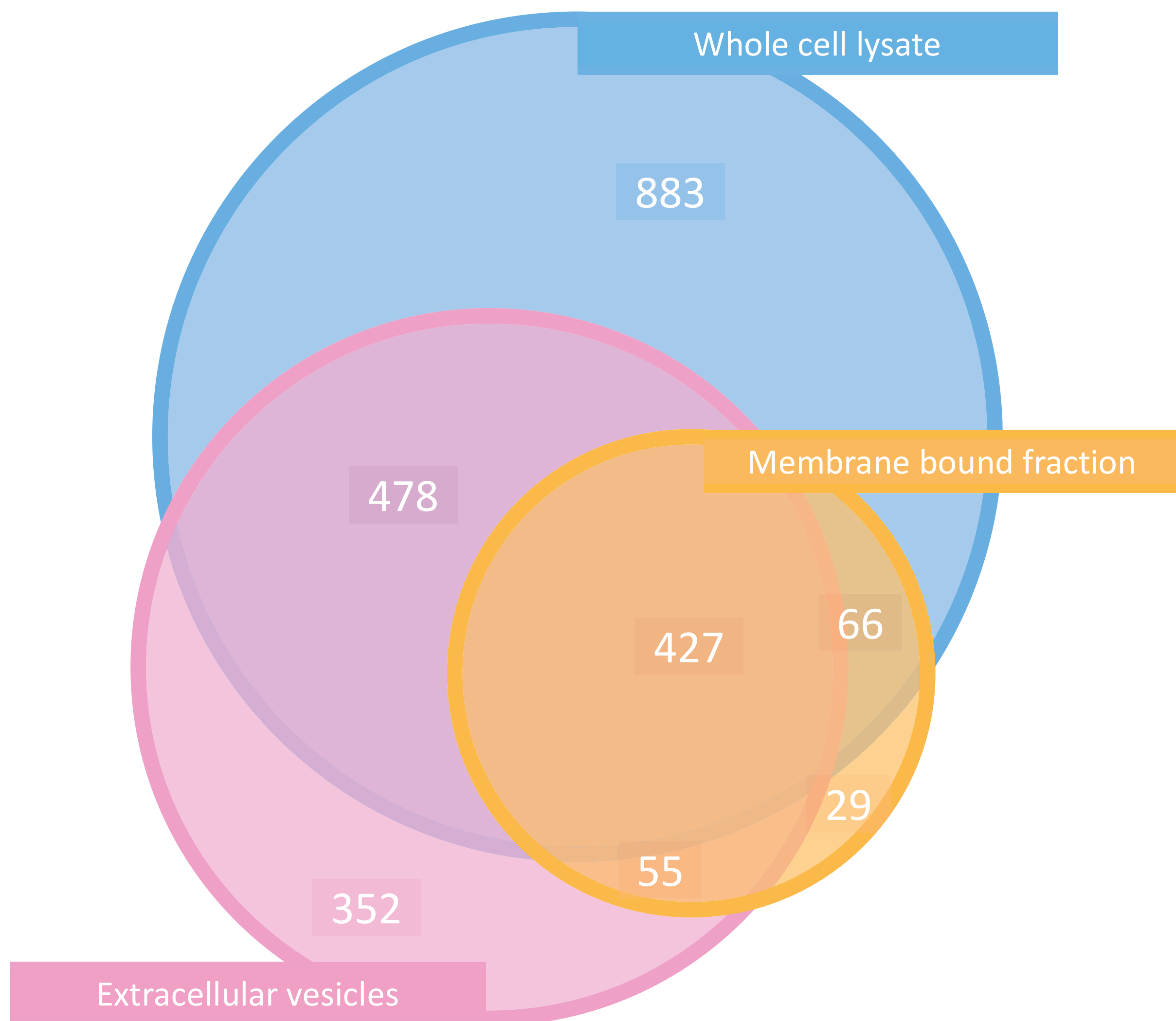


Figure 3: Proportional Venn diagram of ASC whole cell lysate, membrane bound fraction and extracellular vesicles.

Figure 3 demonstrates that the majority of proteins were only detected in the whole cell lysate with 883 unique proteins. Extracellular vesicles had 352 unique proteins that were not detected in other fractions, and the membrane bound fraction had 29 unique proteins that were not detected in other fractions. 599 proteins were found in two fractions (478 between the whole cell lysate and extracellular vesicles, 66 between the whole cell lysate and membrane bound fraction, and 55 between the membrane bound fraction and extracellular vesicles) and 427 proteins were identified as being common in all three fractions.

These proteins were further analysed by the PANTHER Classification System and the detected proteins categorised by molecular function (Figure 4), biological process (Figure 5), cellular compartment (Figure 6), protein class (Figure 7) and protein pathway (Figure 8).

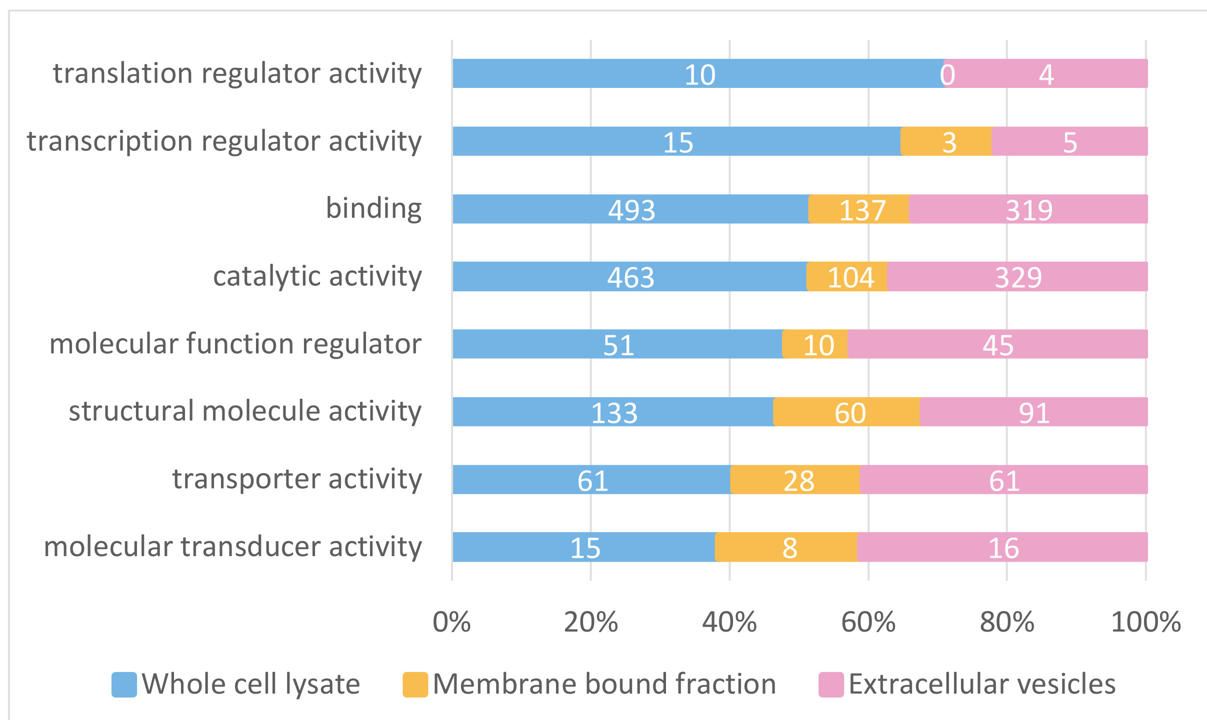


Figure 4: ASC proteins from the whole cell lysate, membrane bound fraction and extracellular vesicles sorted by molecular function.

The PANTHER Classification System detected proteins from 8 different categories of molecular functions with the majority of detected proteins in the categories of binding and catalytic activity.

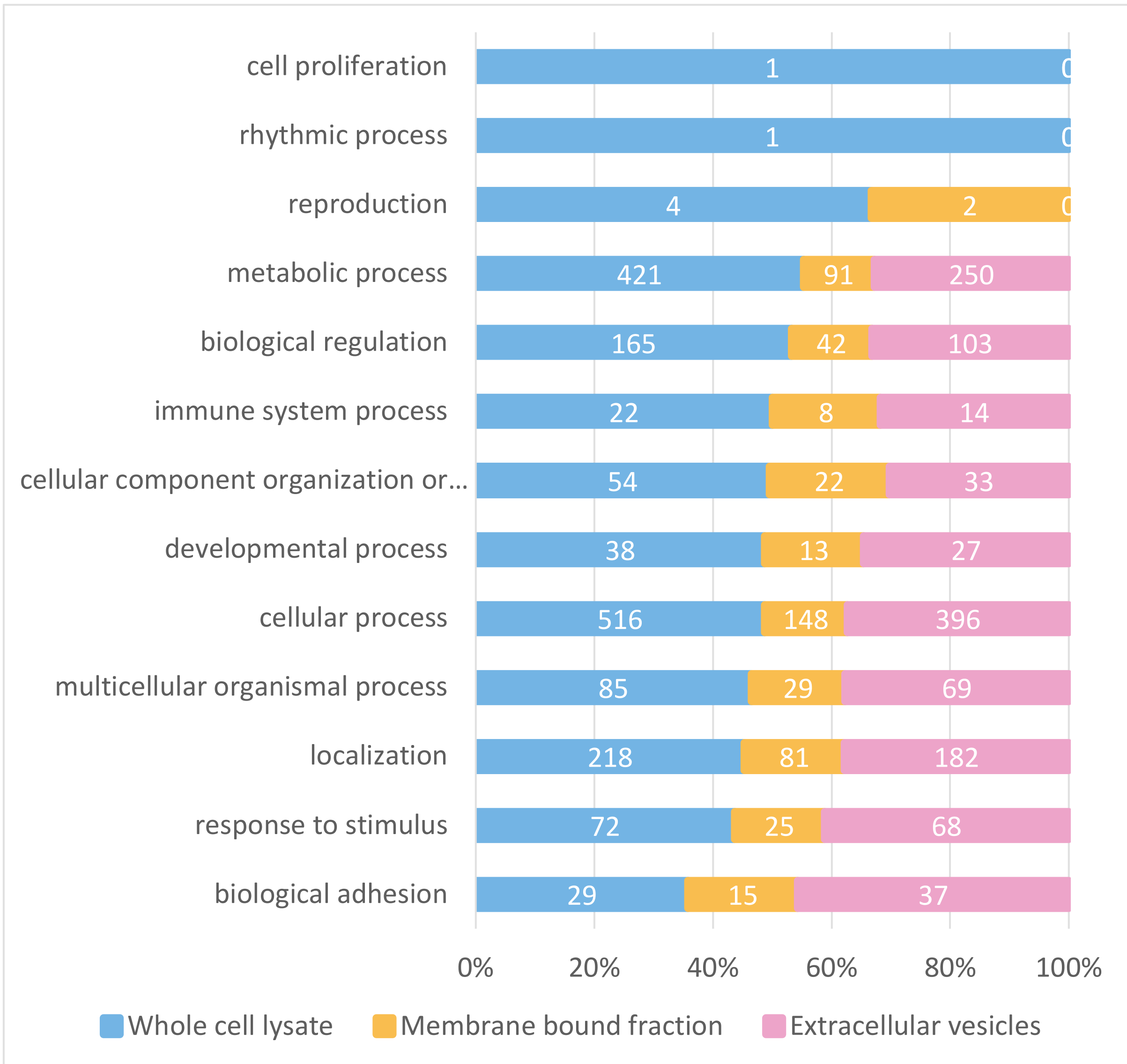


Figure 5: ASC proteins from the whole cell lysate, membrane bound fraction and extracellular vesicles sorted by biological process.

The PANTHER Classification System detected proteins from 13 different categories of biological processes with the majority of detected proteins in the category of cellular process.

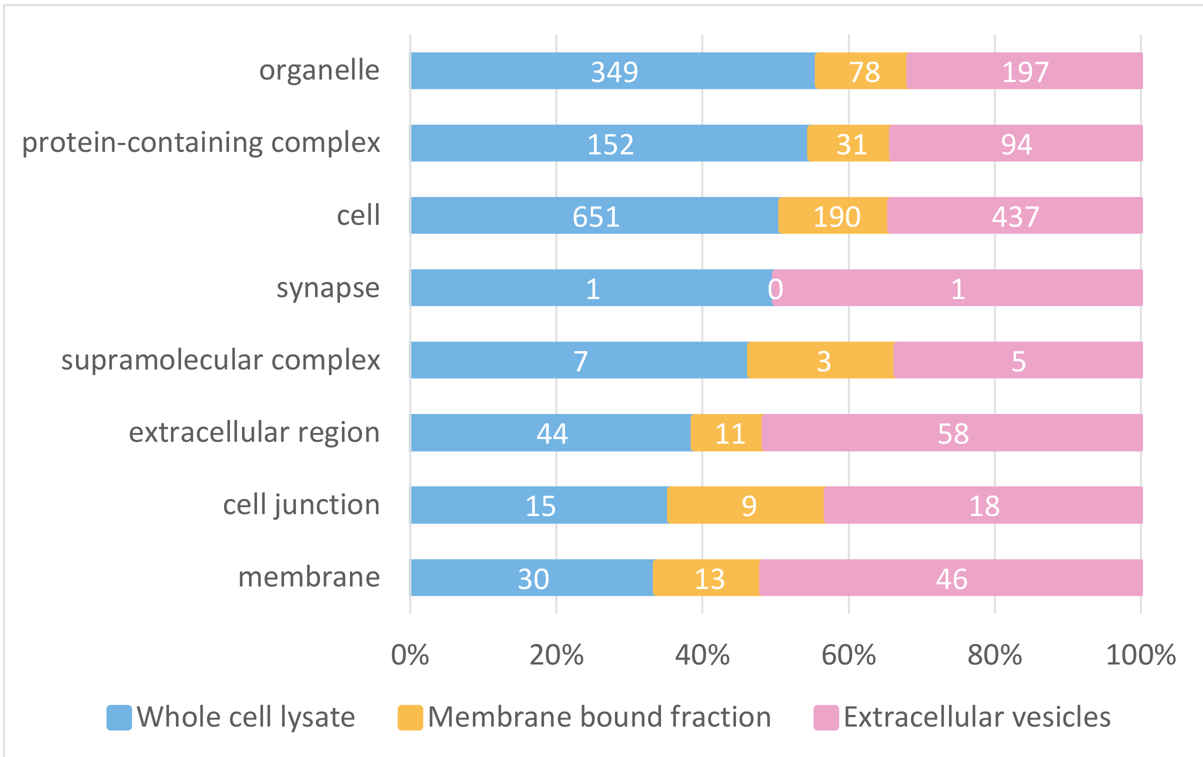


Figure 6: ASC proteins from the whole cell lysate, membrane bound fraction and extracellular vesicles sorted cellular compartment.

The PANTHER Classification System detected proteins from 8 different categories of cellular compartments with the majority of detected proteins the category of compartment cell.

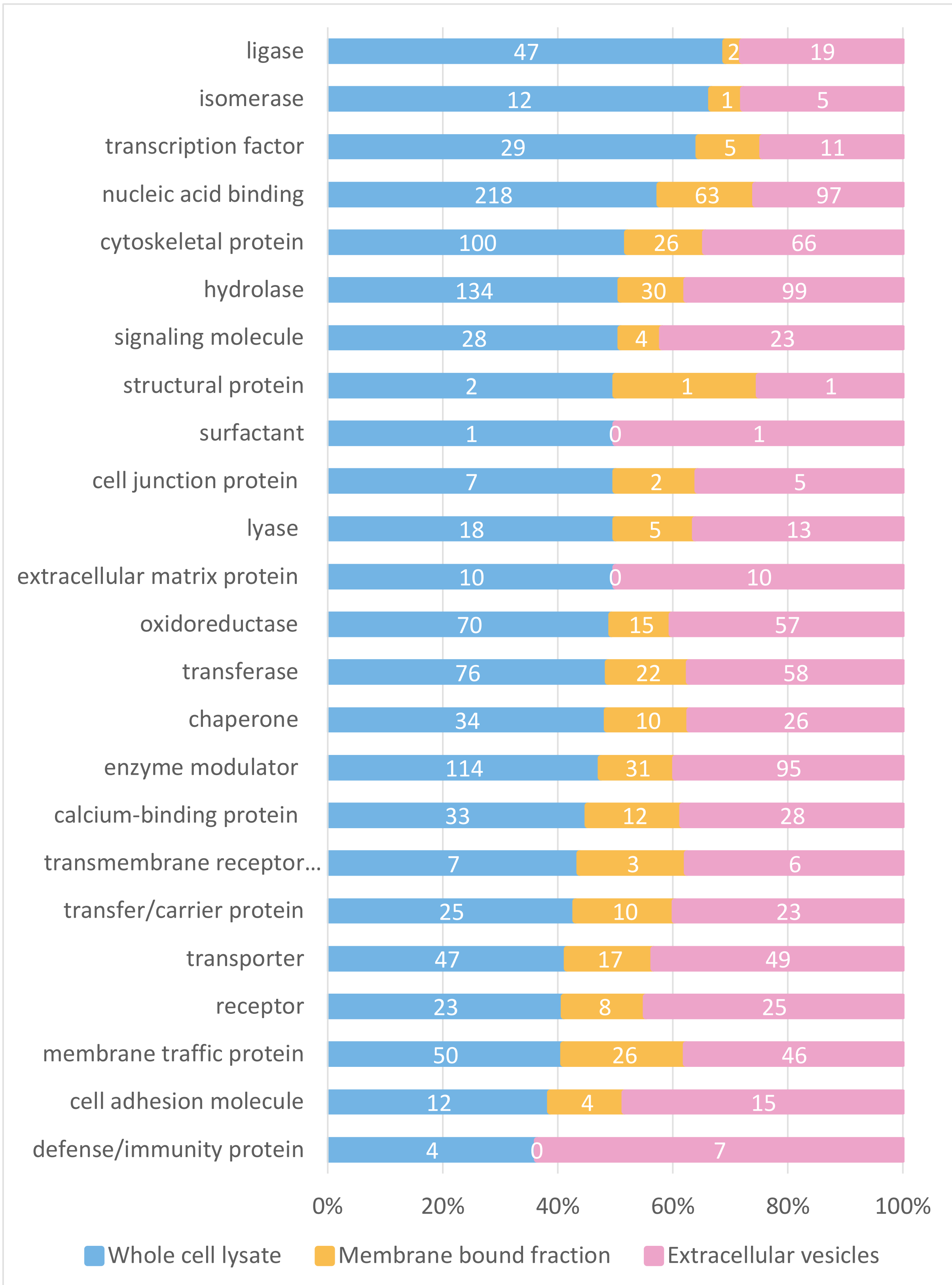
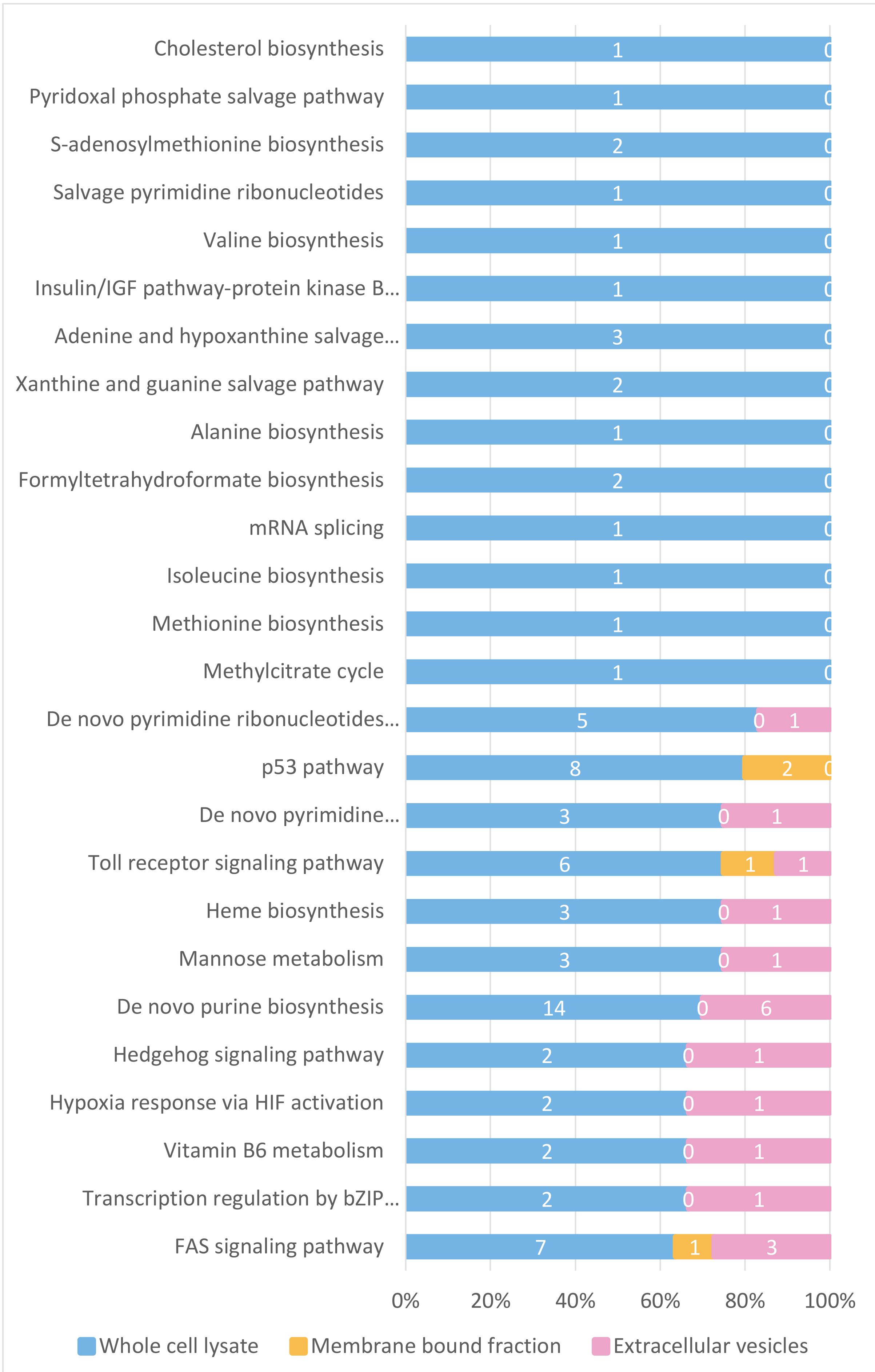
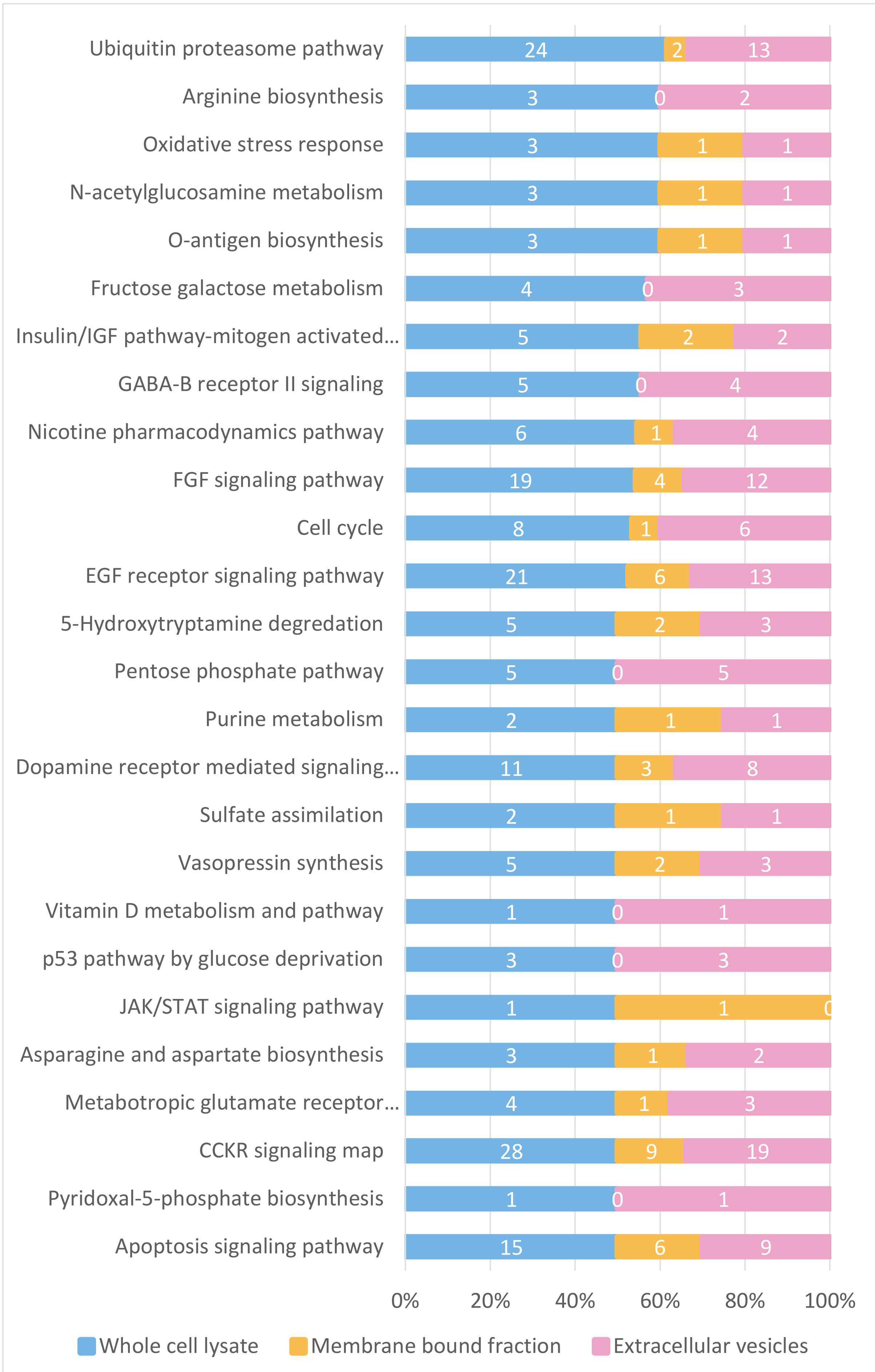


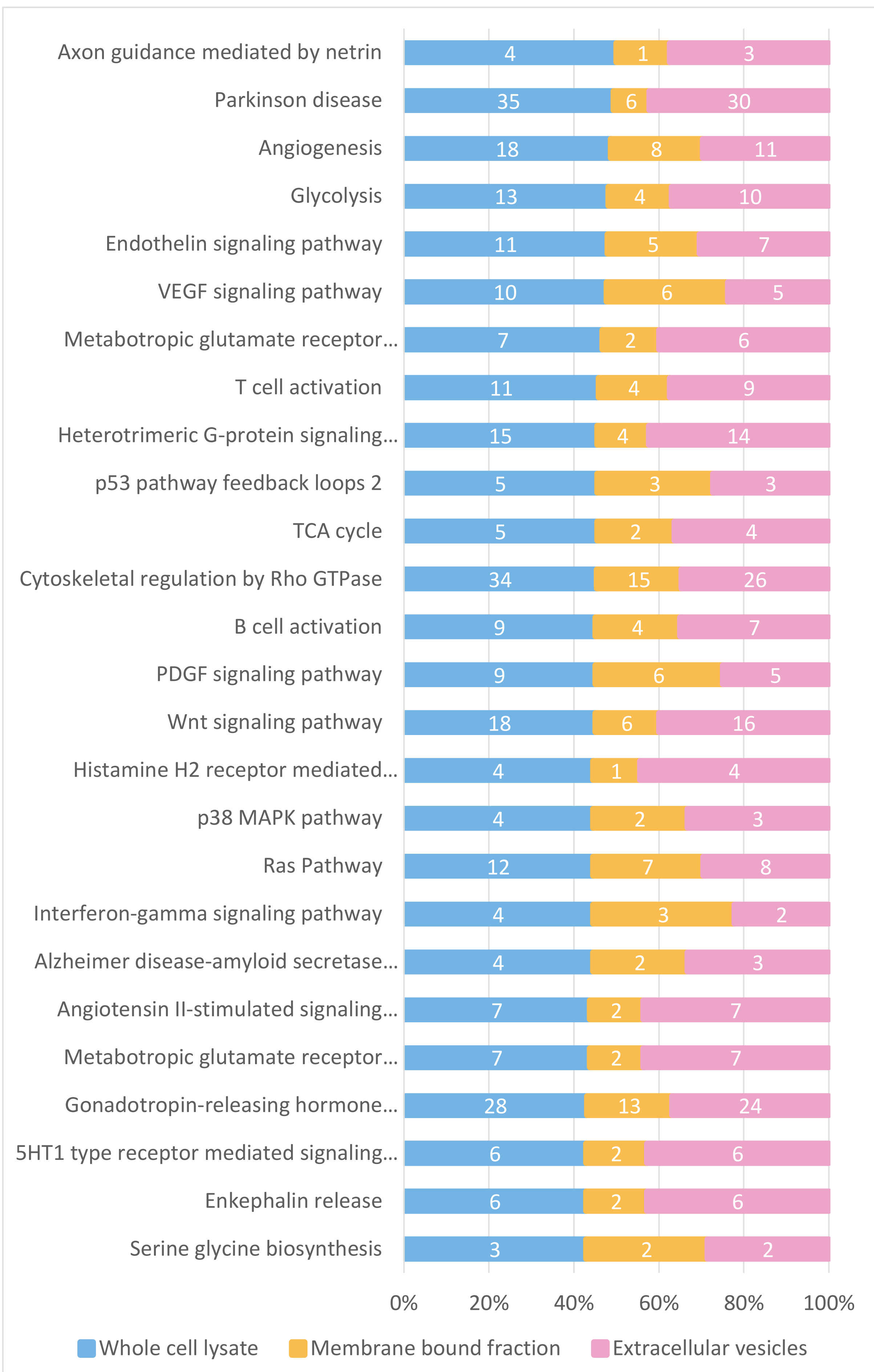
Figure 7: ASC proteins from the whole cell lysate, membrane bound fraction and extracellular vesicles sorted protein class.

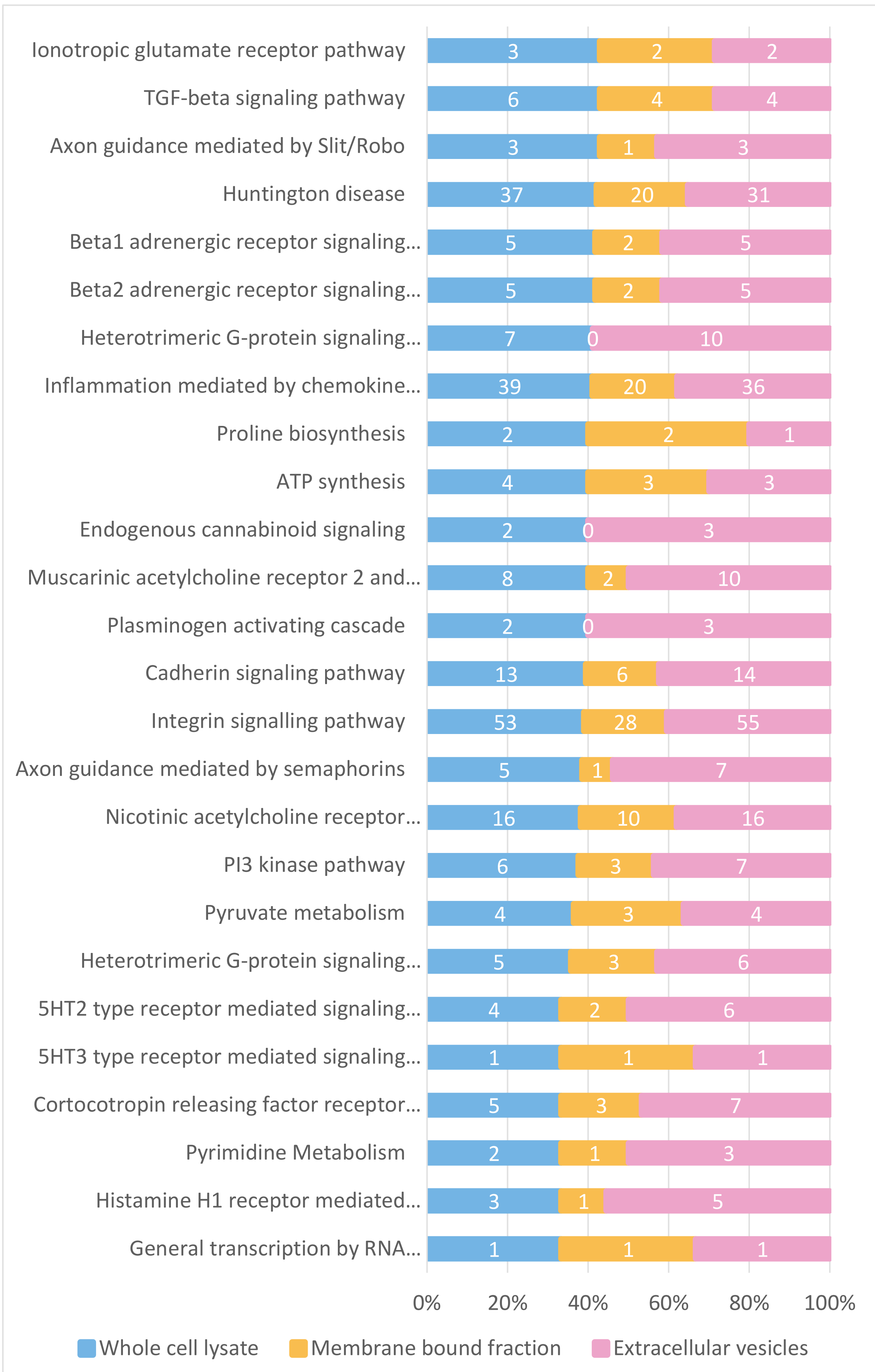
The PANTHER Classification System detected proteins from 24 different categories of protein classes with the majority of detected proteins in the category of nucleic acid binding.


Cholesterol biosynthesis
Pyridoxal phosphate salvage pathway
S-adenosylmethionine biosynthesis
Salvage pyrimidine ribonucleotides
Valine biosynthesis
Insulin/IGF pathway-protein kinase B...
Adenine and hypoxanthine salvage...
Xanthine and guanine salvage pathway
Alanine biosynthesis
Formyltetrahydroformate biosynthesis
mRNA splicing
Isoleucine biosynthesis
Methionine biosynthesis
Methylcitrate cycle
De novo pyrimidine ribonucleotides...
p53 pathway
De novo pyrimidine...
Toll receptor signaling pathway
Heme biosynthesis
Mannose metabolism
De novo purine biosynthesis
Hedgehog signaling pathway
Hypoxia response via HIF activation
Vitamin B6 metabolism
Transcription regulation by bZIP...
FAS signaling pathway
0%
20%
40%
60%
80%
100%
Whole cell lysate
Membrane bound fraction
Extracellular vesicles



Ubiquitin proteasome pathway
Arginine biosynthesis
Oxidative stress response
N-acetylglucosamine metabolism
O-antigen biosynthesis
Fructose galactose metabolism
Insulin/IGF pathway-mitogen activated...
GABA-B receptor II signaling
Nicotine pharmacodynamics pathway
FGF signaling pathway
Cell cycle
EGF receptor signaling pathway
5-Hydroxytryptamine degredation
Pentose phosphate pathway
Purine metabolism
Dopamine receptor mediated signaling...
Sulfate assimilation
Vasopressin synthesis
Vitamin D metabolism and pathway
p53 pathway by glucose deprivation
JAK/STAT signaling pathway
Asparagine and aspartate biosynthesis
Metabotropic glutamate receptor...
CCKR signaling map
Pyridoxal-5-phosphate biosynthesis
Apoptosis signaling pathway
0%
20%
40%
60%
80%
100%
Whole cell lysate
Membrane bound fraction
Extracellular vesicles


Axon guidance mediated by netrin
4
1
3
Parkinson disease
35
6
30
Angiogenesis
18
8
11
Glycolysis
13
4
10
Endothelin signaling pathway
11
5
7
VEGF signaling pathway
10
6
5
Metabotropic glutamate receptor...
7
2
6
T cell activation
11
4
9
Heterotrimeric G-protein signaling...
15
4
14
p53 pathway feedback loops 2
5
3
3
TCA cycle
5
2
4
Cytoskeletal regulation by Rho GTPase
34
15
26
B cell activation
9
4
7
PDGF signaling pathway
9
6
5
Wnt signaling pathway
18
6
16
Histamine H2 receptor mediated...
4
1
4
p38 MAPK pathway
4
2
3
Ras Pathway
12
7
8
Interferon-gamma signaling pathway
4
3
2
Alzheimer disease-amyloid secretase...
4
2
3
Angiotensin II-stimulated signaling...
7
2
7
Metabotropic glutamate receptor...
7
2
7
Gonadotropin-releasing hormone...
28
13
24
5HT1 type receptor mediated signaling...
6
2
6
Enkephalin release
6
2
6
Serine glycine biosynthesis
3
2
2
0%
20%
40%
60%
80%
100%
Whole cell lysate
Membrane bound fraction
Extracellular vesicles



Ionotropic glutamate receptor pathway
TGF-beta signaling pathway
Axon guidance mediated by Slit/Robo
Huntington disease
Beta1 adrenergic receptor signaling...
Beta2 adrenergic receptor signaling...
Heterotrimeric G-protein signaling...
Inflammation mediated by chemokine...
Proline biosynthesis
ATP synthesis
Endogenous cannabinoid signaling
Muscarinic acetylcholine receptor 2 and...
Plasminogen activating cascade
Cadherin signaling pathway
Integrin signalling pathway
Axon guidance mediated by semaphorins
Nicotinic acetylcholine receptor...
PI3 kinase pathway
Pyruvate metabolism
Heterotrimeric G-protein signaling...
5HT2 type receptor mediated signaling...
5HT3 type receptor mediated signaling...
Cortocotropin releasing factor receptor...
Pyrimidine Metabolism
Histamine H1 receptor mediated...
General transcription by RNA...
3 2 2
6 4 4
3 1 3
37 20 31
5 2 5
5 2 5
7 0 10
39 20 36
2 2 1
4 3 3
2 0 3
8 2 10
2 0 3
13 6 14
53 28 55
5 1 7
16 10 16
6 3 7
4 3 4
5 3 6
4 2 6
1 1 1
5 3 7
2 1 3
3 1 5
1 1 1
0% 20% 40% 60% 80% 100%
Whole cell lysate
Membrane bound fraction
Extracellular vesicles

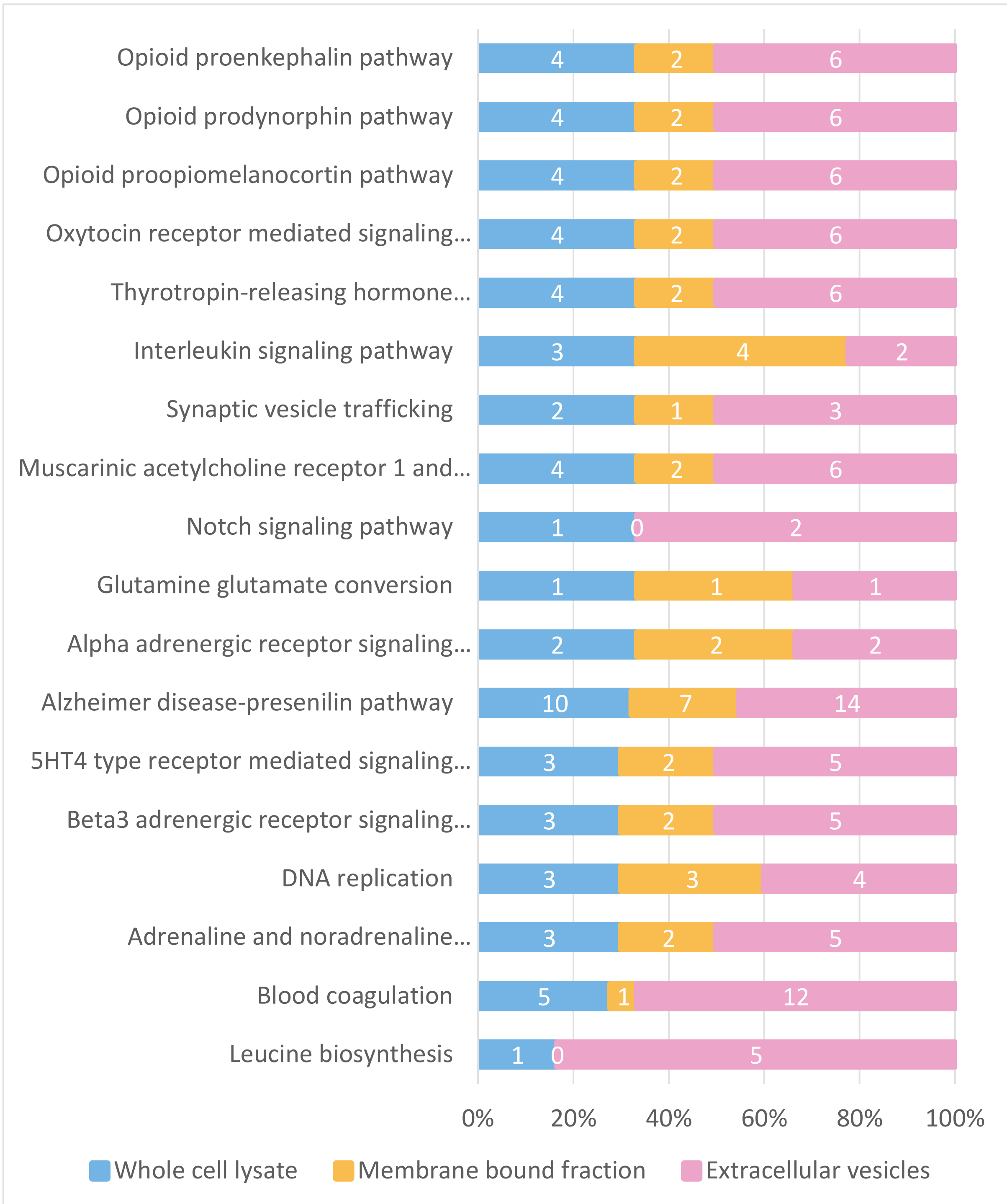


Figure 8: ASC proteins from the whole cell lysate, membrane bound fraction and extracellular vesicles sorted protein pathway.

The PANTHER Classification System detected proteins from 121 different categories of protein pathways with the majority of detected proteins in the category of integrin signalling pathway.

Proteins that were present in a single fraction and not in another were analysed using STRING to identify network interactions and pathways of interest. STRING is a useful tool to visualise the proteins of interest which are shown as nodes and their interactions which are shown as edges. The thicker the edge the more evidence there is for the interaction, and nodes are highlighted based on selective functional enrichments.

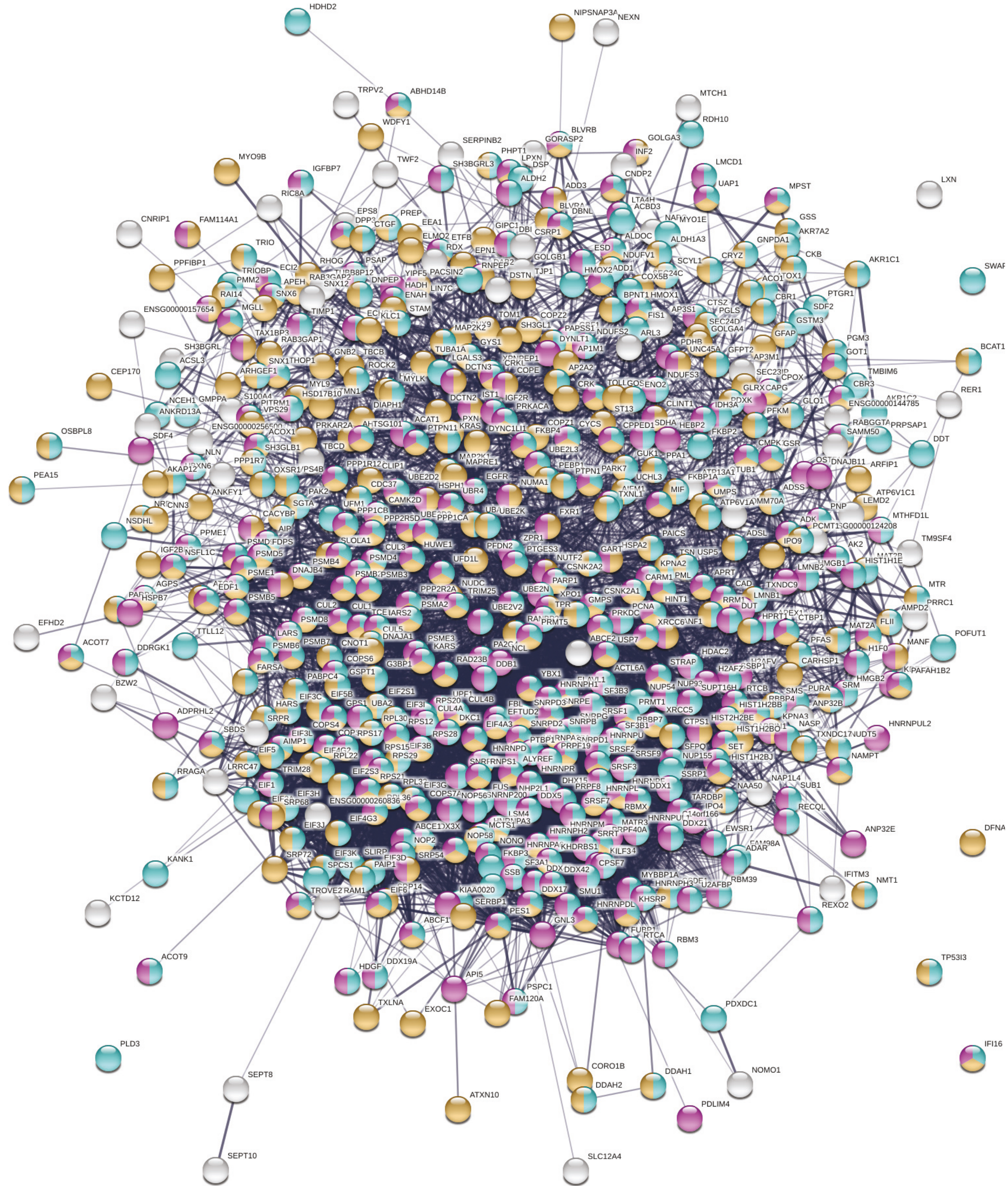


Figure 9: STRING network of detected proteins unique to ASC whole cell lysate.

STRING detected 593 nodes and 7688 edges with an average local clustering coefficient of 0.353. The biological process category metabolic process is shown in blue (424 with a false discovery rate of 2.98e-25), the cellular component category cytosol is shown in orange (375 with a false discovery rate of 3.08e-78) and cellular component category intracellular organelle lumen is shown in pink (316 with a false discovery rate of 2.64e-40).

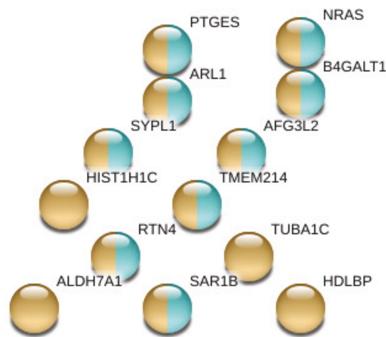


Figure 10: STRING network of detected proteins unique to ASC membrane bound fraction. STRING detected 13 nodes and 0 edges with an average local clustering coefficient of 0. The following cellular components are highlighted; the category organelle membrane is shown in blue (9 with a false discovery rate of 5.4e-03) and the category membrane-bounded organelle is shown in orange (13 with a false discovery rate of 3.60e-02).

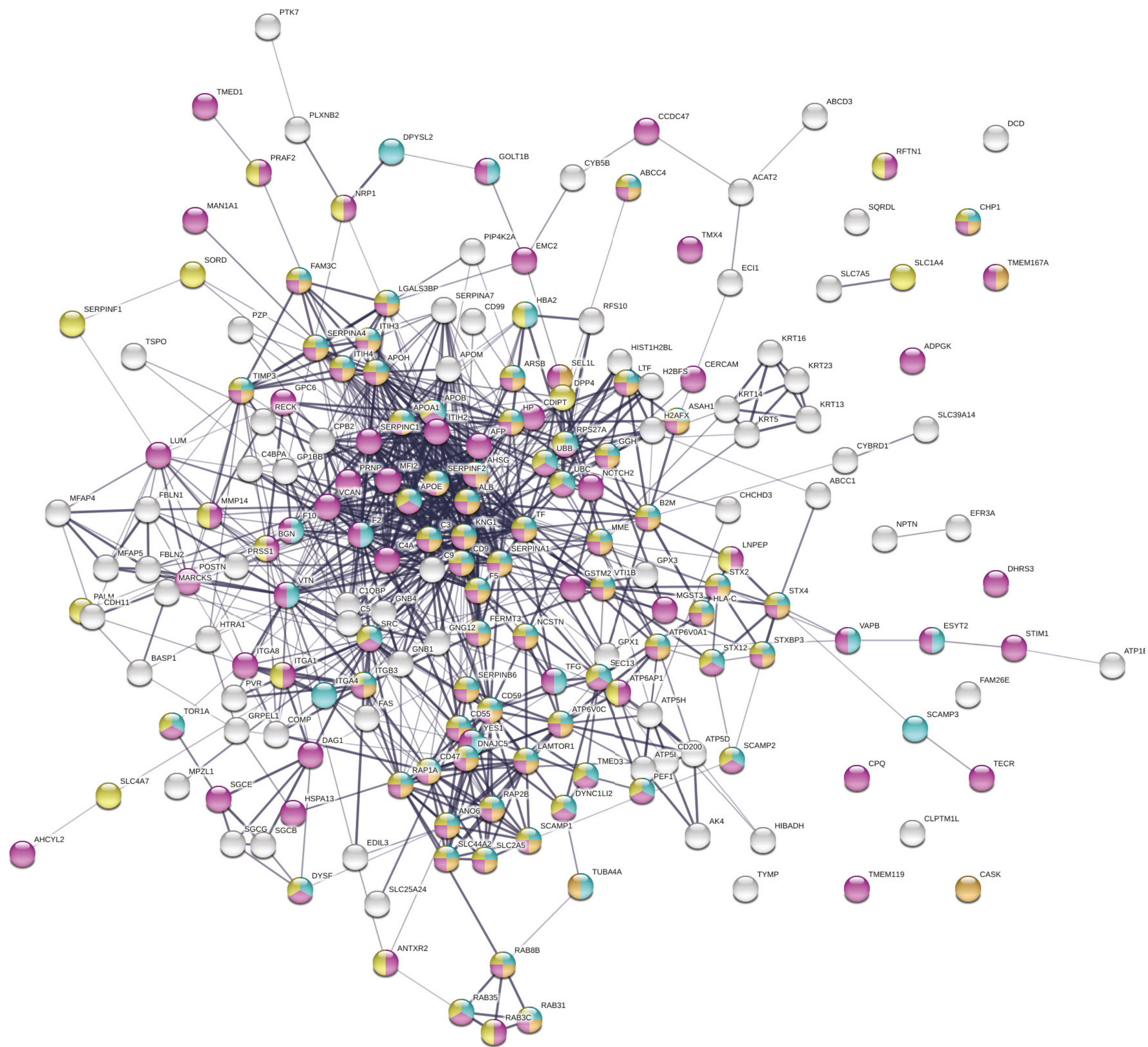


Figure 11: STRING network of detected proteins unique to ASC extracellular vesicles.

STRING detected 198 nodes and 880 edges with an average local clustering coefficient of 0.486. The biological process category of vesicle-mediated transport is shown in blue (78 with a false discovery rate of 1.15e-27), the category of biological process secretion by cell is shown in orange (54 with a false discovery rate of 6.72e-22), the category cellular component endomembrane system is shown in pink (116 with a false discovery rate of 3.04e-25) and the category cellular component vesicle is shown in yellow (82 with a false discovery rate of 2.09e-23).

Proteins that were detected in two fractions were compared using a clustered heatmap. The first compares the whole cell lysate and membrane bound fraction. Proteins are shown on the vertical axis, and patients along the horizontal axis. The horizontal axis forms two distinct clusters in each heatmap, where each horizontal cluster reflects the fraction being analysed. This data does this independently. However, the vertical axis is set to form 4 clusters, and these are generated based on trends in Z scores. The Z score a statistical measurement of variation from the mean: 0 indicates the value is the same as a mean, a positive score indicates a value above the mean, and a negative indicates a value bellow the mean.

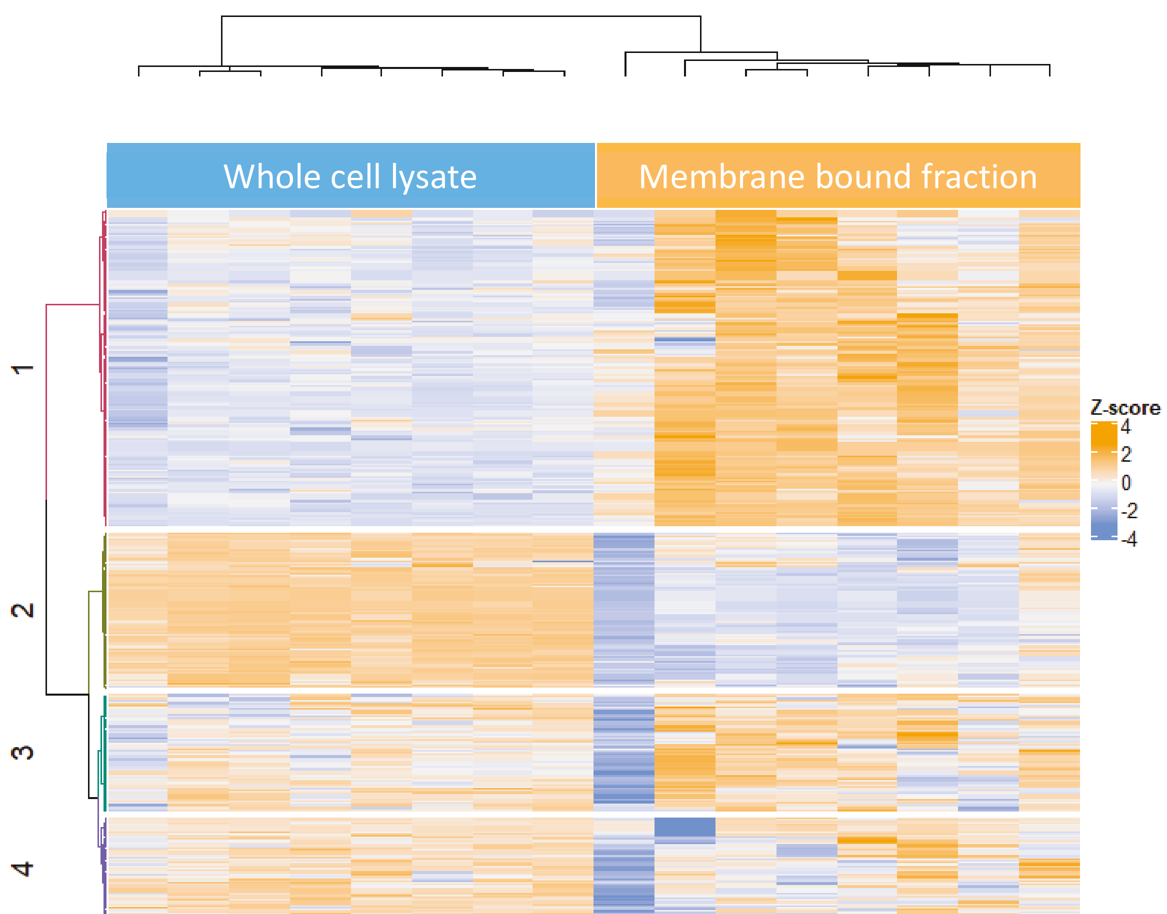


Figure 12: Clustered heatmap of proteins present in both ASC whole cell lysate and membrane bound fraction.

Whole cell lysate and membrane bound fractions formed two different horizontal clusters, with each patient sample represented in a column. Each row is a protein and there are 4 vertical clusters. Cluster 1 appears to have predominantly negative Z scores in the whole cell lysate and predominantly positive Z scores in the membrane bound fraction. Cluster 2

appears to have predominantly positive Z scores in the whole cell lysate and predominantly negative Z scores in the membrane bound fraction. Cluster 3 has less distinct visual trends with more neutral Z scores in the whole cell lysate and more positive Z scores in the membrane bound fraction. Cluster 4 also does not have particularly distinct visual trends; the whole cell lysate cluster appears to have more positive Z scores while the membrane bound fraction has a mix of both positive and negative Z scores.

The 4 vertical clusters are investigated in their own individual STRING networks (Figures 13-16) to identify the proteins present, their interactions and to highlight functional enrichments of interest.

Figure 13: STRING network of proteins detected in Cluster 1 of ASC whole cell lysate and membrane bound fraction.

STRING detected 138 nodes with 864 edges and an average local clustering coefficient of 0.5. The following biological process categories are highlighted: establishment of localization in cell shown in blue (55 with 9.36e-21 false discovery rate), cotranslational protein targeting to membrane shown in orange (18 with a 2.28e-17 false discovery rate) and signal-recognition particle-dependent cotranslational protein targeting to membrane shown in pink (17 with a 2.24e-16 false discovery rate). Proteins detected in this cluster are more abundant in the membrane bound fraction than the whole cell lysate.

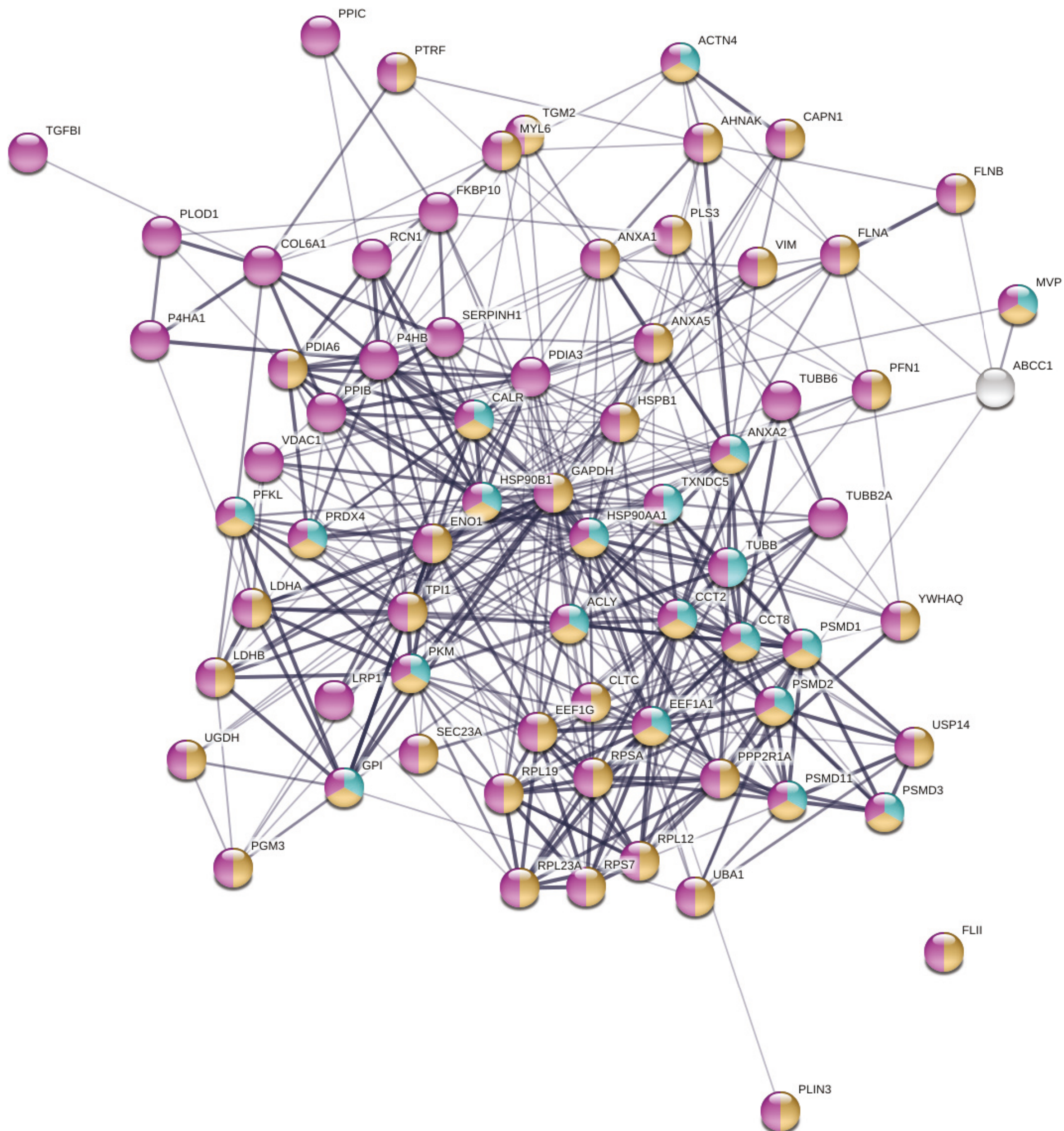


Figure 14: STRING network of proteins detected in Cluster 2 of ASC whole cell lysate and membrane bound fraction.

STRING detected 71 nodes with 445 edges and an average local clustering coefficient of 0.529. The following cellular component categories are highlighted: cytoplasmic vesicle lumen shown in blue (20 with a false discovery rate of 3.62e-16), cytosol shown in orange (53 with a false discovery rate of 6.26e-16), and cytoplasm shown in pink (70 with a false discovery rate of 2.70e-14). Proteins detected in this cluster are more abundant in the whole cell lysate than the membrane bound fraction.

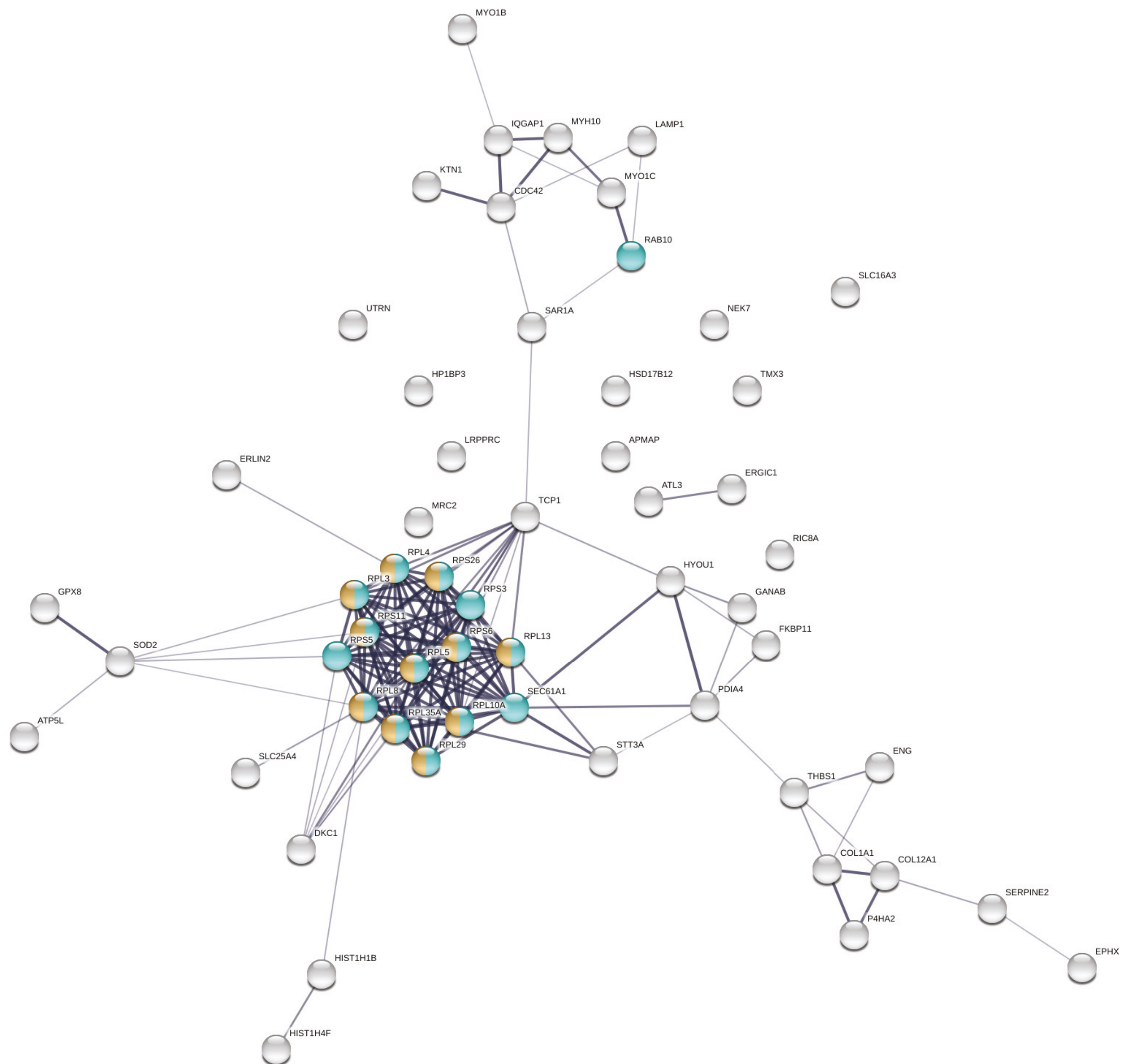


Figure 15: STRING network of proteins detected in Cluster 3 of ASC whole cell lysate and membrane bound fraction.

STRING detected 56 nodes with 156 edges and an average local clustering coefficient of 0.572. The biological process categories establishment of protein localisation to endoplasmic reticulum is shown in blue (15 with a false discovery rate of 6.59e-18) and structural constituent of ribosome is shown in orange (11 with a false discovery rate of 2.11e-10). Proteins from this cluster are detected at varying levels across both fractions, but overall are detected in higher levels in the membrane bound fraction than the whole cell lysate.

Figure 16: STRING network of proteins detected in Cluster 4 of ASC whole cell lysate and membrane bound fraction.

STRING detected 50 nodes with 98 edges and an average local clustering coefficient of 0.517. The following cellular component categories are shown: organelle part in blue (41 with 2.08e-05 false discovery rate), intracellular organelle in orange (46 with 9.84e-05) and membrane bound organelle in pink (42 with a 1.1e-03 false discovery rate). Proteins found in this cluster appear to be detected in greater abundance in the whole cell lysate with a mixture of high and low abundance in the membrane bound fraction.

The same analysis was conducted on the whole cell lysate and extracellular vesicle fraction to investigate proteins found in both of these fractions and to see how they differ in abundance. As with the last heatmap, proteins are on the vertical axis and patient samples across the horizontal axis. The horizontal axis self-assembled into the two respective fractions, while the vertical axis was set to form 4 clusters based on observed Z scores.

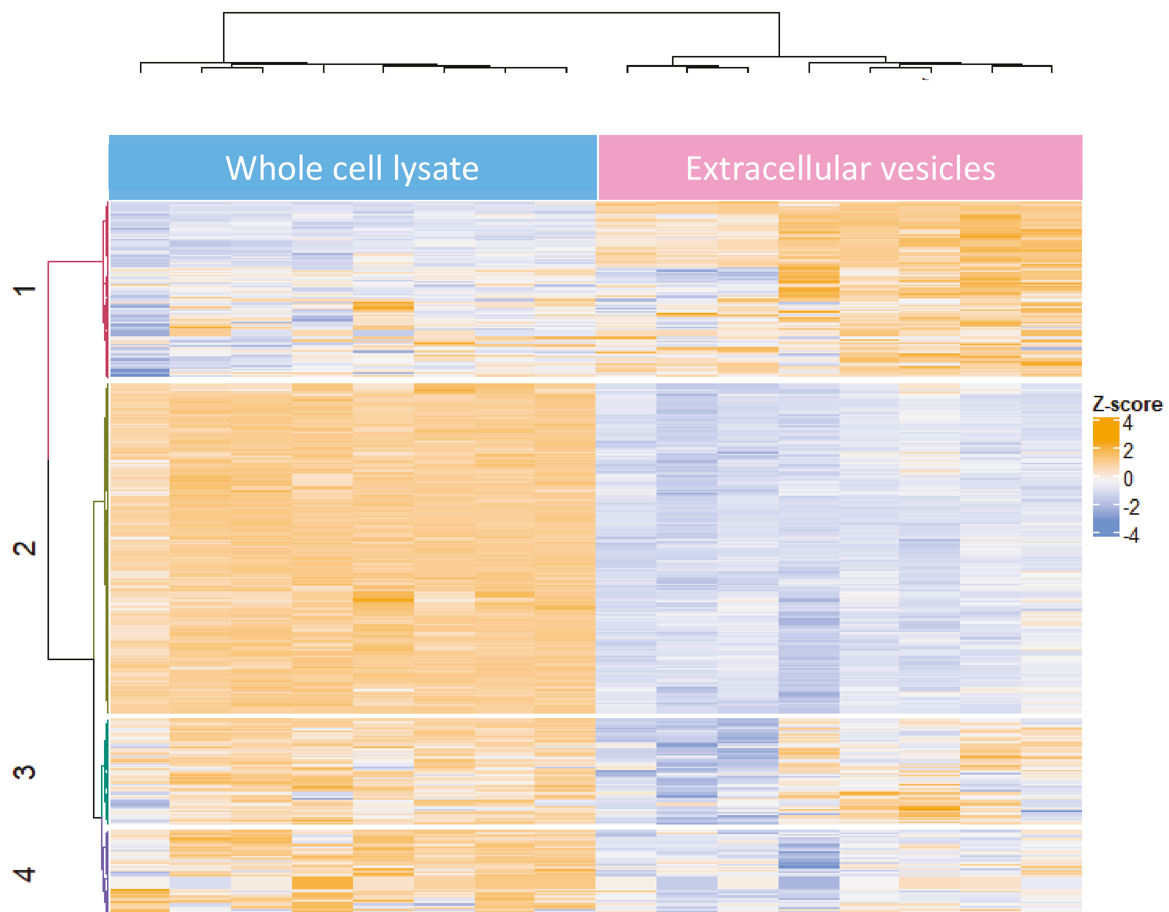


Figure 17: Clustered heatmap of proteins present in both ASC whole cell lysate and extracellular vesicles.

Whole cell lysate and extracellular vesicle fractions formed two different horizontal clusters, with each patient sample represented in a column. Each row is a protein and there are 4 vertical clusters. Cluster 1 has predominantly negative Z scores in the whole cell lysate and predominantly positive Z scores in the extracellular vesicle fraction. Cluster 2 has very clear visual trends that consist largely of positive Z scores in the whole cell lysate and negative Z scores in the extracellular vesicles. Cluster 3 has less distinct grouping but overall, the whole cell lysate has predominantly positive Z scores, extracellular vesicles has a mix of both positive and negative Z scores. Cluster 4 has a mix of Z scores however predominantly positive Z scores in the whole cell lysate and negative Z scores in extracellular vesicles.

STRING networks were utilised to investigate the proteins in each of these clusters (Figure 18-21) by showcasing their interactions and highlighting particular functional enrichments of interest.

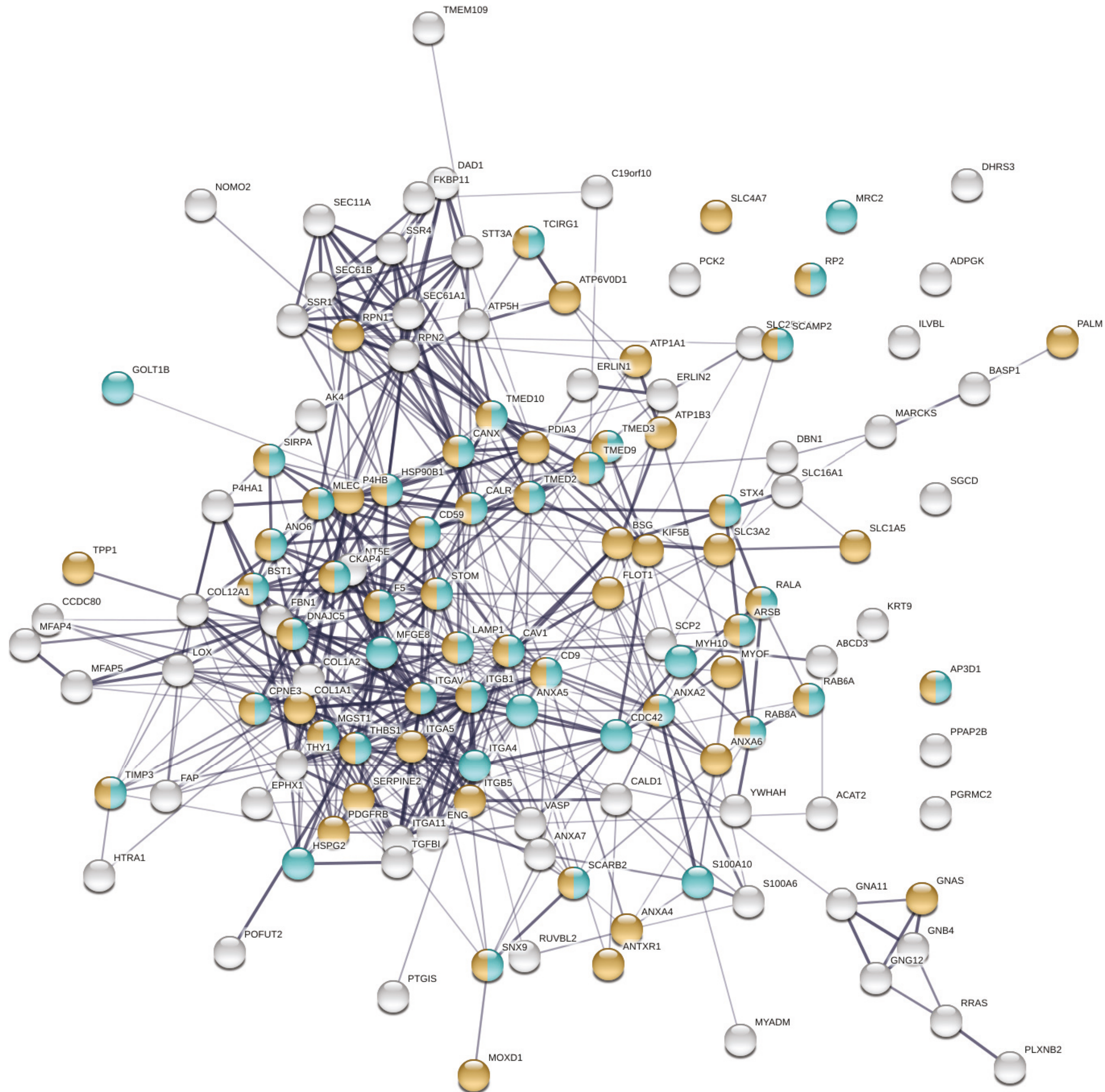


Figure 18: STRING network of proteins detected in Cluster 1 of ASC whole cell lysate and extracellular vesicle fraction.

STRING detected 133 nodes with 563 edges and an average local clustering coefficient of 0.4333. The biological process category vesicle-mediated transport is shown in blue (46 with a false discovery rate of 6.80e-14) and cellular component category vesicle is shown in orange (62 with a false discovery rate of 6.89e-21). Proteins in this cluster are detected at varying levels but overall appear to be detected in lower abundance in the whole cell lysate and higher abundance in the extracellular vesicle fraction.

Figure 19: STRING network of proteins detected in Cluster 2 of ASC whole cell lysate and extracellular vesicle fraction.

STRING detected 262 nodes with 5018 edges and an average local clustering coefficient of 0.55. Categories describing cellular components are highlighted: cytoplasm in blue (257 with a false discovery rate of 1.13e-51), vesicles shown in orange (90 with a false discovery rate of 1.32e-19) and ribonucleoprotein complex shown in pink (49 with a false discovery rate of 8.30e-18). Proteins found in this cluster have a high abundance in the whole cell lysate and a low abundance in the extracellular vesicle fraction.

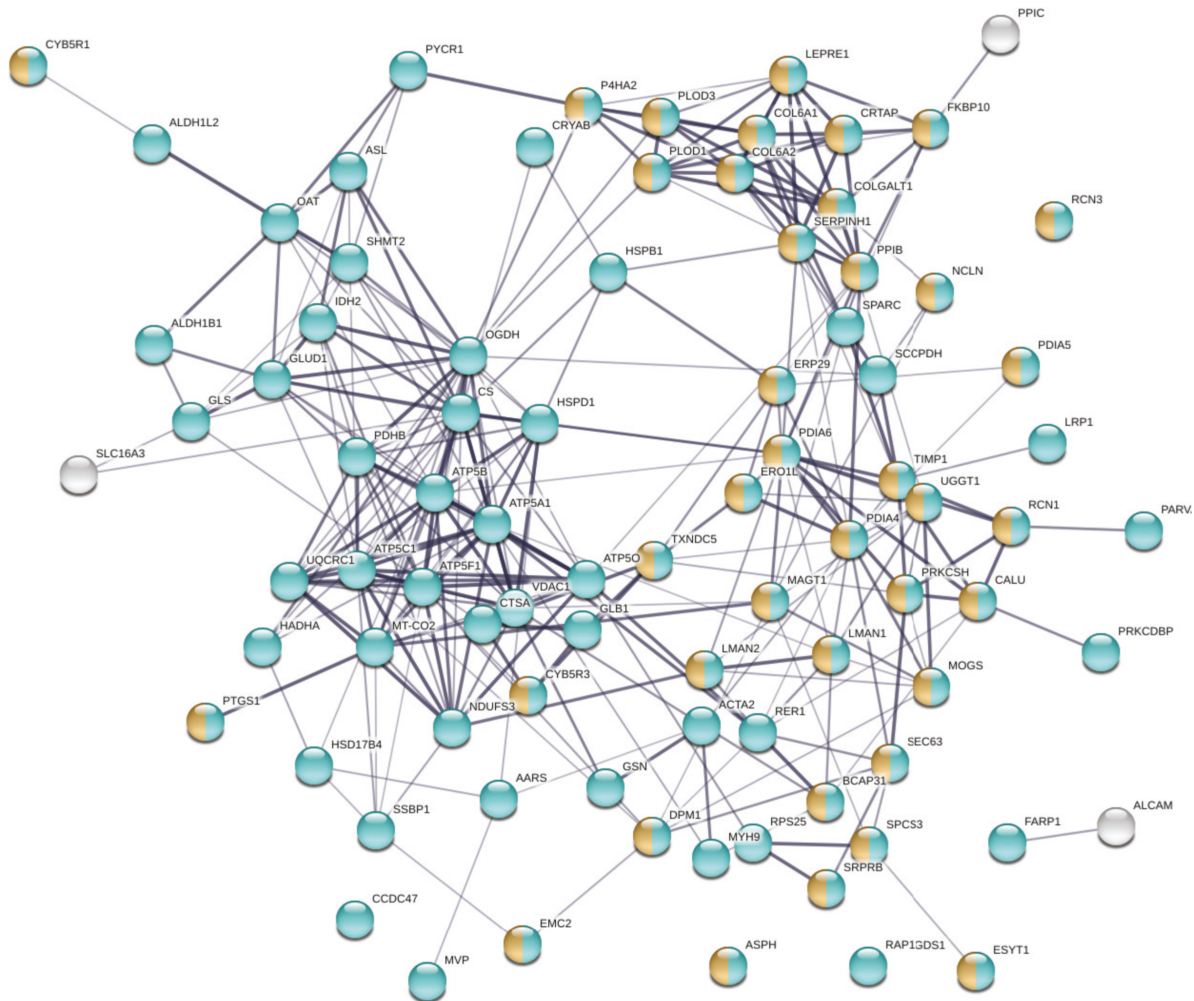


Figure 20: STRING network of proteins detected in Cluster 3 of ASC whole cell lysate and extracellular vesicle fraction.

STRING detected 86 nodes with 297 edges and an average local clustering coefficient of 0.47. The cellular component categories cytoplasmic part is shown in blue (83 with a false discovery rate of 1.09e-20) and endoplasmic reticulum part shown in orange (39 with a false discovery rate of 1.09e-20). Proteins in this cluster are detected at varying levels but overall are more abundant in the whole cell lysate and have both high and low abundance in the extracellular vesicle fraction.

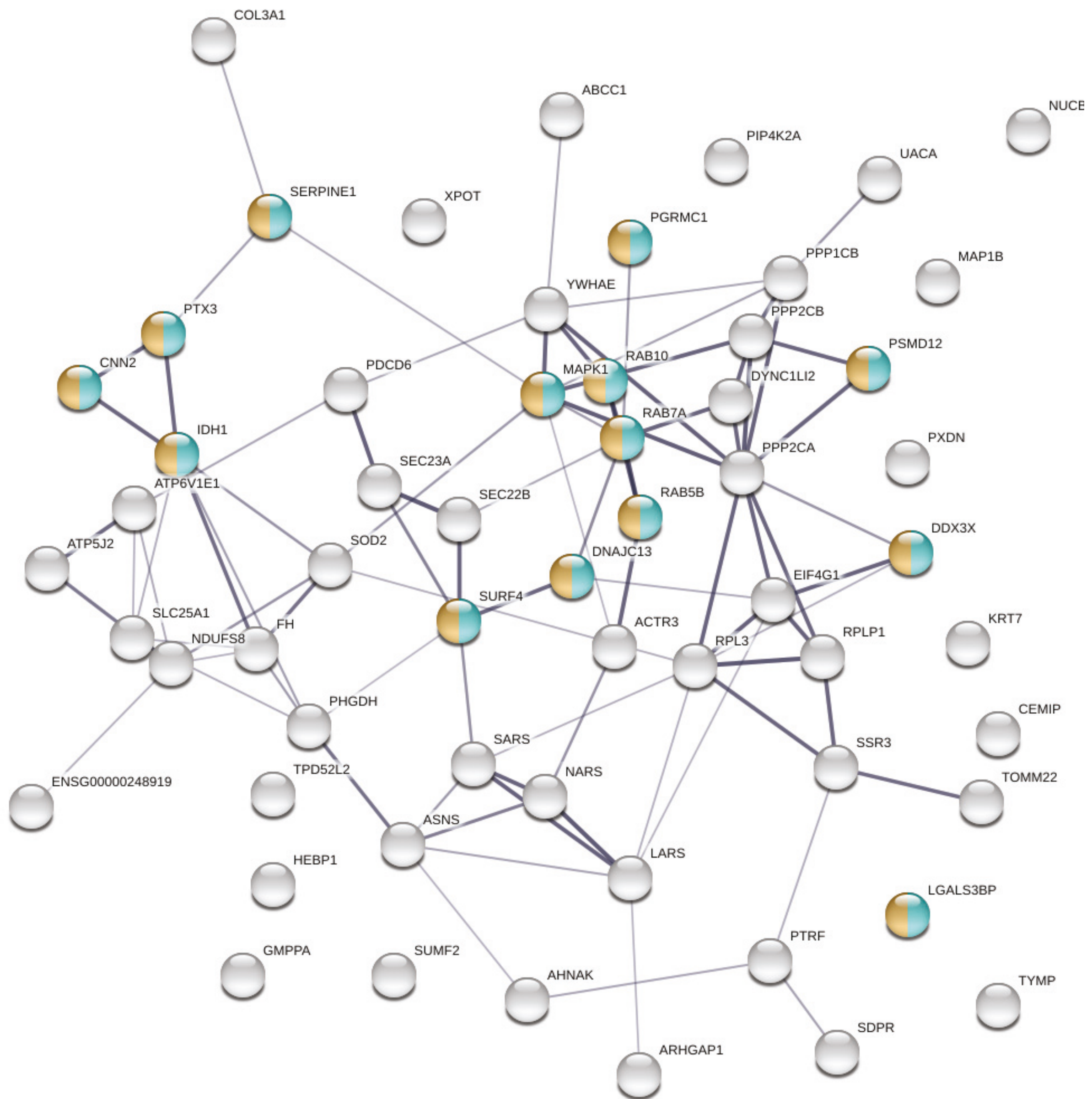


Figure 21: STRING network of proteins detected in Cluster 4 of ASC whole cell lysate and extracellular vesicle fraction.

STRING detected 59 nodes with 87 edges and an average local clustering coefficient of 0.357. The biological process category regulated exocytosis is shown in blue (14 with a false discovery rate of 2.96e-05) and cellular component category secretory granule is shown in orange (14 with a false discovery rate of 9.62e-06). Proteins detected in this cluster have predominantly higher abundance in the whole cell lysate and lower abundance in extracellular vesicles.

The final heatmap compares the extracellular vesicle fraction against the membrane bound fraction. Patient samples are across the horizonal axis and proteins are down the vertical axis. The quantitative changes of proteins between fractions is shown by the Z score.

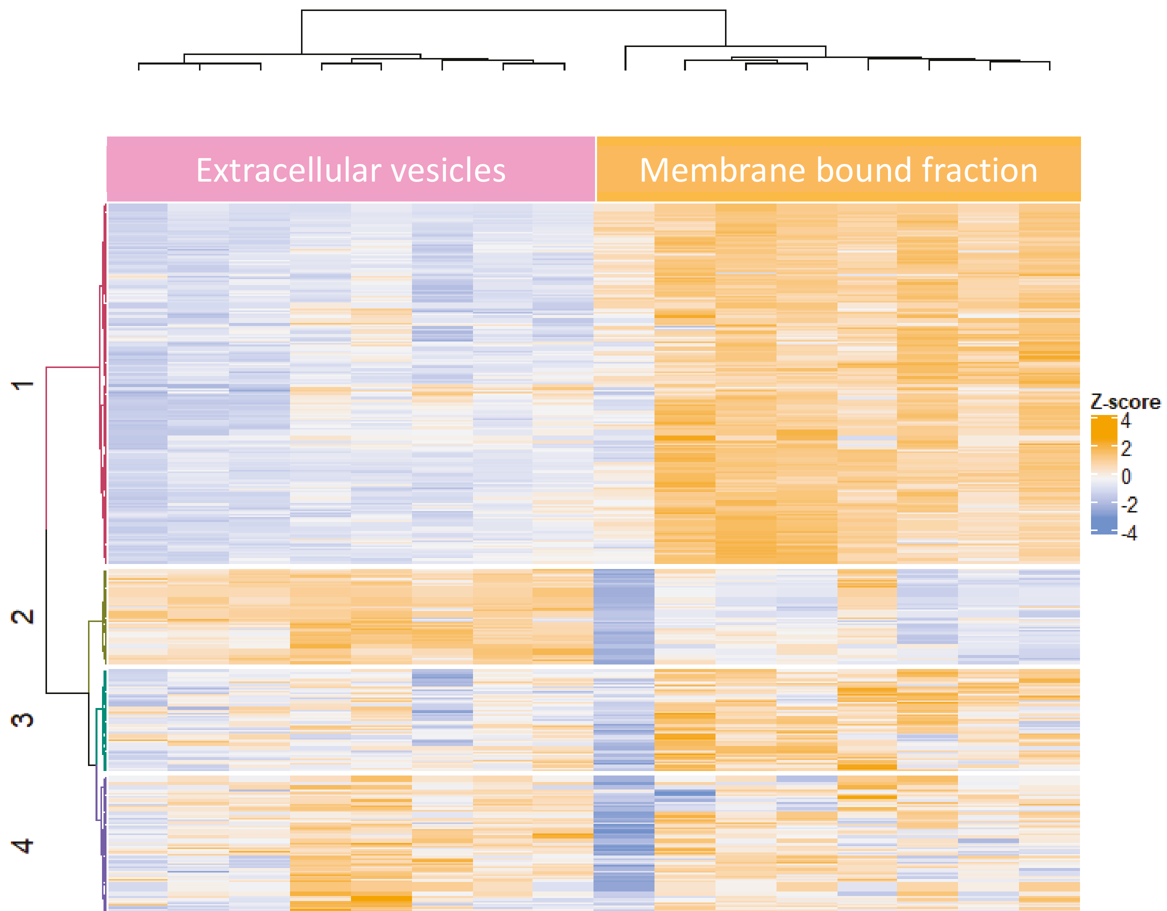


Figure 22: Clustered heatmap of proteins present in both ASC extracellular vesicles and membrane bound fraction.

Membrane bound and extracellular vesicle fractions formed two different horizontal clusters, with each patient sample represented in a column. Each row is a protein and there are four vertical clusters. Cluster 1 has predominantly negative Z scores in the extracellular vesicles and predominantly positive Z scores in the membrane bound fraction. Cluster 2 has predominantly positive Z scores in the extracellular vesicle fraction and a mixture of both positive and negative Z scores in the membrane bound fraction with overall more negative Z scores. Cluster 3 does not have distinct patterns, however overall the extracellular vesicle fraction has more negative Z scores and the membrane bound fraction has more positive Z scores. Cluster 4 does not have distinct patterns, with a mixture of both positive and negative

Z scores throughout both fractions; the extracellular vesicle fraction appears to have slightly more positive Z scores and the membrane bound fraction appears to have slightly more negative Z scores.

The 4 protein clusters are investigated using STRING networks (Figures 23-26) to showcase the individual proteins, their connections, and to highlight functional enrichments of interest within the cluster.

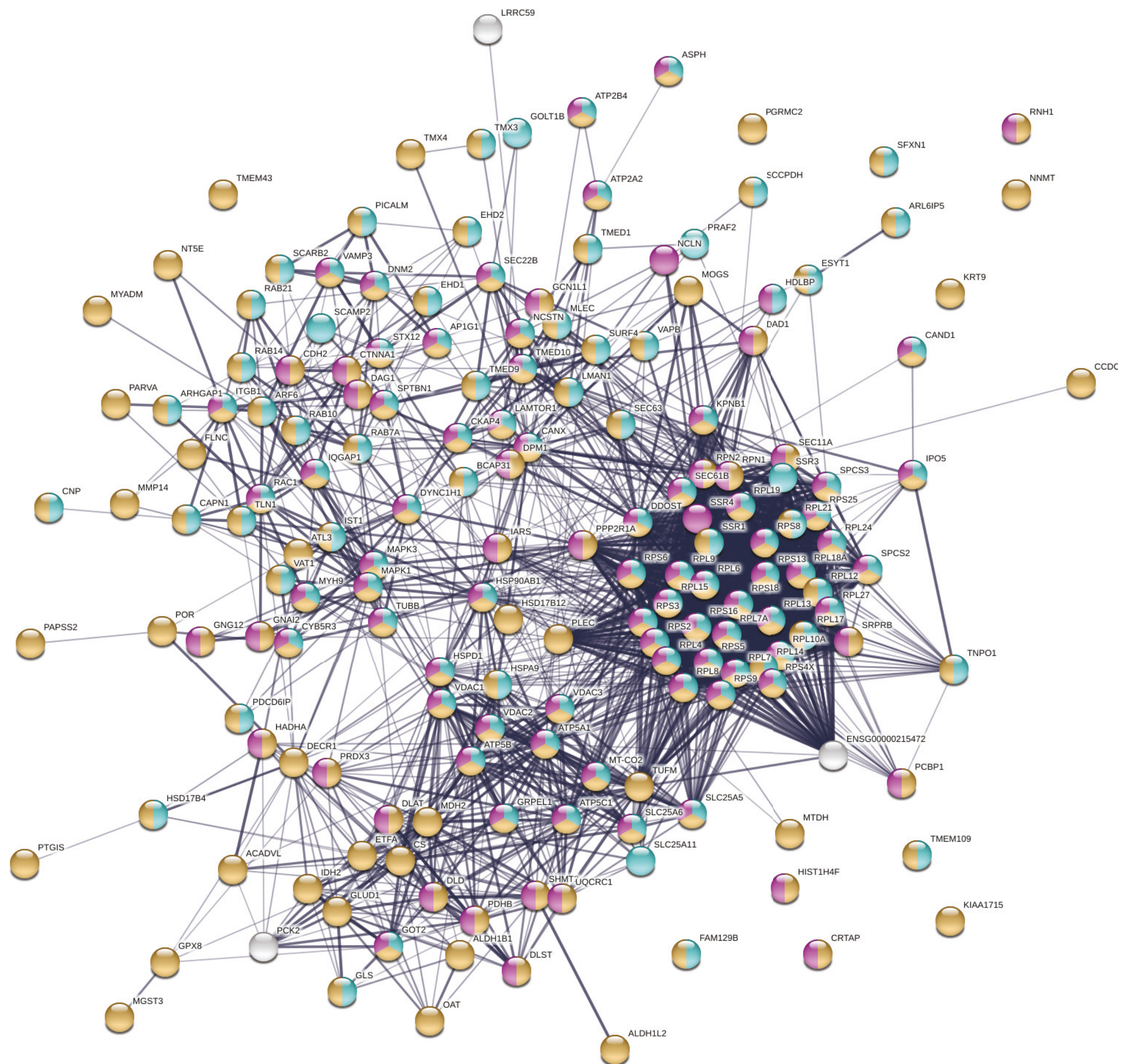


Figure 23: STRING network of proteins detected in Cluster 1 of ASC extracellular vesicle and membrane bound fraction.

STRING detected 177 nodes with 1756 edges and an average local clustering coefficient of 0.54. The biological process categories transport is shown in blue (113 with a false discovery rate of 2.28e-31), cellular process is shown in orange (166 with a false discovery rate of 3.11e-09) and cellular component protein-containing complex is shown in pink (96 with a false discovery rate of 8.58e-16). Proteins detected in this cluster are more abundant in the membrane bound fraction, with a lower abundance observed in extracellular vesicles.

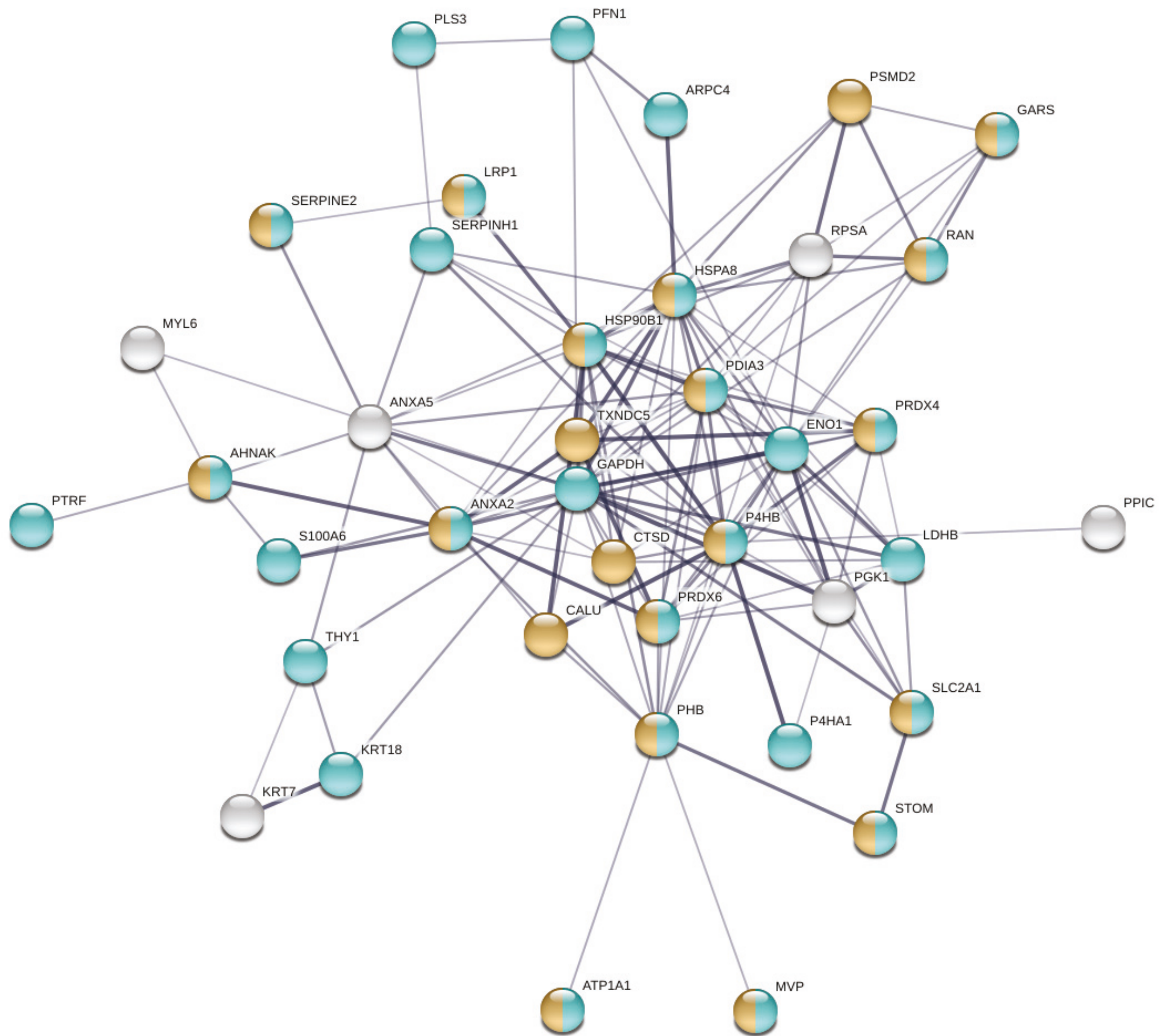


Figure 24: STRING network of proteins detected in Cluster 2 of ASC extracellular vesicle and membrane bound fraction.

STRING detected 39 nodes and 138 edges and an average local clustering coefficient of 0.553. The molecular function categories protein binding is shown in blue (29 with a false discovery rate of 8.44e-05) and cellular component vesicle is shown in orange (21 with a false discovery rate of 1.95e-08). Proteins detected in this cluster are more abundant in extracellular vesicles with a lower abundance observed in the membrane-bound fraction.

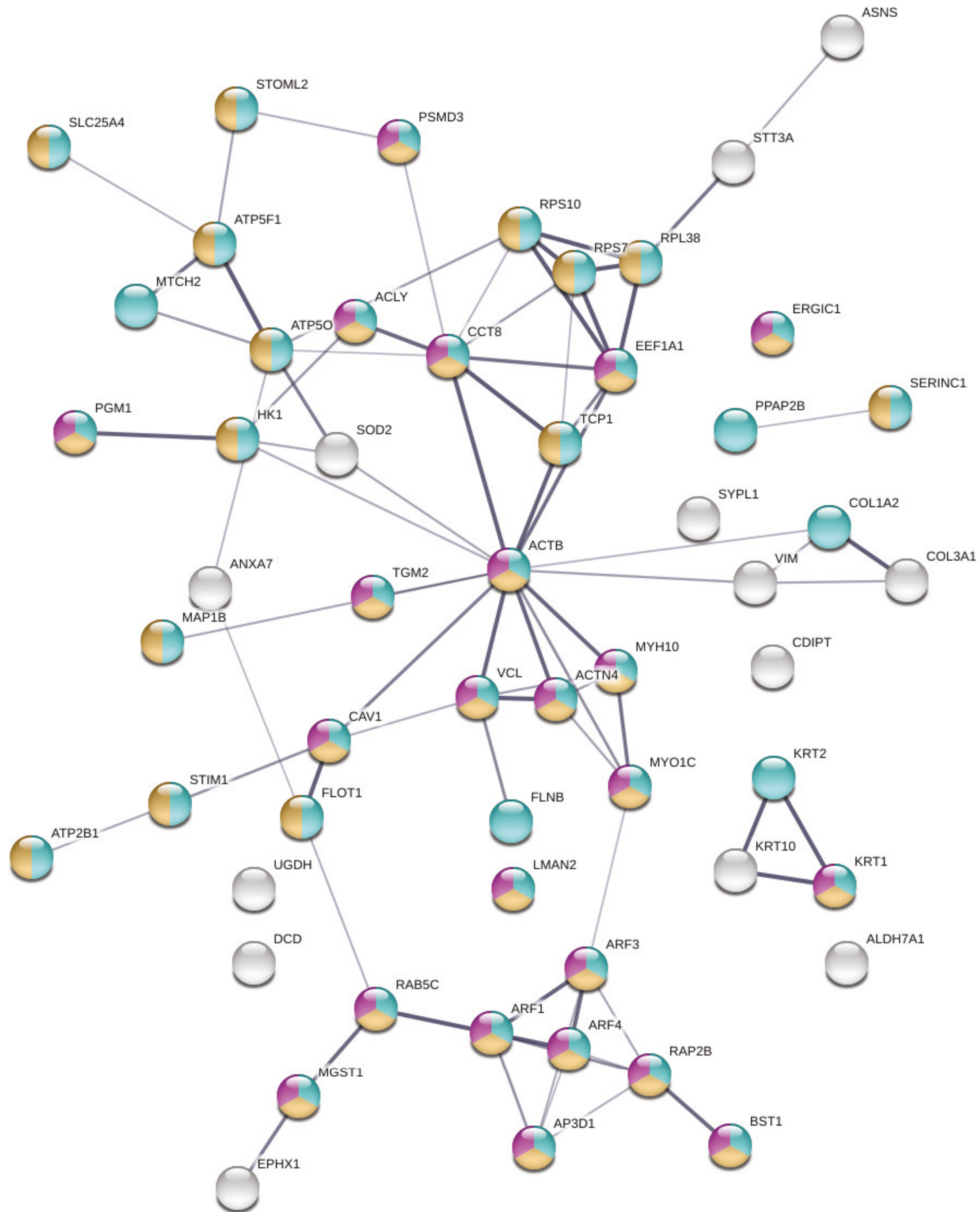


Figure 25: STRING network of proteins detected in Cluster 3 of ASC extracellular vesicle and membrane bound fraction.

STRING detected 55 nodes with 77 edges and an average local clustering coefficient of 0.515. The following biological process categories are highlighted; localisation in blue (42 with a false discovery rate of 5.39e-11), establishment of localisation in orange (37 with a false discovery rate of 6.21e-10) and vesicle-mediated transport in pink (23 with a false discovery rate of 2.47e-08). Proteins detected in this cluster have differing levels of abundance but overall have

slightly higher abundance in the membrane bound fraction and slightly lower abundance in the extracellular vesicles.

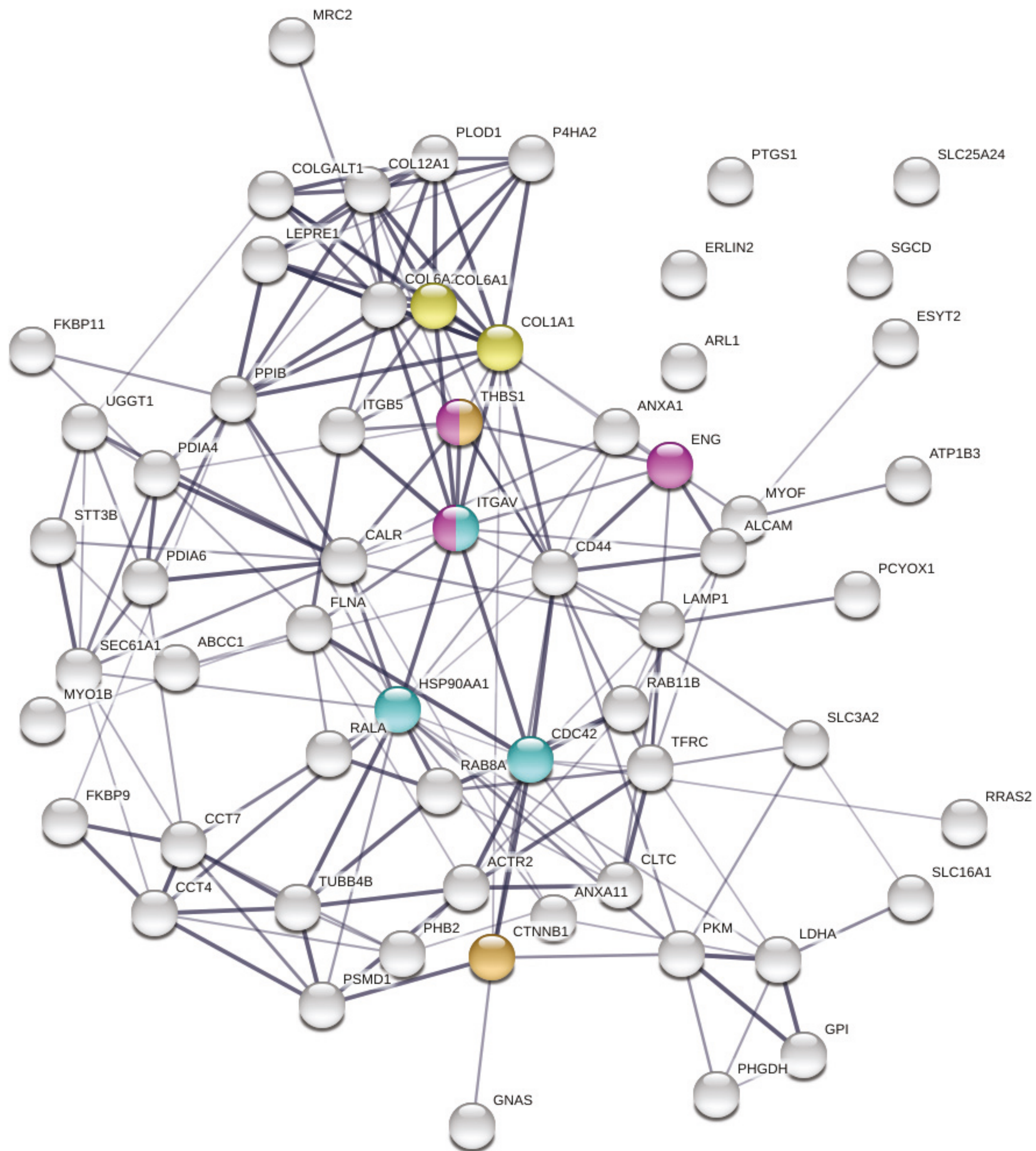


Figure 26: STRING network of proteins detected in Cluster 4 of ASC extracellular vesicle and membrane bound fraction.

STRING detected 61 nodes with 176 edges and an average local clustering coefficient of 0.47. The biological process category vascular endothelial growth factor receptor signalling pathway is shown in blue (3 with a false discovery rate of 1.49e-02), biological process category regulation of fibroblast growth factor signalling pathway is shown in orange (2 with a false discovery rate of 3.38e-02), molecular function category transforming growth factor

beta binding is shown in pink (3 with a false discovery rate of 8.2e-04) and PDGF binding is shown in yellow (2 with a false discovery rate of 5.3e-03). Proteins detected in this cluster have differing levels of abundance, but overall have a slightly higher abundance in the extracellular vesicles and a slightly lower abundance in the membrane bound fraction.

Cytokines are often below the detectable limit for the shotgun LC-MS/MS methods used in this work and that of others. Therefore, a quantitative multiplex immunoassay was utilised to detect the presence of and provide insight into the cytokines secreted by ASCs.

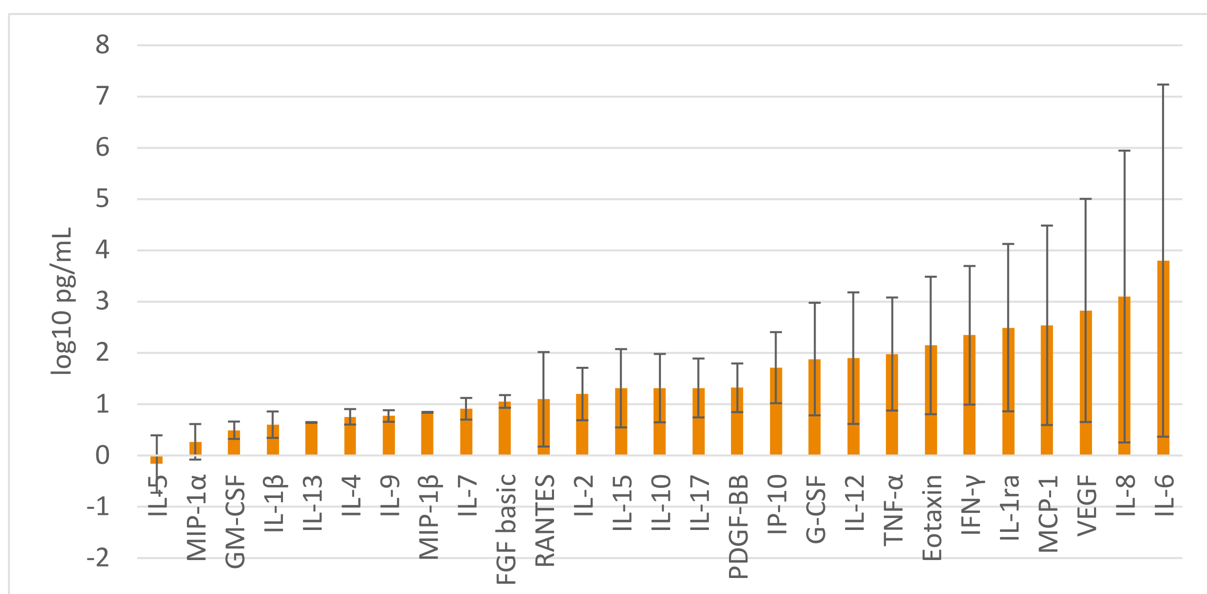


Figure 27: ASC secreted cytokines.

27 cytokines were measured and the standard deviation of the measurements across patients is indicated by the error bars. The standard deviation is large but reflects the expected patient to patient variation. It is important to note that these samples were blanked against the neat media as the media itself contains cytokines. The secreted cytokines are ordered from lowest to highest levels of detection.

4. Discussion

4.1 Proteomic analysis of ASCs

ASCs are currently utilised in unproven stem cell therapies around the world, yet surprisingly little is known about the proteomic phenotype of these cells. In order to develop a comprehensive understanding of the proteome of ASCs, cells were isolated from liposuction aspirates of 8 individual patients. These cells were expanded for 5 passages and the whole cell lysate and membrane bound fraction was obtained after cellular disruption, whilst extracellular vesicles were isolated from collected culture media. All fractions were isolated from the same cell culture flask per patient. These fractions underwent proteomic analysis using a 'shotgun' LC-MS/MS strategy, where peptides generated by trypsin digestion were separated by nanoflow reverse-phase chromatography and analysed by electrospray tandem mass spectrometry using a benchtop Orbitrap system (Thermo Q Exactive Plus). This type of shotgun LC-MS/MS experiment allows the identification and quantification of the products of open reading frames, hence referred to as 'proteins' rather than intact proteoforms. This project provides a sound proteomic catalogue of ASCs by investigating the whole cell lysate, membrane bound fraction, extracellular vesicles and secreted cytokines. The whole cell lysate gives the most complete catalogue of the ASC proteome in a single fraction, which is further complimented by the membrane bound fraction that shows proteins found in any cellular membrane, and the proteome of extracellular vesicles and measurement of secreted cytokines which provide insight into cellular communication. There is evidence that suggests secreted proteins are also important for stem cell differentiation, supporting the need to quantify secreted cytokines and to investigate the proteome of ASC produced extracellular vesicles [209] Furthermore, it is known that sorting of proteins into extracellular vesicles can affect the cells from which they originated [210,211]. It is important to remember when reading this chapter that while these findings may seem commonplace and expected of ASCs, this is the most comprehensive proteomic analysis of ASCs to date. This novel information is essential as ASCs are being utilised in clinics across the globe and despite their limited understanding. Furthermore, developing this knowledge could further their use as a research model.

The average number of unique proteins detected across all 8 patient samples was 4379 for the whole cell lysate, 1583 for the membrane bound fraction and 2582 for the extracellular vesicles. Only 2290 of these proteins were found to be conserved in the ASCs of all 8 patients. This is demonstrated in Table 1 which shows that while an average of 4379 proteins were detected per patient in the whole cell lysate, this is reduced by 57.67% to 1854 proteins when examining only the conserved proteins. The membrane bound fraction saw a similar reduction of 63.55% with an average protein number per patient of 1583 being reduced to 577 proteins conserved in all patients. The extracellular vesicles had an average number of proteins per patient of 2582 that underwent a reduction of 49.19% to 1312 proteins conserved in all patients. While this reduction of proteins is substantial, it is not unexpected when considering the variation that is likely to occur between patients as it is immensely difficult to define what a 'healthy' patient is. It is also possible that the 'missing' proteins are simply below the limit of detection and that further fractionation of each cellular fraction would increase the number of commonly detected proteins. This fractionation was not possible due to the demands and lack of capacity of the instrumentation being accessed. For the purposes of beginning to understand the proteome of ASCs, the focus of this work is on the conserved proteins to enable their quantification across the cohort. The considerable reduction in the number of proteins when restricting to those that are common highlights the scope of patient to patient variation that is seen. The correlation plots of normalised protein abundances for each patient can be seen in Supplementary Figure 1. Overall the correlation values are quite high showing that proteins in the compared samples have relatively similar abundance. As this study is focussed on building a broader understanding of the ASCs themselves, the emphasis is on proteins present in all patient samples, rather than those in individual patients.

The distribution of these conserved proteins in the cellular fractions is shown in Figure 3 with a proportional Venn diagram. The greatest number of proteins were detected in the whole cell lysate, followed by the extracellular vesicles and then the membrane bound fraction. Of the total 2290 proteins, 883 were unique to the whole cell lysate and were not detected in the other fractions. 352 proteins were uniquely detected in the extracellular vesicles and 29 proteins were unique to the membrane bound fractions. This demonstrates that each of the

fractions provides a different insight into the proteome of ASCs and collectively contributes to the overall picture. Analysis of all three fractions allows for a more comprehensive picture of the ASC proteome, not only through the identification of more unique proteins as seen here, but also because these fractions increase the number of detected peptides for each individual protein, allowing for more reliable and robust quantification of proteins. 44.80% of the detected proteins were found in two or more of the fractions, 599 proteins were found in two fractions (478 between the whole cell lysate and extracellular vesicles, 66 between the whole cell lysate and membrane bound fraction, and 55 between the membrane-bound fraction and extracellular vesicles) and 427 proteins were identified in all three fractions.

In order to gain insight into the distribution of proteins within these fractions, the proteins were placed into the PANTHER Classification System and categorised by molecular function, cellular compartment, biological process, protein class and protein pathway [202]. This type of analysis allows for an unbiased view of the way proteins are distributed across categories and for comparison between fractions. The diagrams' percentage distribution of proteins for individual categories are shown across the x axis of the figure with the number of detected proteins displayed on the corresponding fraction. As seen in Figure 4, the PANTHER Classification System detected eight different molecular functions and proteins are not equally distributed across these molecular functions. For example, in the category molecular transducer activity (GO:0060089) it can be seen that the majority of proteins were located in the extracellular vesicles, while in the category translation regulator activity (GO:0045182) the majority of proteins were from the whole cell lysate. There were also no proteins from the membrane-bound fraction detected by PANTHER that were involved in this molecular function, which is likely because translation regular activity refers to molecular functions involved in initiation, activation, perpetuation, repression or termination of synthesis of a polypeptide at the ribosome. This makes sense, as the ribosome is not a membranous structure. What is consistent across molecular functions is that the membrane-bound fraction had the lowest percentage of proteins. Unsurprisingly, the largest number of proteins detected were from the molecular function catalytic activity (GO:0003824) and binding (GO:0005488). Catalytic activity refers to enzymes which are almost entirely composed of protein binding to substrates, whilst binding denotes interaction of specific sites between molecules. It is therefore logical that so many proteins belonged to these categories.

Figure 5 is the Panther Classification of all detected proteins sorted by biological process. Of these 13 biological process categories, most of them have a relatively similar distribution of proteins across all three fractions. This can be seen in cellular process (GO:0009987) where 48.68% of the proteins are found in the whole cell lysate, 13.96% in the membrane bound fraction and 37.36% in the extracellular vesicles. This distribution is what is typically expected as it reflects what is observed in the proportional Venn diagram in Figure 3. While the categories rhythmic process (GO:0048511) and cell proliferation (GO:0008283) account for 100% of proteins from the whole cell lysate, it is important to recognise that each of those biological processes consist of one single protein. This could infer that other proteins from this process category was below the limit of detection for other fractions, or alternatively were simply not annotated into this category in PANTHER.

Proteins sorted by cellular compartment through PANTHER are shown in Figure 6. 1278 of these proteins are annotated as being in the category cellular component cell (GO:0005623). Again, a distribution of proteins is observed that reflects that of all detected proteins across all 3 fractions. This is not evident in the cellular compartment membrane (GO:0016020) where 51.69% of the identified proteins are from extracellular vesicles, 33.71% from the whole cell lysate, and only 14.61% from the membrane bound fraction. Considering the membrane bound fraction will contain a significantly greater number of membranous proteins, in spite of any non-specific interactions, it would be expected that this number be higher. However, this finding could be reflective of the methods used, as the whole cell lysate and extracellular vesicle fraction utilised single-pot, solid-phase-enhanced sample proteomics which is capable of solubilising some membrane bound proteins [200]. Or alternatively the method used to isolate the membrane bound fraction did not allow for proper coverage of the membrane proteome [212,213].

ASC proteins from the whole cell lysate, membrane bound fraction and extracellular vesicles were sorted into PANTHER categories of protein class and this is displayed in Figure 7. This resulted in the classification of proteins into 24 different protein classes categories, the majority of proteins being classified to the nucleic acid binding class (PC00171), 378 proteins to be exact. Nucleic acid binding proteins are proteins that bind DNA or RNA. These proteins

assist with processing, packing or metabolism, and it is therefore reasonable for most proteins detected to be involved with this class of proteins. The protein class extracellular matrix protein (PC00102) has 10 whole cell lysate proteins and 10 extracellular vesicle proteins, but no membrane bound proteins. These proteins form the extracellular matrix that cells are embedded in and can be both produced and secreted by cells. This is unexpected as the extracellular matrix interacts with the plasma membrane, but again could be due to the proteins being below the detectable range or the detected membrane proteins not being annotated in the PANTHER database to these categories.

122 protein pathways were highlighted from the PANTHER Classification System for the proteins detected in ASCs from the following fractions; whole cell lysate, membrane bound and extracellular vesicles. This is visualised in Figure 8 where it can be seen that most highlighted pathways only contain a few proteins. CCN family member 1 is a highly abundant protein found in BMSCs that contributes to angiogenesis-promoting activity and is important for regeneration and repair of damaged tissues [214], but it was not detected in these ASCs. However, 18 proteins in the whole cell lysate, 8 proteins in the membrane bound fraction and 11 proteins in extracellular vesicles were classified as being involved in the pathway angiogenesis (P00005) were identified. The angiogenic capacity of stem cells is very important for their use in clinical settings, as there is evidence of stem cells releasing angiogenic factors [215]. These presence of these angiogenic proteins in ASCs warrants further investigation. The ubiquitin proteasome pathway (P00060) is a complex pathway that is crucial for protein degradation [216]. Ubiquitin carboxyl-terminal hydrolase isozyme L1 was detected in the whole cell lysate and extracellular vesicles, and has previously been detected in extracellular vesicles [217,218] as well as showing intracellular actions [219]. The Wnt signalling pathway (P00057) has a total of 49 components, 18 of which were detected in the whole cell lysate, 6 in the membrane-bound fraction and 16 in extracellular vesicles. The Wnt signalling pathway regulates many cellular functions [220], but has been shown to control stem cells and assist in their maintenance in their undifferentiated state [221]. Identification of proteins from this pathway in these ASCs is to be expected based on the literature.

Proteins identified in the samples analysed in this work correlate with what would be expected for ASCs. For example surface markers that have been characterised as being

positive such as CD44 (detected in the whole cell lysate and membrane bound fraction), CD166 (detected in the whole cell lysate and extracellular vesicles) and CD59 (detected in extracellular vesicles) were successfully detected [222]. Additionally, negative cell surface markers such as CD31, CD56, CD146, CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR were not detected in any of the cellular fractions [222,223]. It must also be noted that because the ASCs were detached with TrypLE the membranes protruding out of the cell could have been shaved off. However, as a portion of these proteins are still located within the membrane this is unlikely to affect the number of protein identifications, rather it would impact the detected sequence coverage. This is supported by the detection of cell surface markers in this dataset. Further evidence of proteins known to be essential in stem cell function that were detected in this data set include proteins involved in self-renewal of stem cells via growth factor pathways, such as platelet-derived growth factor receptor beta in the whole cell lysate and extracellular vesicles, epidermal growth factor receptor in the whole cell lysate, and cation-independent mannose-6-phosphate receptor in the whole cell lysate [224]. Proteins involved in the Wnt signalling pathway, as highlighted above in Figure 8, are known to be involved in both self-renewal and differentiation of stem cells and were also detected. These include catenin beta-1, which was detected in the whole cell lysate, membrane-bound fraction and extracellular vesicles, ras-related C3 botulinum toxin substrate 1, which was detected in the whole cell lysate, membrane-bound fractions and extracellular vesicles, serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform, which was detected in whole cell lysate and extracellular vesicles, calcineurin B homologous protein 1, which was detected in extracellular vesicles, calcium/calmodulin-dependent protein kinase type II subunit delta, which was detected in the whole cell lysate, and cAMP-dependent protein kinase catalytic subunit alpha which was detected in the whole cell lysate [224].

STRING is a platform to showcase functional protein association networks and was used to illustrate the interactions of the detected proteins [203]. These interactions are based on direct and indirect associations [204], but are also inferred from computational predictions, knowledge from other organisms, and aggregates from primary databases [205,206]. The main databases used by STRING are genomic context predictions, high-throughput lab experiments, co-expression, automated text mining and previous knowledge in databases [207,208]. STRING contains information on 24,584,628 proteins and will only create networks for proteins that

it is able to detect from its own database [208], the assembly of which is reliant on other sources of information, for example gene ontology. It must be noted that the gene ontology terms used in STRING are relevant at the time of analysis, but the databases and annotation of entries evolve and as such information informing interactions will change over time. It is important to investigate proteins and their interactions as these are the components of cellular function and ultimately phenotype. The generated interaction networks of proteins allows for the elucidation of biological function of the cells being analysed. Within the network each node represents a protein and the edges show their interactions with other proteins/nodes. The STRING networks in this chapter have edges set to represent confidence of interactions, the thicker the edge the more evidence there is for the interaction. Utilising STRING allows for a greater understanding of detectable proteins without introducing bias, however some bias is introduced when only particular functional enrichments are highlighted, and this is the case in this chapter. These functional enrichments are categorised by molecular function (based on gene ontology), molecular function (based on gene ontology), cellular component (based on gene ontology), reference publications, KEGG pathways, reactome pathways, UniProt keyword, PFAM protein domains, INTERPRO protein domains and featured, and SMART protein domains. The functional enrichments chosen were based on nodes that aligned with specific zones, and it must be acknowledged that this introduces personal bias. To mitigate this personal bias only functional enrichments with low false discovery rates were chosen. Bias is also introduced by the aforementioned potential incompleteness of the databases because discoveries are always being made that need to be imputed onto the proteins in the database, if the protein or, more importantly, the proteoform is present. This bias cannot be overcome and so any inferences made by these analyses needs to be validated in properly designed experiments. Additionally a lot of the information on the functional enrichments is made available through STRING and is dependent on the databases that it uses, information taken from here does not come from a single publication, for example biological processes are associated the gene ontology annotation, and therefore will not contain a specific reference.

Proteins that were only detected in a single fraction provide unique insight to that fraction and were consequently analysed using a STRING network. When analysing these 883 proteins unique to the whole cell lysate with STRING, only 593 nodes were detected. This is a limitation

of STRING, as not all of the accession numbers of the proteins identified through PEAKS have recorded proteins within STRING as mentioned above. This speaks of a broader limitation of utilising these bioinformatic systems for analysis, as the conclusions drawn can only be those that have previously been observed, often in a different cell type. These missing proteins are not because of duplicate ID's, as they simply did not have an annotation in STRING. It is clear that the databases that these bioinformatic inference systems rely on are not complete, however they still provide valuable guidance for data analysis. Figure 9 of the ASC whole cell lysate shows a dense STRING network with 593 nodes and 7688 edges however it has a relatively low average local clustering coefficient of only 0.353. A high average local clustering coefficient is not necessarily expected in this case, as the many proteins present in the whole cell lysate are unlikely to all be connected to or interact with each other. The very broad biological process category metabolic process (GO:0008152) is shown in blue. These 424 nodes are used by living organisms to transform a wide variety of chemical substances [225]. The nodes that representing proteins involved in metabolic processes are spread out across the entirety of the network but are most prominent in the dense cluster on the lower half of the network. It is to be expected that proteins unique to the whole cell lysate are overwhelmingly involved in metabolism and that these are connected as the products of one reaction are substrates for others. Proteins annotated as being in the category cellular component cytosol (GO:0005829) are shown in orange with 375 nodes. These highlighted proteins are found in the part of the cytoplasm that does not contain organelles but does contain interacting proteins and protein complexes. These cytosolic proteins appear throughout the network but are mostly present in the upper, less dense half of the network. It is expected that many of the proteins unique to the whole cell lysate are found in the cytosol and, as anticipated, many of the detected proteins are both a part of the cytosol and involved in metabolic processes as shown by the nodes coded both blue and orange. Proteins involved in the cellular component category intracellular organelle lumen (GO:0070013) are indicated in pink, and these 316 proteins are present throughput the entire network but are mostly present in the dense lower half of the network. The term intracellular organelle lumen refers to the internal volume that is enclosed within membranes within an organelle. It is therefore expected that many proteins from this cellular compartment were detected only in the whole cell lysate rather than the membrane bound fraction or extracellular vesicles, as during protein extraction the soluble proteins within the lumen are released from the organelles.

There were 29 proteins detected in the membrane-bound fraction that were not present in the other fractions, as shown in Figure 10. As with every STRING network only particular proteins were identified, in this case 13. These nodes have no edges and the average local clustering coefficient therefore is 0. This STRING network indicates that the proteins that are unique to the membrane-bound fraction do not interact with one another. However, as shown in blue, the cellular component categories they comprise are the organelle membrane (GO:0031090) shown in blue and membrane-bounded organelle (GO:0043227) shown in orange. As this network represents proteins only found in the membrane-bound fraction it is expected that they are annotated as organelle membrane and membrane-bounded organelles. This is also indicative of the membrane isolation method utilised which extracts all membranes of the cell and does not separate different membranes (plasma, nuclear etc) or parts of those membranes.

ASCs produce extracellular matrix, metabolic and cytoskeletal proteins, demonstrating that they have the mechanisms required for secreting cellular proteins [226]. 730 proteins were previously identified in BMSC microvesicles and successfully demonstrated that microvesicles are not just useful for their secreted factors, but are likely vital for cellular function [217]. For example, Rab proteins, a number of which being detected in this work, are membrane organizers, which coordinate formation of vesicles, motility of organelles and vesicles, and tethering between vesicles and membranes [227]. In fact, the initial specific tethering that occurs between a vesicle and the target membrane is mediated by Rab proteins[227]. It appears that Rab proteins are specialised proteins and that they are tailored to particular organelles and transport systems [227]. Rab proteins are vital for microvesicle docking, fusion and targeting to specific cellular compartments. Rab proteins have also been detected in cancer cells [228,229] and BMSCs [217]. Rab proteins are a part of the Ras superfamily of small GTPases [230], and as shown in Figure 8 there are 12 proteins in the whole cell lysate, 7 in the membrane bound fraction and 8 in extracellular vesicles from the Ras pathway (P04393). Some examples of Rab proteins that were also present in the ASC proteome detected in this project are Ras-related protein Rab-1A in all fractions, Ras-related protein Rab-1B which was detected in whole cell lysate and extracellular vesicle and is utilised in transporting vesicles from the endoplasmic reticulum to the golgi-plasma membrane in colorectal cancer cells [228], Rab3 GTPase-activating

protein non-catalytic subunit was identified in the whole cell lysate, Rab3 GTPase-activating protein catalytic subunit was also found in the whole cell lysate, Ras-related protein Rab-5A in which was detected in extracellular vesicles and whole cell lysate , Ras-related protein Rab-5B was detected in whole cell lysate, membrane bound fraction and extracellular, and Ras-related protein Rab-5C was also detected in the whole cell lysate, membrane bound fraction and extracellular vesicles. Ras-related proteins Rab-5B and Rab-5C regulate clathrin-coated vesicle transport between the plasma membrane and early endosomes in human colorectal cancer cells [228]. Ras-related protein Rab-6A was also detected in the whole cell lysate, membrane bound fraction and extracellular vesicles, and this protein is utilised in transporting vesicles from the endoplasmic reticulum to the golgi-plasma membrane in colorectal cancer cells [228]. Ras-related protein Rab-7a was detected in all fractions, this protein is essential for transporting vesicles between early endosomes and multivesicular bodies in human colorectal cancer cells [228]. Ras-related protein Rab-8A was detected in extracellular vesicles and whole cell lysate and is likely important in basolateral microvesicle biogenesis as displayed through their function in epithelial and intestinal epithelial cells [231,232]. Ras-related protein Rab-10 was found in whole cell lysate, membrane bound fraction and extracellular vesicles and is utilised in transporting vesicles from the endoplasmic reticulum to the golgi-plasma membrane in colorectal cancer cells [228]. Ras-related protein Rab-11B was detected in whole cell lysate, membrane bound fraction and extracellular vesicles and is utilised in transporting vesicles from the endoplasmic reticulum to the golgi-plasma membrane in colorectal cancer cells [228]. Ras-related protein Rab-31 was detected in the extracellular vesicles, as was Ras-related protein Rab-35 which is important for the regulation of an endocytotic recycling pathway [233]. While BMSCs and ASCs are different, the detection of proteins in extracellular vesicles in this work and in BMSCs in other published work reinforces the importance of Rab proteins in vesicles derived from stem cell.

The following proteins have previously been detected in stem cell secretions and their detection in this data set suggests they are important for stem cell function [186]. Extracellular matrix proteins and proteins involved in cell secretion include biglycan, cadherin-11, fibulin-1, fibulin-2, lumican, periostin, versican core protein and vitronectin were detected in extracellular vesicles. Fibrillin-1, laminin subunit γ1 were detected in the whole cell lysate and extracellular vesicles and has previously been detected in other stem cell secretions, and

collagen alpha-2(I) chain, serpin H1 and fibronectin were detected across all three fraction and have also previously been detected in secretions of other stem cells [186]. Enzymes and enzyme inhibitors detected in the extracellular vesicle fraction include metalloproteinase inhibitor 3 and serine protease HTRA1. Glia-derived nexin Plasminogen activator inhibitor 1 was detected in our whole cell lysate and extracellular vesicles and metalloproteinase inhibitor 1 and plasminogen activator inhibitor 2 was detected in our whole cell lysate. All of these proteins are likely vital for stem cell extracellular vesicles to exert their function, but the functional roles of these proteins are yet to be investigated.

When examining the proteins only detected in the extracellular vesicles and not in the other fractions, a STRING network with 198 nodes and 880 edges was produced. This has a reasonable average local clustering coefficient of 0.486 and it can be observed in Figure 11 that these nodes are relatively well connected, indicating that the proteins that are exclusive to this fraction interact with one another. 78 of these proteins are involved in the biological process category vesicle-mediated transport (GO:0016192), shown in blue and are distributed across the network, particularly being present in the denser areas. The process inferred by proteins in this category involves a substance being directed to the forming vesicle, which then buds and is coated. This vesicle is then targeted to a membrane that it will fuse with. It is therefore not surprising that many of the proteins unique to the extracellular vesicles are involved in this biological process, validating the method used to isolate the vesicles. The biological process category secretion by cell (GO:0032940) is shown in orange, and these 54 proteins are largely found in the outer dense regions of the network. Proteins involved in the controlled release of substances by cells are logically detected in the extracellular vesicle fraction. The cellular compartment category endomembrane system (GO:0012505) is shown in pink with 116 proteins present throughout the entire network, particularly in its most dense regions. The endomembrane system includes membranous structures within the cell that facilitate cellular transport such as the endoplasmic reticulum, golgi bodies, nuclear envelope, cell membrane and vesicles. The materials moving within this system pass through the membranous structures themselves, or through vesicles. This suggests that the extracellular vesicles that were isolated from this experiment were a part of the endomembrane system. This is supported by the previously discussed biological process category secretion by cell and many nodes can be observed highlighted with both orange and

pink. The 82 proteins shown in yellow are annotated as belonging to the cellular component category vesicles (GO:0031982), which is to be expected as this network is for proteins unique to the extracellular vesicle fraction. It must also be reiterated that these extracellular vesicles were isolated by differential centrifugation like many stem cell vesicles are [186]. It is typically recommended to expand stem cells in the absence of FBS prior to extracellular vesicle extraction as highly abundant serum proteins such as albumin will make it mask low abundance proteins [186]. However, serum starvation causes stem cells to respond differently to how they normally would, such as membrane blebbing and growth arrest [194,195]. Serum starvation has been shown to effect the transcription of over 100 genes in ASCs [196], consequently these vesicle were not serum starved. It must be noted that FBS contain extracellular vesicles [234], however because the proteomic search is limited to human proteins, the bovine proteins are likely not impacting this study. Furthermore, FBS extracellular vesicles are shown to persist even after serum starvation [234].

The proteins that were detected in multiple ASC fractions were then compared in clustered heatmaps. These heatmaps were created using the output from PEAKS which provides quantitative proteomics data based on area under the curve of peptide signal in the MS1 scan. The first heatmap displayed in Figure 12 included the whole cell lysate and membrane bound fractions. Each column represents a patient sample and each row a protein. The patient samples have formed two vertical distinct clusters, one for the whole cell lysate and one for the membrane bound fraction, as expected as the same sample type from different patients should cluster together to further validate that the data is robust and reliable. The colours shown are indicative of Z scores, a statistical measurement of variation from the mean. A Z score of 0 indicates the value is the same as a mean, while a positive score indicates a value above the mean, and a negative indicates a value bellow. There are four distinct vertical clusters of proteins, and STRING networks were created for each of these protein clusters.

Cluster 1 of the ASC whole cell lysate and membrane bound fraction is shown in Figure 13. The STRING network has 138 nodes with 864 edges and a relatively high average local clustering coefficient of 0.5, which can be observed by the dense clusters of proteins with many thick connections. Biological processes were highlighted within this network. The category establishment of localisation in cell (GO:0051649) is shown in blue with 55 proteins

scattered throughout but focussed in the three dense regions of the network. The highlighted proteins within this cluster are involved in any process within a cell that localises a substance or cellular component. The proteins within this cluster are more abundant in the membrane bound fraction than the whole cell lysate, reinforcing that membranes are important for the establishment of intracellular localisation within ASCs. Within the densest cluster of proteins on the upper left quarter of the network, two more biological process categories were highlighted. In orange, with 18 proteins, is the category cotranslational protein targeting to membrane (GO:0006613) and of those 18, 17 proteins indicated in pink, belong to the process signal-recognition particle-dependent cotranslational protein targeting to membrane (GO:0006614). Cotranslational protein targeting to membrane is a process that targets proteins to membranes during translation. For example, this process commonly transports most secretory proteins into the endoplasmic reticulum lumen or imports proteins to the mitochondria [235,236]. The biological process to which these 17 proteins are a part of (signal-recognition particle-dependent cotranslational protein targeting to membrane) is reliant on the presence of two components, the signal-recognition particle and signal-recognition particle receptor. This cytosolic-located particle transiently binds to the endoplasmic reticulum signal sequence which is located in a nascent protein; the complex then binds to the large ribosomal unit and then to the signal-recognition particle receptor within the endoplasmic reticulum membrane [237]. This shows that not only are ASC proteins involved in these biological processes of cotranslational protein targeting to the membrane, but specifically that those are signal-recognition particle-dependent, and that these are more abundant in the membrane bound fraction than the whole cell lysate.

Cluster 2 of the ASC whole cell lysate and membrane bound fraction in Figure 14 contains only 71 nodes with 445 edges, however it has a relatively high average local clustering coefficient of 0.529, and there is only one node present in the network that is without any connections. The proteins within this cluster are more abundant in the whole cell lysate than the membrane bound fraction. Three different cellular component categories are highlighted in the STRING network, with the cytoplasmic vesicle lumen shown in blue (GO:0060205) cytosol (GO:0005829) shown in orange and cytoplasm (GO:0005737) shown in pink, with 20, 53 and 70 proteins respectively. Many of these proteins are a part of two or more of these cellular components. This is expected as proteins that have higher abundance in the whole

cell lysate are likely to be those found in the cytosol and cytoplasm. This is confirmed in the data as all but 1 protein within this cluster are shown to be components of the cytosol or cytoplasm.

The STRING network for Cluster 3 of ASC whole cell lysate and membrane bound fraction, while dispersed with many nodes without interactions, still has a reasonably high average local clustering coefficient of 0.572 (Figure 15). This is likely due to the dense mass of proteins towards the centre of the network that share multiple connections. The network itself has 56 nodes with 156 edges. The proteins in this cluster have variable Z scores but overall are more abundant in the membrane-bound fraction than the whole cell lysate. The dense section is made up of 15 proteins shown in blue that are a part of the biological process category establishment of protein localisation to endoplasmic reticulum (GO:0072599). This suggests that membranes within ASCs are utilised for the movement of proteins to specific locations in the endoplasmic reticulum. Within this collection of 15 proteins are 11 proteins shown to be structural constituents of ribosomes (GO:0003735). The ribosome itself is a complex molecule comprising of proteins and ribosomal RNA. While ribosomes are found in the cytoplasm, they preferentially associate with the membrane of the endoplasmic reticulum. It is therefore logical to find these membranous proteins in the membrane-bound fraction and recognise that membrane bound proteins can still be detected in the whole cell lysate.

The final cluster of the comparison between ASC whole cell lysate and membrane bound fraction is Cluster 4 (Figure 16). Proteins in this cluster have higher abundance in the whole cell lysate with both high and low abundance in the membrane bound fraction. This cluster has 50 nodes with 98 edges and a relatively high average local clustering coefficient of 0.517, despite the cluster appearing quite dispersed. The cellular components highlighted within this STRING network are not specific to a single region, rather they are all spread throughout the network. The cellular components categories are organelle part (GO:0044422) shown in blue with 41 nodes, intracellular organelle (GO:0043229) shown in orange with 46 nodes and membrane-bound organelle (GO:0043227) shown in pink with 42 nodes. Consequently, this demonstrates that most of the proteins within this cluster constituted a part of an organelle, a group of structures within a cell that have individual functions and morphology. Examples of organelles that are membrane-bound include the nucleus and mitochondria. This

ultimately indicates that proteins involved in organelles are detected in both the whole cell lysate and membrane bound fraction, with overall a greater abundance in the whole cell lysate, and varying abundance within the membrane bound fraction.

ASC whole cell lysate and extracellular vesicles were compared in a heatmap (Figure 17), with each row representing a protein and each column a patient. There are four clusters of proteins and the patient samples formed two distinct clusters, the whole cell lysate and extracellular vesicles, as expected for the aforementioned reasons. This provides independent validation of the robustness of this experiment as each of the four protein clusters were visualised using a STRING network, with functional enrichments highlighted within each of these clusters.

The STRING network for the ASC whole cell lysate and extracellular vesicle Cluster 1 is shown in Figure 18 which has a low average local clustering coefficient of 0.4333 as, despite some dense network interactions, many of the nodes are not connected. This cluster has a total of 133 nodes with 563 edges. The highlighted functional enrichments are distributed throughout the network but are more concentrated in the denser areas. The biological process category vesicle-mediated transport (GO:0016192) is shown in blue with 46 nodes and the cellular component category vesicle is shown in orange (GO:0031982) with 62 nodes, indicating that the proteins in this cluster are involved in the formation, maintenance and docking of the vesicle rather than the cargo of the vesicle. Many of these nodes, particularly those in the dense core, are both blue and orange. Vesicles themselves are membrane covered organelles that are spherical in shape and filled with fluid. These vesicles transport substances by enclosing them within the lumen of the vesicle or in the membrane of the vesicle itself. Once the vesicle has budded and is coated, it is targeted to fuse with an acceptor membrane. Proteins within Cluster 1 have varying detected protein abundance between the two fractions but are more abundant in the extracellular vesicle fraction, as expected due to these proteins being involved in vesicles.

The largest of the ASC whole cell lysate and extracellular vesicle clusters is shown as a STRING network in Figure 19. Cluster 2 is extremely dense and well connected with an average local clustering of 0.55 made up of 262 nodes with 5018 edges. The proteins detected in this

fraction have high abundance in the whole cell lysate and low abundance in the extracellular vesicles. of these nodes are highlighted blue showing the cellular component category cytoplasm (GO:0005737). The detection of proteins from the category cytoplasm in the whole cell lysate and extracellular vesicle fractions is expected as these both contain cytoplasm, while the higher abundance in the whole cell lysate is also expected, with cytoplasmic proteins in the vesicle being the cargo. 90 proteins are from the cellular component category vesicle (GO:0031982) which are shown in orange; these 90 proteins are scattered throughout the less dense regions of the network. Whilst it wouldn't typically be expected to see a higher abundance of proteins from vesicles in the whole cell lysate than the extracellular vesicles themselves, it is important to recognise that there are also intracellular vesicles that are not released except in the event of cell lysis. The final cellular component highlighted is the ribonucleoprotein complex (GO:1990904) shown by the 49 pink nodes. These proteins along with RNA molecules make up the macromolecular complex known as the ribonucleoprotein complex. Proteins involved in this complex are concentrated in the dense core located in the lower right quadrant of the network. Ribonucleoproteins have been shown to bind and load miRNA into extracellular vesicles [238], so their presence is anticipated. In addition, as most ribonucleoprotein complexes are intracellular, it is reasonable for these proteins to be more abundant in the whole cell lysate. In other studies, BMSC microvesicles have been shown to sort selected miRNAs, suggesting that stem cells are likely partially dependent on miRNAs shuttled in microvesicles. Furthermore, miRNAs derived from these BMSC microvesicles were able to suppress specific targets, demonstrating their ability to mediate cellular communication [239].

Cluster 3 of the whole cell lysate and extracellular vesicle fraction is not particularly dense, with 89 nodes that have 297 edges and an average local clustering coefficient of 0.47 (Figure 20). The proteins in this cluster have variable abundance across fractions but are more abundant in the whole cell lysate, with both high and low abundance in the extracellular vesicle fraction. The cellular compartment category cytoplasmic part (GO:0044444) is shown in blue with 83 nodes, and it is expected that more proteins from this compartment would be detected in the whole cell lysate. The endoplasmic reticulum part (GO:0044432) is shown in orange in the less dense parts of the network. Whilst the cargo of extracellular vesicles are often sorted by the endoplasmic reticulum, proteins from that cellular component are not

expected to be detectable within the extracellular vesicles themselves. This may be considered unusual, but it is important to recall that these proteins are more abundant in the whole cell lysate.

Figure 21 shows Cluster 4 of the ASC whole cell lysate and extracellular vesicle fraction, and it is relatively sparse with a low average local clustering coefficient of 0.357. The 59 nodes have 87 edges. 14 nodes have functional enrichments of the biological process regulated exocytosis (GO:0045055) shown in blue, and the cellular component secretory granule (GO:0030141) shown in orange. Secretory granules are vesicles formed in the golgi apparatus, and the term regulation of exocytosis refers to storing these vesicles for later release [240]. Upon stimulation, these vesicles migrate to the periphery of the cell where their membranes fuse with the cell membrane, leading to exocytosis. The proteins within this cluster were found to have low abundance in extracellular vesicles, but high abundance in the whole cell lysate. This is consistent with what would be expected, as vesicles are formed at the surface of the membrane within ASCs.

The membrane-bound fraction and extracellular vesicle proteins were compared using a clustered heatmap (Figure 22). The patient samples formed two horizontal clusters, one for the membrane-bound fraction and one for the extracellular vesicles, as expected. There are four vertical clusters, with each row representing a protein. Compared to the two previous heatmaps, this one has less distinctive patterns regarding Z scores within their respective clusters. Each of these clusters were examined in a STRING network.

The first cluster of the ASC extracellular vesicle and membrane-bound fraction has a dense well-connected core and is a highly interactive network, with an average local clustering coefficient of 0.54. As seen in Figure 23, Cluster 1 has 177 nodes with 1756 edges. Proteins within this cluster are more abundant in the membrane bound fraction than the extracellular vesicles. There are three functional enrichments in this cluster, the biological process category transport (GO:0006810) shown in blue, the biological process category cellular process (GO:0009987) shown in orange, and the cellular component category protein containing complex (GO:0032991) shown in pink. 113 proteins detected in the membrane bound fraction and extracellular vesicles are involved in the biological process category

transport, meaning they direct substances or cellular components in and out of a cell through a variety of mechanisms. This indicates that within this cluster there is a higher abundance of proteins involved in transport within the membrane bound fraction than the extracellular vesicles. 166 proteins were identified as being a part of cellular processes and there is a greater abundance of these in the membrane than the extracellular vesicles. 96 of the proteins from this cluster are involved in protein-containing complexes, where different macromolecules such as nucleic acids, carbohydrates or lipids function together with a protein. These findings indicate that within ASCs there is a greater abundance of proteins involved in these protein-containing complexes within the membrane bound fraction.

The STRING network of Cluster 2 of the extracellular vesicle and membrane bound fraction only has 39 nodes, but they have 138 edges and therefore a relatively high average local clustering coefficient of 0.553 (Figure 24). The molecular function category protein binding (GO:0005515) is shown in blue on 29 proteins, showing that these proteins interact non-covalently and selectively with proteins and protein complexes. There is a greater abundance of these bound proteins in the extracellular vesicles than the membrane-bound fraction in ASCs within this cluster which could potentially indicate specific packaging into the vesicle. In orange are 21 proteins involved in the cellular component category vesicle (GO:0031982), and it is not surprising that these have greater abundance in the extracellular vesicle fraction.

55 nodes with 77 edges make up Cluster 3 of ASC extracellular vesicle and membrane bound fraction STRING network shown in Figure 25. These nodes are well connected with a relatively high average local clustering coefficient of 0.515. Proteins in this fraction have differing levels of abundance but overall have a slightly higher abundance in the membrane bound fraction and a slightly lower abundance in the extracellular vesicles. The first functional enrichment is the biological process category localisation (GO:0051179) shown in blue with 42 proteins. Of these 42 proteins, 37 are of the biological process category establishment of localisation shown in orange (GO:0051234). This refers to the transport and ability to maintain a process, substance or entity of the cell in a specific location. Within this cluster, proteins from the membrane bound fraction related to localisation appear to have a higher abundance, indicating the role of membranes in localisation. The biological process category vesicle mediated transport (GO:0016192) is shown in pink with 23 nodes, demonstrating that while

membrane bound and extracellular vesicle proteins are involved in the transport of vesicles, they have greater abundance in the membrane bound fraction than the extracellular vesicle fraction, inferring that these proteins have more roles than what their annotations indicate.

Figure 26 shows Cluster 4 of the ASC extracellular vesicle and membrane bound fraction STRING network that had 61 nodes with 176 edges and an average local clustering coefficient of 0.47. The biological process category VEGF receptor signalling pathway is shown in blue with 3 proteins, the biological process categories regulation of FGF signalling pathway in orange with 2 proteins, molecular function transforming growth factor beta binding in pink with 3 proteins and PDGF binding in yellow with 2 proteins. The proteins in this cluster have differing levels of abundance but overall have a slightly higher abundance in the extracellular vesicles and slightly lower abundance in the membrane bound fraction. All of these proteins are involved in different signalling pathways. The proteins in blue are any signals that are instigated by the binding of VEGF receptor to an extracellular ligand, the orange proteins modulate the activity of the FGF signalling pathway, the proteins in pink interact with transforming growth factor beta, a peptide which controls cellular proliferation and differentiation, and in yellow are proteins that interact with platelet derived growth factor. All these signalling pathways are associated with cytokine production and are understandably found in the membrane-bound and extracellular vesicle fraction, as these are utilised for cellular secretion. Other cytokines and chemokines detected in this proteomic data but not within this cluster include macrophage migration inhibitory factor which was identified in the whole cell lysate and has also previously been detected in stem cell secretions [186].

As many cytokines are below the detectable limit for LC-MS/MS a multiplex-immunoassay was utilised. This quantified the following cytokines that were secreted from the ASCs into the media: FGF basic, eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF. As seen in Figure 27, the 27 detected cytokines vary in secretion levels, however consistent across almost all cytokines is the large standard deviation as shown by error bars. This is to be expected, as it is exceptionally difficult to define a 'normal' or 'healthy' patient, and this variation reflects the large variation seen between patient to patient proteomes earlier in this chapter.

This project is the first to identify a number of these cytokines in ASCs, while some of these cytokines have previously been detected in ASCs. For example MCP-1, IL-6 and IL-8 have previously been detected in human ASCs and appear to have relatively high secretion levels in comparison to the other measured cytokines in this project [189]. These cytokines have also been identified in other stem cell secretions. MCP-1 is a chemokine that has been seen in many other stem cell secretions[189,241-249], while IL-6 is a pleiotropic cytokine that has been detected in the secretions of numerous stem cells [170,219,241,243,244,246,249-251]. The IL signalling pathway (P00036) was highlighted in Figure 8 and it can be seen that there are 3 proteins from this pathway in the whole cell lysate, 4 in the membrane bound fraction and 2 in extracellular vesicles. IL-8, which is a chemokine, was quantified in this project and has also been detected the secretions of numerous stem cells [189,241-244,250]. Another cytokine with relatively high secretions levels is the angiogenic factor VEGF that has been detected in stem cell secretions in a substantial amount of literature [243-246,249,251-256]. Furthermore, in Figure 8 it was shown that 10 proteins in the whole cell lysate, 6 in the membrane bound fraction and 5 in extracellular vesicles are involved in the VEGF signalling pathway (P00056).

The following hematopoietic cytokines have varying secretion levels in this data, but have been identified in the secretions of other stem cells; G-CSF [241,243], GM-CSF [241,243,249,250] and IL-7 [243,250]. Pro-inflammatory cytokines IL-12, TNF-α and IFN-γ have similar secretion levels in ASCs as seen in Figure 27 and have also been detected in other stem cell secretions [241,243,249,250,256]. IL-2 is also a pro-inflammatory cytokine but it had lower secretion levels than the aforementioned cytokines, however it too has been detected in stem cell secretions [243]. In fact, the category inflammation mediated by chemokine and cytokine signalling pathway (P00031) can be seen in Figure 8, represented by 39 proteins in the whole cell lysate, 20 in the membrane bound fraction and 36 in the extracellular vesicles. Many of these stem cell secretions are from BMSCs, and it has been shown that ASC cytokine profiles, much like their extracellular vesicle profiles, are similar to those of BMSCs [250]. Anti-inflammatory cytokines IL-10 and IL-13 were also detected in other stem cell secretions [243]. IL-1β is a pleiotropic cytokine that was found to have low secretion levels in this project, but has previously been detected in stem cell secretions [243,249]. RANTES is a chemokine that has been detected in stem cell secretions [243,246,249], and eotaxin, another cytokine, has also been detected [243]. IFN-γ and

IP-10 were secreted by ASCs and quantified in this study, and additionally the IFN-γ signalling pathway (P00035) was shown in Figure 8 with 4 proteins in the whole cell lysate, 3 in the membrane bound fraction and 2 in extracellular vesicles. PDGF-BB has been shown to increase osteogenic differentiation of BMSCs while inhibiting adipogenic differentiation [257]. PDGF-BB was measured in this multiplex immunoassay and proteins involved in the PDGF signalling pathway (P00047) were identified in Figure 8, specifically 9 proteins in the whole cell lysate, 6 in the membrane bound fraction and 5 in extracellular vesicles.

The analysis of the whole cell lysate, membrane bound fraction, extracellular vesicles and secreted cytokines have collectively revealed a comprehensive ASC proteome. This immense data set provides much needed clarity on proteins expressed by ASCs isolated from fresh patient lipoaspirates. This project showcased the complexity of ASC proteins, the way that proteins between fractions cluster, how different proteins interacted with one other in STRING networks as well as across different analysis. While this is the most comprehensive proteomic analysis to date it is far from complete.

4.2 Future directions

This project aimed to develop a comprehensive catalogue of the proteome of adipose stem cells and to introduce as little bias as possible while doing so, the main source of bias being patient-to-patient variation. However, the functional networks highlighted in the STRING analysis is an area where bias could not be overcome because of the lack of completeness of entries within the supporting databases used to construct the network. Therefore, one of the first things to examine in the future is to validate the inferences made by these analyses. For example, in Figure 15 the STRING network of proteins detected in Cluster 3 comparing the abundance of proteins in the ASC whole cell lysate and membrane bound fraction, the main functional enrichment found was for the biological process categories establishment of protein localisation to endoplasmic reticulum shown in blue and constituent of ribosome shown in orange. It is known that while ribosomes are found in the cytoplasm, they preferentially associate with the membrane of the endoplasmic reticulum, and therefore it is logical to detect these membranes associated proteins in higher abundance in the

membrane-bound fraction while also detecting them in the whole cell lysate. This could be validated by conducting a microscopy experiment that tracks these proteins of interest to validate that these proteins were in fact involved in establishing protein localisation to the endoplasmic reticulum or were part of the ribosome. This should be done for all inferences made from STRING networks, particularly as and as stated, these are dependent on databases and the detected proteins may have other functional roles within ASCs that are yet to be elucidated.

When examining the secreted proteins there are more experiments that could be conducted to build on the data collected from this project. For example most secreted proteins and cell surface markers are glycosylated and therefore it would be ideal to collect N-glyocosylated proteins from the media, consequently allowing for enrichment of proteins that are undergoing post-translational glycosylation [186]. This approach could also be used to identify better surface markers, as there is no single marker or adequate panel of markers to identify ASCs[180]. The minimal criteria to define mesenchymal stem cells, a term that should no longer be used, was set in 2006 by Dominici *et al.* for the International Society for Cellular Therapy and required that, to be defined as a stem cell, the cells be able to adhere to plastic in standard culture conditions, have the capacity to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts, express the surface molecules CD73 and CD90, and lack the expression of CD45, CD34, CD14 or CD11B, CD79a, or CD19 and HLA-DR [12]. However, it was discovered that cells of the stromal vascular fraction prior to culture did not express markers CD45, CD235A, CD31 but positively expressed CD34, which directly contradicted with previous guidelines [258]. This positive expression of CD34 on ASCs at passage 0, has also been shown to have a correlation with the yield and doubling time of ASCs [259]. CD34 expression still remains during early culture of ASCs, however after long-term culture the expression ceases [260]. Bourin *et al.* identified that cultured ASCs express CD90, CD73, CD105 and CD44, and are negative for CD45 and CD31, and consequently suggest using CD13, CD73, CD90 and CD105 to assist in defining the stromal vascular fraction [258]. ADSCs have also been found to express CD9, CD10, CD13, CD29, CD34, CD44, $CD49_d$, $CD49_e$, CD54, CD55, CD59, CD105 and CD106 [261], while ADSCs grown in human platelet lysate have shown non-classical markers such as CD36, CD163, CD271, CD200, CD273, CD274, CD146, CD248 and CD140B [262]. These inconsistencies

likely arise from the lack of standardisation of isolation and culturing protocols, which will be investigated in the next chapter [260].

This project did attempt to solve this issue by analysing the membrane bound fraction. The method used extracted the cellular membrane that ASCs are encased in, along with other membranous structures. It would be beneficial to look at cell surface markers rather than just the membrane bound fraction. An approach such as combining trypsin shaving and surface biotinylation could be used [263]. Once an adequate set of cell surface markers have been established, the work presented in this chapter should be repeated with cells sorted to have these specific markers, as in this project ASCs were isolated from the stromal vascular fraction by growing them in tissue culture to eliminate non-adherent cells [193]. Further purification of cells to achieve a more homogenous cell population would improve the quality of the proteomics data, as this would more accurately reflect the cell population. Alternatively, a more comprehensive proteome could be catalogued by conducting single cell proteomics using methodologies such as SCoPE-MS [264]. This single cell approach would allow the proteomes of cell populations to be deconstructed and for protein abundance relationships to be inferred across individual cells. If the technical issue of isolating a single vesicle were to be overcome, this could even be conducted on single extracellular vesicles. This would provide an immense amount of insight into the biology and mechanism of signalling by secreted vesicles and warrants further investigation.

An easier and more readily available way of expanding our understanding of ASC extracellular vesicle proteins would be to investigate the membranes of extracellular vesicles. This would use the same extracellular vesicle extraction method, followed by the membrane protein extraction method. The analysis of the membrane bound fraction increased the number of ASC proteins detected by 4.5% when compared to the whole cell lysate. Hopefully investigating membrane bound extracellular vesicle proteins will increase the number of extracellular vesicle proteins by a similar percentage, and ultimately increase our understanding of these vesicles and how they are targeted to other cells distant to the cell of origin. There are also other published methods that specifically investigate extracellular vesicle membranes that could be utilised [212].

It's also important to recognise that the extracellular vesicles used in this work were isolated by differential centrifugation [186]. While this method is common, it is also quite crude. It has the benefit of being cheap and accessible and enables the collection of a sufficient number of extracellular vesicles for proteomic analysis from what is a resource intensive methodology, rather than performing further purification which risks a loss of vesicles and therefore material. However, should this experiment be expanded to collect a greater quantity of extracellular vesicles, different isolation methods should be used, particularly ones that can differentiate between different classes of extracellular vesicles, for example apoptotic bodies, microvesicles and exosomes. Furthermore, the method used in this project involved freezing the media from the cells before isolating extracellular vesicles. In future fresh, extracellular vesicles should also be studied, as the process of freezing and thawing likely effects the extracellular vesicles. The impact of cryopreservation on ASCs is investigated in the next chapter.

5. Conclusions

The work presented in this chapter successfully created the most comprehensive catalogue of ASC proteins to date. Proteins were quantified from the ASC whole cell lysate, membrane bound fraction, extracellular vesicles and select secreted cytokines. This analysis was desperately needed as ASCs are already being used in the clinic, both in controlled clinical trials and in unproven stem cell treatments. This project was necessary to increase the overall understanding of ASCs and the data from this project can shape the use of ASCs in the clinic. Furthermore, this will serve as a thorough baseline library of proteins that can be used to support the use of ASCs as a research model.

It is important for studies like this to be conducted as complex scientific questions can only be answered when there is a well-established baseline of understanding. This project has achieved this by detecting a total of 2290 proteins which were conserved across all patients. A PANTHER analysis was conducted on all of these proteins to further tease out how proteins from particular categories, specifically molecular function, cellular compartment, biological process, protein class, and protein pathway, were allocated between the different fractions. Proteins that were unique to a single fraction were placed into a STRING network with functional enrichments of interest highlighted. Proteins detected across multiple fractions were quantified and clustered in a heatmap. Each individual cluster of proteins was analysed in its own STRING network with particular functional enrichments shown. Select secreted cytokines were also quantified. Together all of this revealed a comprehensive ASC proteome. This provides clarity on proteins expressed by ASCs isolated from fresh patient lipoaspirates. This immense data set showcases the complexity of ASC proteins and their interactions.

6. Supplementary figures

Supplementary Figure 1: Correlation plots of normalised protein abundances.

Normalised protein abundance values for each group of technical replicate samples was individually compared to each other group of samples. If the abundance of the same protein from each group is the same, the dot representing that protein will fall on the diagonal line. If all proteins in the compared samples have the same abundance, the correlation value will equal 1. Individual patient samples represent biological replicates and the correlation value for the same sample from each patient should be as close as possible to 1.

Chapter Three

Trick or treatment: the effects of xeno free isolation and cryopreservation on human adipose stem cells

1. Introduction

1.1 Background

Adult stem cells, such as adipose stem cells (ASCs), are the most common stem cell used in clinical trials, in addition to both ESCs and iPSCs [36]. To date there has been success with stem cell treatments for cancers such as leukaemia and multiple myeloma. However, with the exception of hematopoietic stem cells, all other stem cell therapies are still in the research, pre-clinical or clinical phases [37]. This has not prevented unproven stem cell treatments being offered to patients with chronic and debilitating conditions. For vulnerable populations, hope is a crucial part of their self-identity and agency to control their future, but the stem cell therapies being promoted to these groups come with extremely high risks [73]. In Australia, autologous ASCs are being offered outside of clinical trials for the treatment of osteoarthritis, cartilage repair, stroke, multiple sclerosis, retinal neuropathy, spinal cord injury, amyotrophic lateral sclerosis and autism [83]. The number of private clinics offering autologous stem cell therapies is continuing to grow in Australia [84] and these predatory stem cell clinics are offering stem cell treatments that have little to no scientific evidence of safety, let alone efficacy [86]. It is extremely unethical to be offering unproven stem cell treatments outside of clinical trials [85]. However, the unfortunate reality is that predatory clinics are continuing to exploit vulnerable patient populations due to a lack of regulation and oversight. Despite regulatory efforts to reduce their commercial promotion, unproven stem cell treatments are still a growing global problem[87]. Australia has the 5th highest total number of stem cell clinics and the 5th highest number of clinics per capita in the world [88].

Despite these facts, ASCs still have potential as therapies. The stromal vascular fraction has even been used without further isolation of ASCs [265]. It has been proposed that the presence of endothelial progenitor cells within the stromal vascular fraction may be advantageous for the treatment of some diseases, however isolating and culturing ASCs provides significantly more cells which is preferable for treatment as higher cell yield likely increases the potential efficacy of the treatment [161]. In addition, the ASC secretome is a complex mixture of secreted factors that have therapeutic potential, comprising of growth factors, cytokines, extracellular matrix molecules, extracellular matrix molecule proteases, hormone and lipid mediators [167,193,266]. For example, there are growth factors that promote angiogenesis and wound healing such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF)β and hepatocyte growth factor [267]. Other growth factors are proinflammatory such as interleukin (IL)-6, IL-7, IL-8, IL-11, granulocyte-macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor (TNF)α [250]. There are also growth factors that are immunosuppressive such as IL-10 and prostaglandin E2 [268,269].

To further support the use of ASCs in regenerative medicine, they have also been shown to accumulate at sites of injury, often in response to an inflammatory reaction [168,169], and have been shown to improve the formation of the extracellular matrix through remodelling [170]. While the mechanism of ASC migration to areas of injury is not completely understood, there is evidence of it occurring, such as in rats with cerebral ischemia [270]. ASC immunosuppressive properties have been demonstrated *in vivo* with mice for graft-versus-host disease [271], experimental colitis [272] and experimental arthritis [273]. ASCs have been demonstrated to be immune privileged both *in vivo* and *in vitro* as they regulate both an inflammatory and immune response due to their ability to suppress T cells and activate antigen-specific regulatory T cells [162-164]. They also have immunosuppressive abilities such as suppressing lymphocyte reactivity, inhibiting production of inflammatory cytokines and the secretion of proteins which assist in immunomodulation and tissue regeneration [165-167]. Activated lymphocyte proliferation is also inhibited by ASCs [274]. The ability of ASCs to modify host tissue responses and overall immunobiological properties suggests minimal immune rejection [10,159].

Like other stem cell treatments, ASCs still face challenges such as immune mediated rejection, problems with cell survival, loss of function and the potential to transform into malignant

cells. Concerningly, stem cells have been isolated from human lipoma tissue [171]. The pro-angiogenic, immunomodulatory and anti-apoptotic properties of ASCs raise concern as these may support tumour growth [275]. Cytokines such as platelet-derived growth factor (PDGF)-BB has also been shown to enhance the transmigration of ASCs toward malignancy [276]. BMSCs have been shown to promote tumour growth through expression of growth factors, increasing vessel formation within tumours and creating a tumour stem cell niche [173]. Stromal cells that are similar to BMSCs have also been isolated from primary osteosarcoma [172], and this evidence suggests that stem cells are involved in tumour development. ASCs cross talk with chemo-residual breast cancer cells, being able to migrate toward chemo-residual triple negative breast cancer cells in a CXCR4/SDF-1α-dependent manner and drive chemo-residual tumour cell proliferation by secreting FGF2 and activating ERK [277]. Despite extensive research into the tumorigenic potential of ASCs, contrasting results have been produced [278-282]. This is partly due to the challenge of creating a model that successfully replicates the complexity of the microenvironment of a tumour [275]. Nevertheless, a definitive conclusion on the tumorigenic potential of ASCs has not yet been achieved. This further highlights the need for extensive research into ASCs.

For regenerative medicine to be successful, it is vital that the research currently being conducted is clinically translatable, meaning that there needs to be an increased focus on safety, efficacy, reproducibility and quality of research [283]. The International Society for Cell and Gene Therapy and the International Federation for Adipose Therapeutics and Science released a joint statement in 2013 that established minimal definitions of stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells [258]. While useful, these minimal definitions barely scrape the surface for what is required. To achieve clinical-grade ASCs, the current lack of standardisation of both isolation and expansion methods needs to be addressed [284,285]. A hindrance to the utilisation of ASCs in regenerative medicine is the lack of standardisation of procedures for isolation [285]. ASC isolation involves enzymatic digestion of lipoaspirates, the removal of mature adipocytes by centrifugation, and the resuspension of the stromal vascular fraction for further expansion in tissue culture to achieve a homogenous cell culture [286]. This process of isolation is not standardised and there are contradictory results on the optimal site and technique for liposuction that produces the greatest yield of ASCs [287-289]. Furthermore,

the time from liposuction to isolation varies amongst different studies, and an increase in this time has been shown to decrease the yield of ASCs [290].There are also a variety of safety issues that will hinder clinical translation that arise from the common practice of using animal-derived reagents, as exposure of human cells to these reagents *in vivo* have resulted in reports of anaphylaxis and immune reactions [284,291,292]. It is vital to address the lack of standardisation for ASC isolation and expansion procedures, and the safety and efficacy of ASCs for regenerative medicine.

A lack of standardisation of procedures and maintenance of clinical standards is also apparent in the methods utilised for increasing the number of ASCs, commonly referred to as expansion. There are a variety of media available for culturing ASCs including, but not limited to: Minimum essential media, Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) and DMEM Glutamax/F12 [10]. Most of these media need to be supplemented with serum, which provides essential nutrients, growth and attachment factors, hormones and a variety of macromolecules [293]. Fetal bovine serum (FBS) is typically utilised, however its animal origin presents safety issues and the risk of inducing an immune response if introduced into the patient [23]. Media containing FBS are highly variable and consequently poorly defined, [294] and the use of bovine derivatives can also result in bacterial, viral and prion infections [295,296]. An alternative is to utilise human and potentially autologous serum-derived products. However, this is often unfeasible in many research conditions, and while they have been shown to have comparable proliferation and differentiation potentials when compared to bovine sources [297-299], it has been shown that the properties of serum that affect stem cell differentiation differ greatly between individuals [300]. In addition, both FBS and human serum are undefined and consequently do not meet the standards of good cell culture practice [301].

There has been a shift towards utilising systems that are xeno free and defined [302]. Initial serum-free and xeno free media were unable to maintain the same growth rate or multipotency of ASCs grown in the presence of serum [303,304]. However, the products have evolved and some serum-free and xeno free media have been shown to provide higher proliferation rates than media containing serum, without affecting differentiation potential [305-307]. The movement toward xeno free media is necessary for consistent and reproducible

study of the isolation and expansion of ASCs [294]. There is a lack of a comprehensive study that compares current available xeno free media for ASCs. It is vital for this comparison to occur as the choice of media has been shown to result in varying proliferation rates and gene expression [32]. Utilisation of xeno free media under good laboratory practice, good manufacturing practice and good cell culture practice will reduce uncertainty in the development and application of *in vitro* procedures and consequently increase the potential for successful clinical translation [23,301,307-310]. However, there is minimal research into the impact that isolation and expansion with xeno free media will have on ASCs, and in particular there is a lack of proteomic characterisation. The examination of proteome differences of cells grown in different media needs to be explored as serum starvation has been shown to affect the transcription of over 100 genes in ASCs [196], so it is highly likely that proteome differences will occur but they are as yet unstudied.

Both fresh and cryopreserved ASCs are utilised as clinical treatments [311]. In a research setting, cryopreserved ASCs would be more common as this allows them to be stored for future use in experiments, an occurrence that is common to nearly all cell biology studies. Cryopreservation of stem cells has been extensively researched because it allows for the cells to be easily utilised in both commercial and clinical applications [312]. An examination of BMSCs cryopreservation showed that it does not impact differentiation capacity, proliferation potential, surface marker expression or morphology, however the impact on colony forming ability, viability, attachment and migration are still not clear [313]. ASCs maintained in serum free media that were frozen and then thawed have been shown to maintain growth and differentiation capacity [314]. The impact of long-term cryopreservation (more than 10 years) has also been investigated. Long-term cryopreservation negatively impacts osteogenic capacity of ASCs, but cell viability, immunophenotype and adipogenic capacity remained relatively unchanged [315]. There is a substantial amount of literature investigating the effect of cryopreservation on ASCs, but to the best of our knowledge the changes in the proteome is yet to be characterised.

1.2 Chapter Proposal

A baseline proteomic characterisation of fresh ASCs was reported in the previous chapter. This chapter aims to compare the isolation and expansion of ASCs in the most commonly used ASC media DMEM/F12 +10% FBS, hereafter referred to as traditional media, and a xeno free media (StemMACS MSC Expansion Media Kit Xeno Free human). This was achieved by isolating and culturing ASCs from 6 patients in both mediums. Cells were expanded to passage 5, where extraction of proteins from the whole cell lysate and membrane bound fraction was performed to examine proteome differences using a 'shotgun' LC-MS/MS approach, and a Bioplex multiplex immunoassay was used to quantify 27 secreted cytokines from the culture media.

The impact of cryopreservation will also be investigated in this chapter. ASCs from 5 individual patients will be isolated in traditional media and a portion will be cryopreserved using FBS + 10% dimethyl sulfoxide, while a portion will continue being cultured without any cryopreservation. The cryopreserved cells will be thawed and cultured for 5 passages. At passage 5, the cells will be isolated, and proteins will be extracted for proteomic analysis of the whole cell lysate using a 'shotgun' LC-MS/MS approach.

2. Methods

The methods are the same as those presented in Chapter 2 with a few modifications explained bellow.

2.1 Xeno free method

Six patient samples were extracted in either traditional or xeno free media. Traditional media is DMEM/F12 + 10% FBS as this is what is most commonly used across the literature, and the xeno free media chosen was StemMACS Mesenchymal Stem Cell Expansion Media Kit Xeno Free, human. This media was chosen from a preliminary study (data not shown) that compared NutriStem (Biological Industries), StemMACS (Miltenyi Biotec), StemPro (ThermoFisher Scientific) and TheraPEAK (LonzaThis preliminary data suggested that StemMACS had superior cell growth compared to all of the other medias across all time-points despite equal loading of cells, consequently suggesting that StemMACS is the most suitable media for xeno free expansion of ASCs. Additionally, extracellular vesicles were not collected or examined in this experiment.

2.2 Cryopreserved method

Five patient samples were extracted as described in section 2.1 and a portion of each of these samples was cryopreserved in dimethyl sulfoxide as it is the most common cryopreservation medium used to store ASCs [316]. These cryopreserved ASCs were revived in tissue culture and expanded to passage 5 for comparison against fresh patient samples. The only modification of the tissue culture protocol was that these were grown in T75s with a comparative cell density, and the entirety of the cell suspension was stored for whole cell lysate analysis. Membrane bound fraction and extracellular vesicles were not collected nor analysed. Media for future multiplex immunoassay analysis were harvested but were not analysed due to funding restrictions within this project.

3. Results

3.1 Traditional and xeno free media

ASCs were isolated and expanded to passage 5 using traditional or xeno free media conditions. Proteomic analysis of the whole cell lysate and membrane bound fraction was conducted using a Q Exactive Plus Mass Spectrometry system. This resulted in the detection of 2645 proteins which were conserved across all patients. The numbers of proteins identified from the whole cell lysate and membrane bound fraction of cells maintained in traditional media and xeno free media are listed before and after filtering for proteins common to all patients in Table 1.

Table 1: Proteins detected from the ASCs of 6 patients.

	Traditional media whole cell lysate	Traditional media membrane bound	Xeno free whole cell lysate	Xeno free membrane bound
Average per patient	4634	1860	4523	2772
Conserved in all patients	1896	700	1800	881

Table 1 demonstrates that the proteome of the whole cell lysate across both culturing methods yielded a similar average number of proteins per sample, however the xeno free membrane bound fraction yielded a higher average number of proteins per patient than the traditional media membrane bound fraction. In a similar manner to the data presented in chapter 2, the number of proteins that are conserved across all patients is significantly reduced, with the traditional media whole cell lysate decreasing by 59.08%, traditional media membrane bound decreasing by 62.36%, xeno free whole cell lysate decreasing by 60.20% and xeno free membrane bound fraction decreasing by 68.21%.

Proteins that were conserved across all patients are the most accurate representation of ASC proteins as this removes the variation observed between patients. The proteins that are conserved across all 6 patient samples will be focussed on as they most confidently represent ASCs. As mentioned in chapter 2, it is likely that numerous proteins are present in a majority of patient samples but fall below the limit of reliable detection in a single patient, removing them from further analysis.

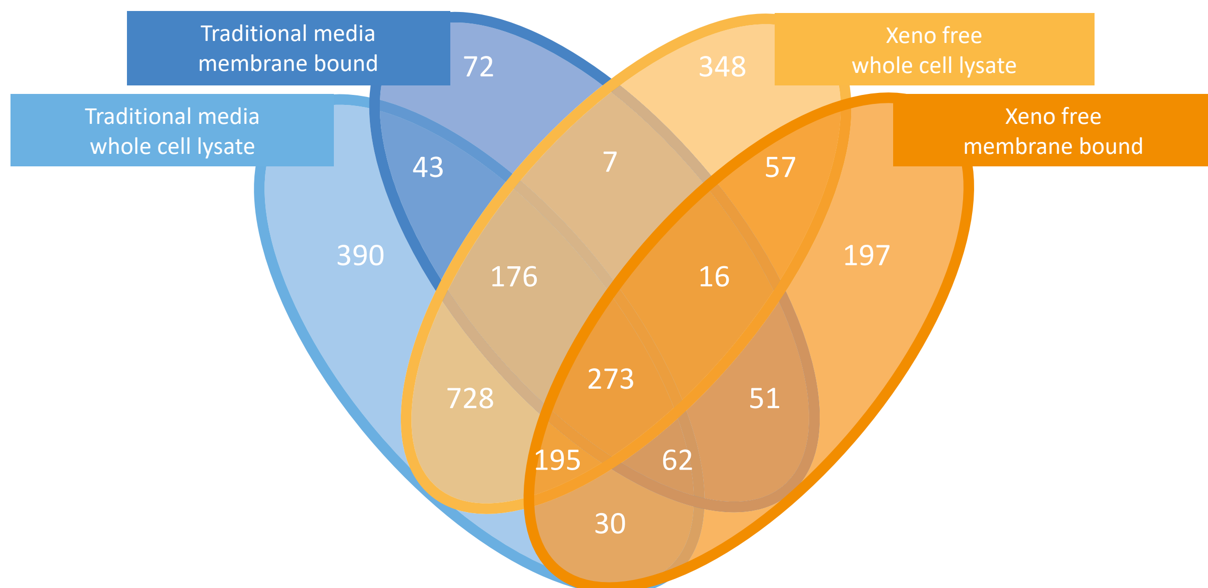


Figure 1: Venn diagram of ASC whole cell lysate and membrane bound fraction from both traditional and xeno free media.

Figure 1 demonstrates that the traditional media whole cell lysate contains the largest number of detected proteins. The traditional media membrane bound fraction contained 146 proteins that were not detected in the traditional media whole cell lysate. The xeno free membrane bound fraction contained 340 proteins that were not detected in the xeno free whole cell lysate. 505 proteins were unique to the traditional media ASCs while 602 proteins were unique to the xeno free ASCs. 1538 proteins were found in both xeno free and traditional media ASCs.

The proteins from these 4 different fractions were further analysed by the PANTHER Classification System and the detected proteins categorised by molecular function (Figure 2), cellular compartment (Figure 3), biological process (Figure 4), protein class (Figure 5) and protein pathway (Figure 6).

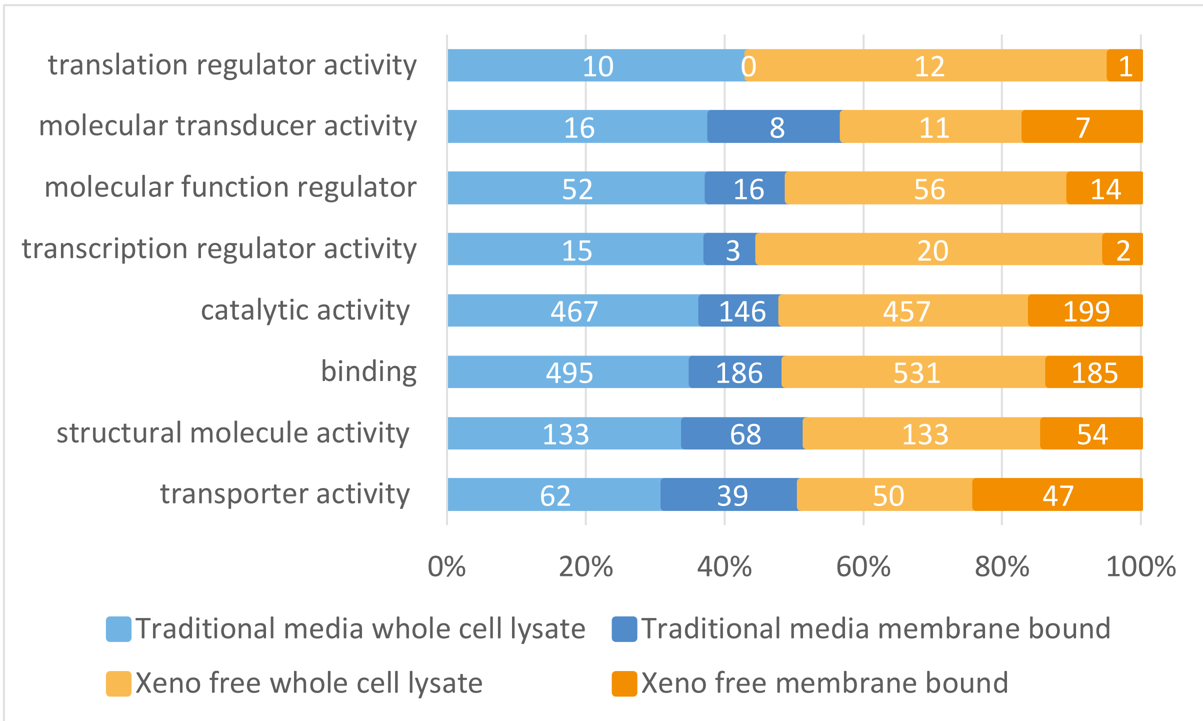


Figure 2: ASC proteins from the traditional media whole cell lysate, traditional media membrane bound fraction, xeno free whole cell lysate and xeno free membrane bound fraction, sorted by molecular function.

The PANTHER Classification System detected proteins from 8 different categories of molecular functions with the majority of detected proteins in the categories of binding and catalytic activity.

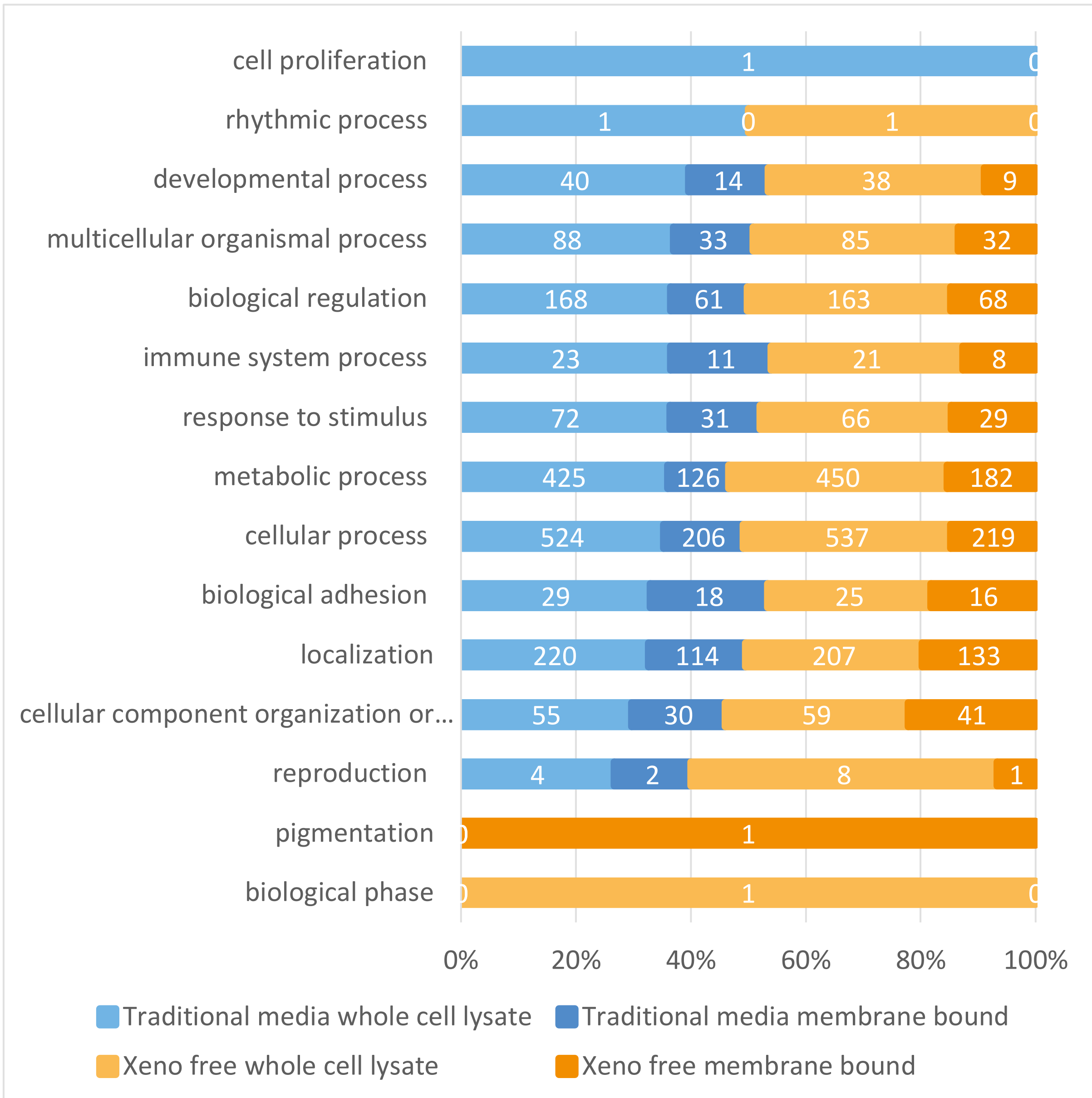


Figure 3: ASC proteins from the traditional media whole cell lysate, traditional media membrane bound fraction, xeno free whole cell lysate and xeno free membrane bound fraction, sorted by biological process.

The PANTHER Classification System detected proteins from 15 different categories of biological processes with the majority of detected proteins in the category of cellular process.

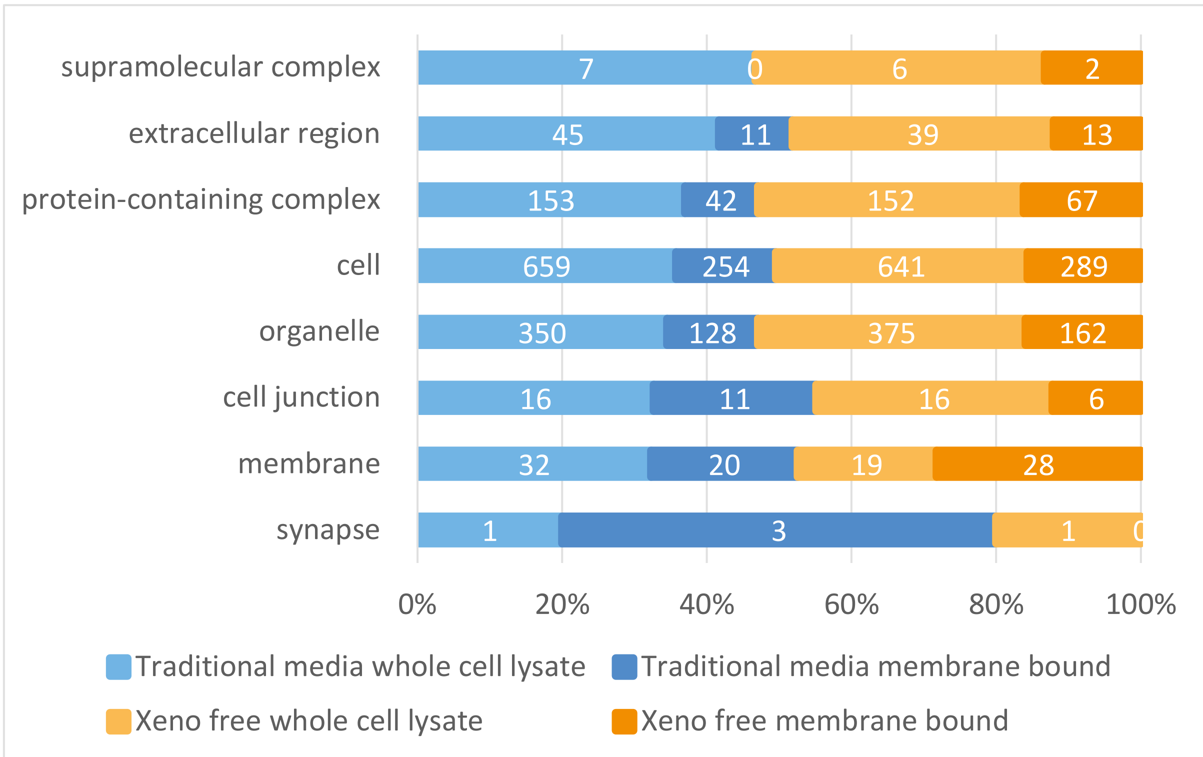


Figure 4: ASC proteins from the traditional media whole cell lysate, traditional media membrane bound fraction, xeno free whole cell lysate and xeno free membrane bound fraction, sorted by cellular component.

The PANTHER Classification System detected proteins from 8 different categories of cellular compartments with the majority of detected proteins the category of compartment cell.

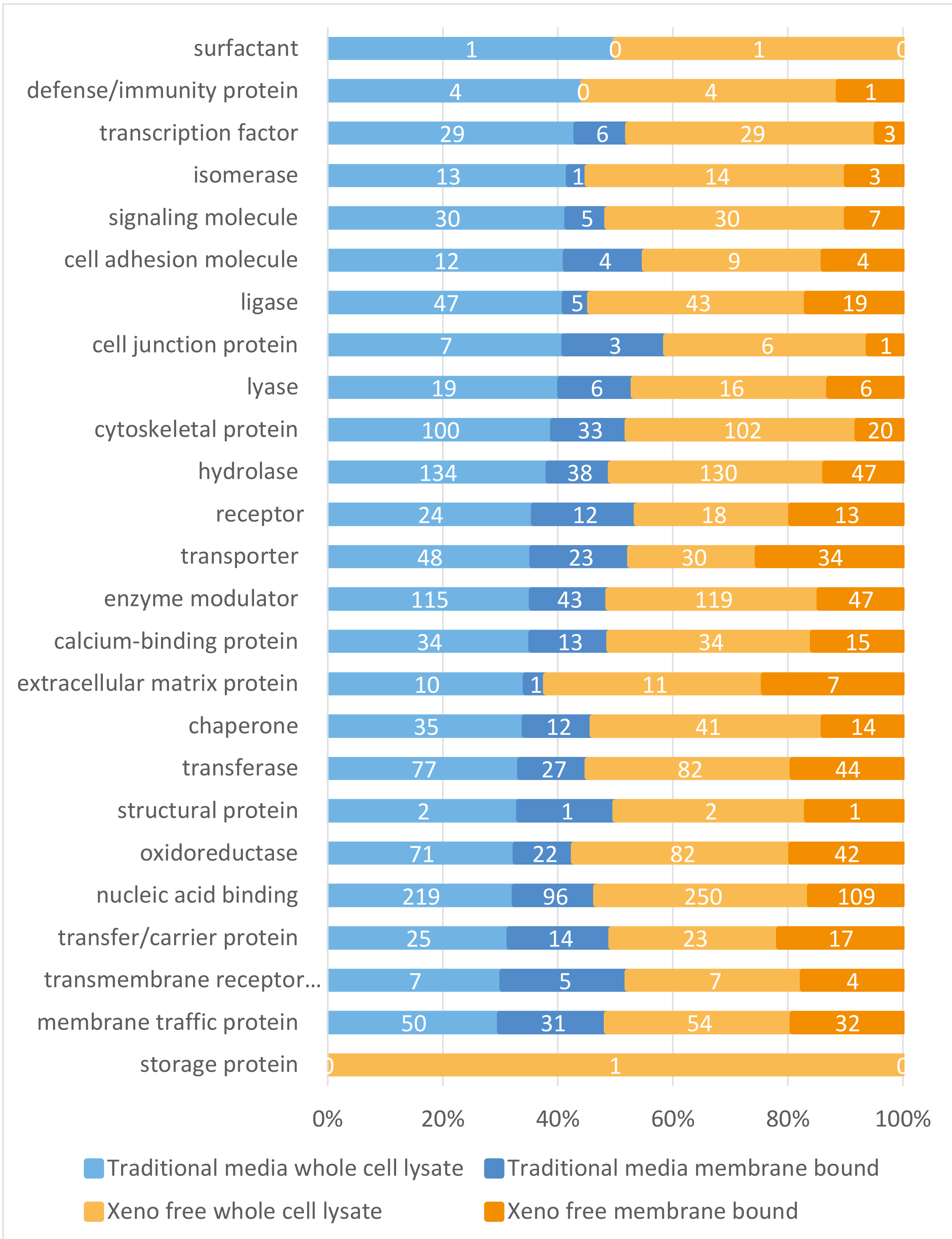
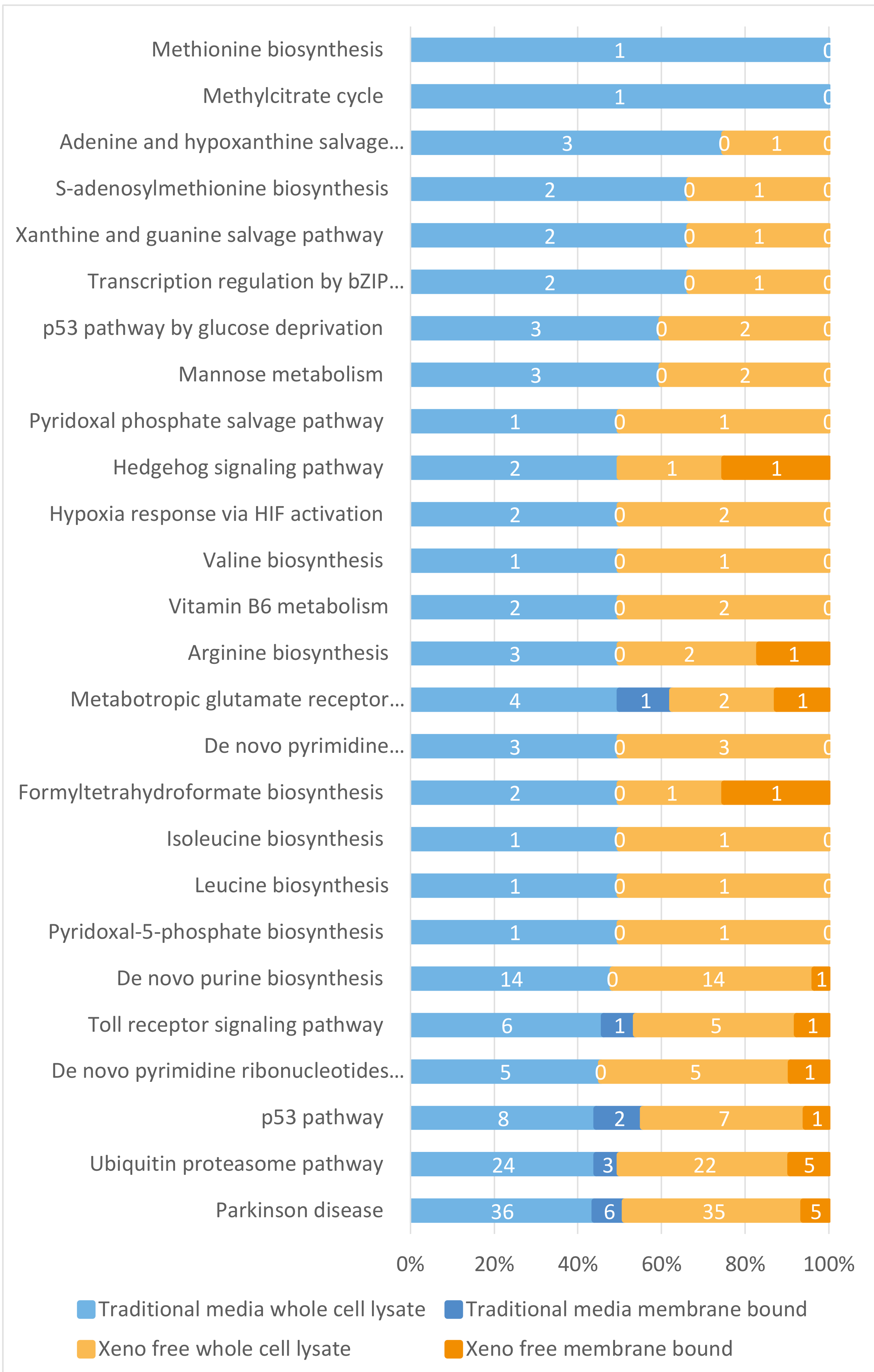
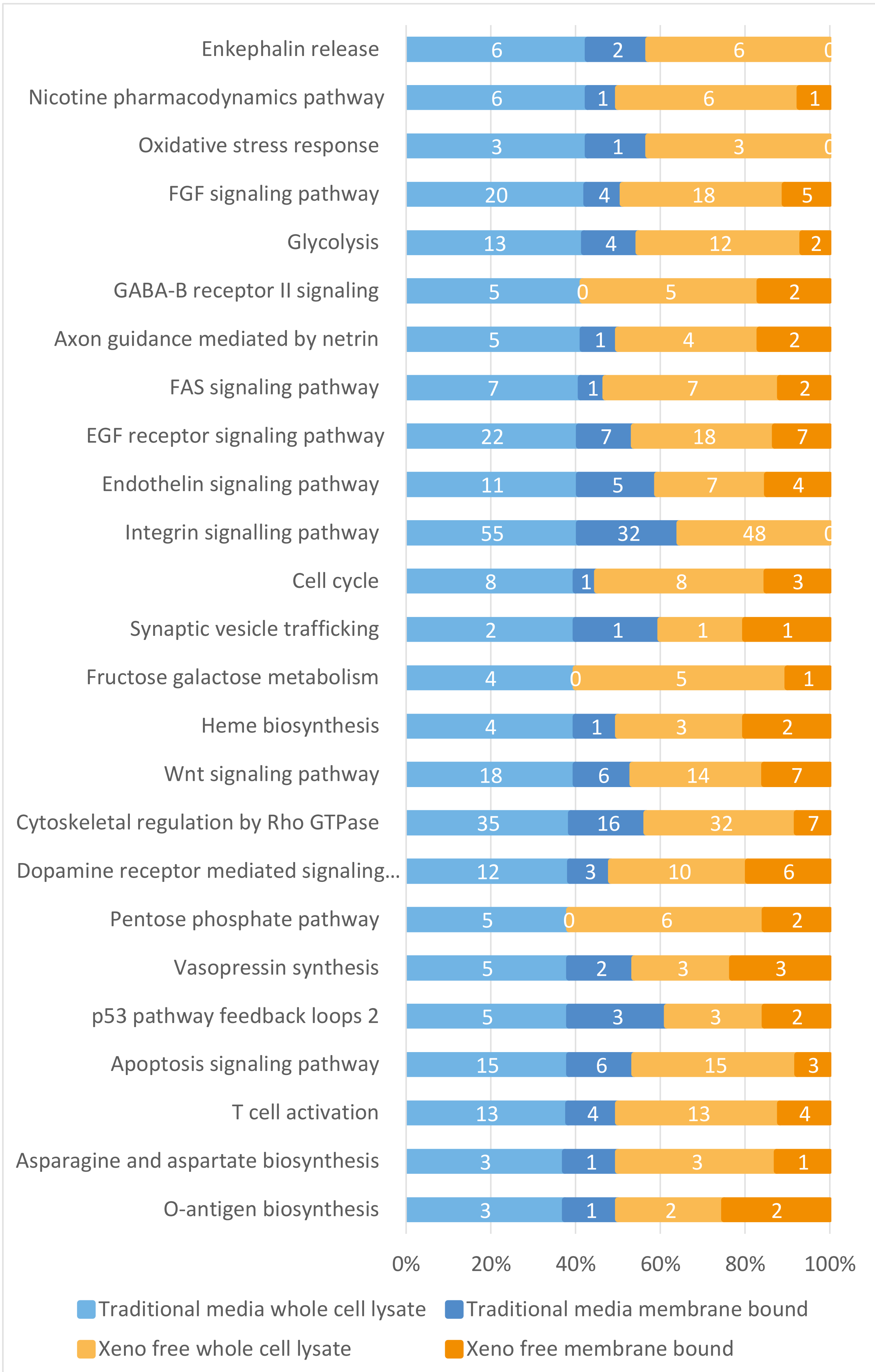


Figure 5: ASC proteins from the traditional media whole cell lysate, traditional media membrane bound fraction, xeno free whole cell lysate and xeno free membrane bound fraction, sorted by protein class.

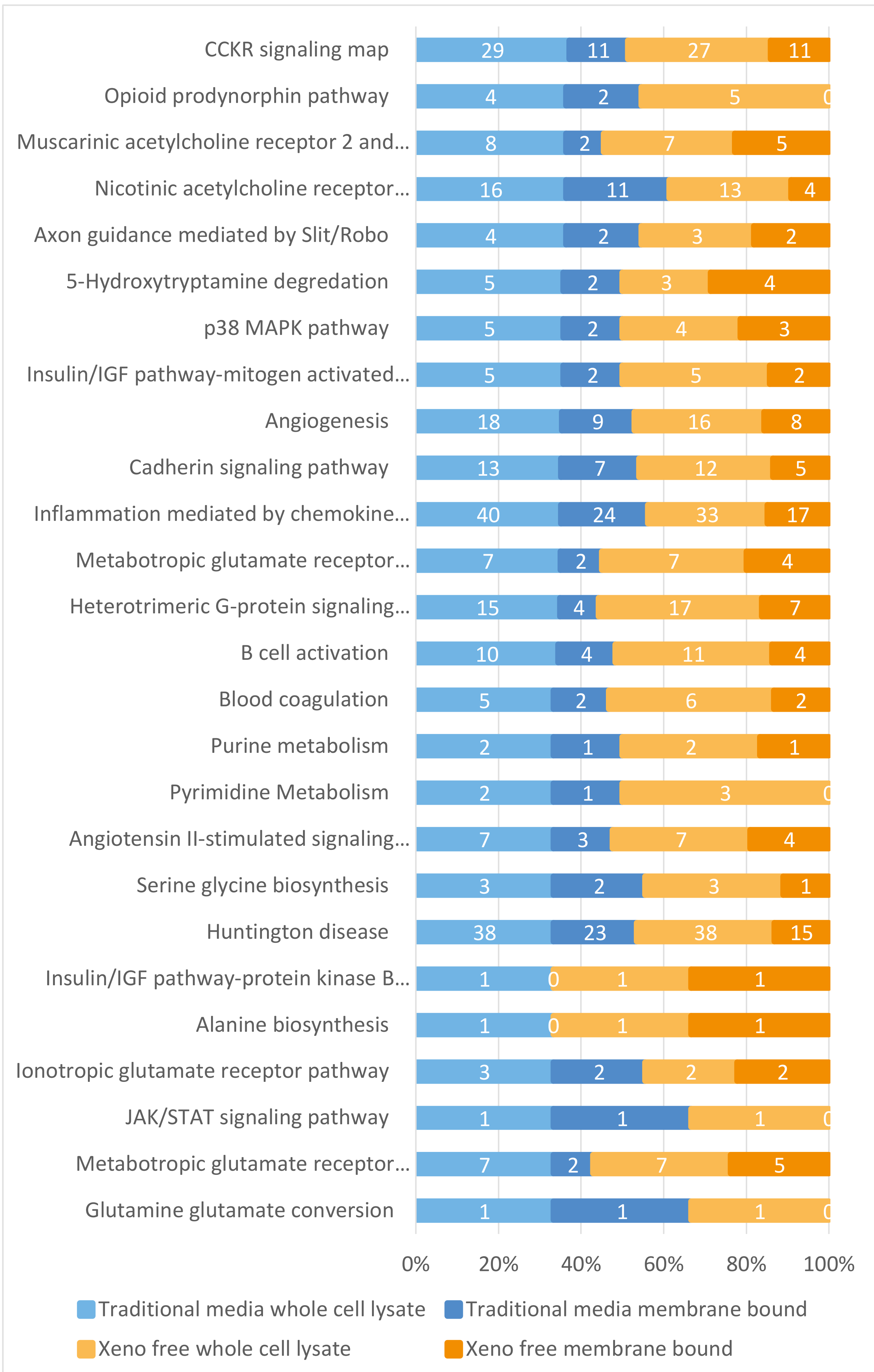
The PANTHER Classification System detected proteins from 25 different categories of protein classes with the majority of detected proteins in the category of nucleic acid.



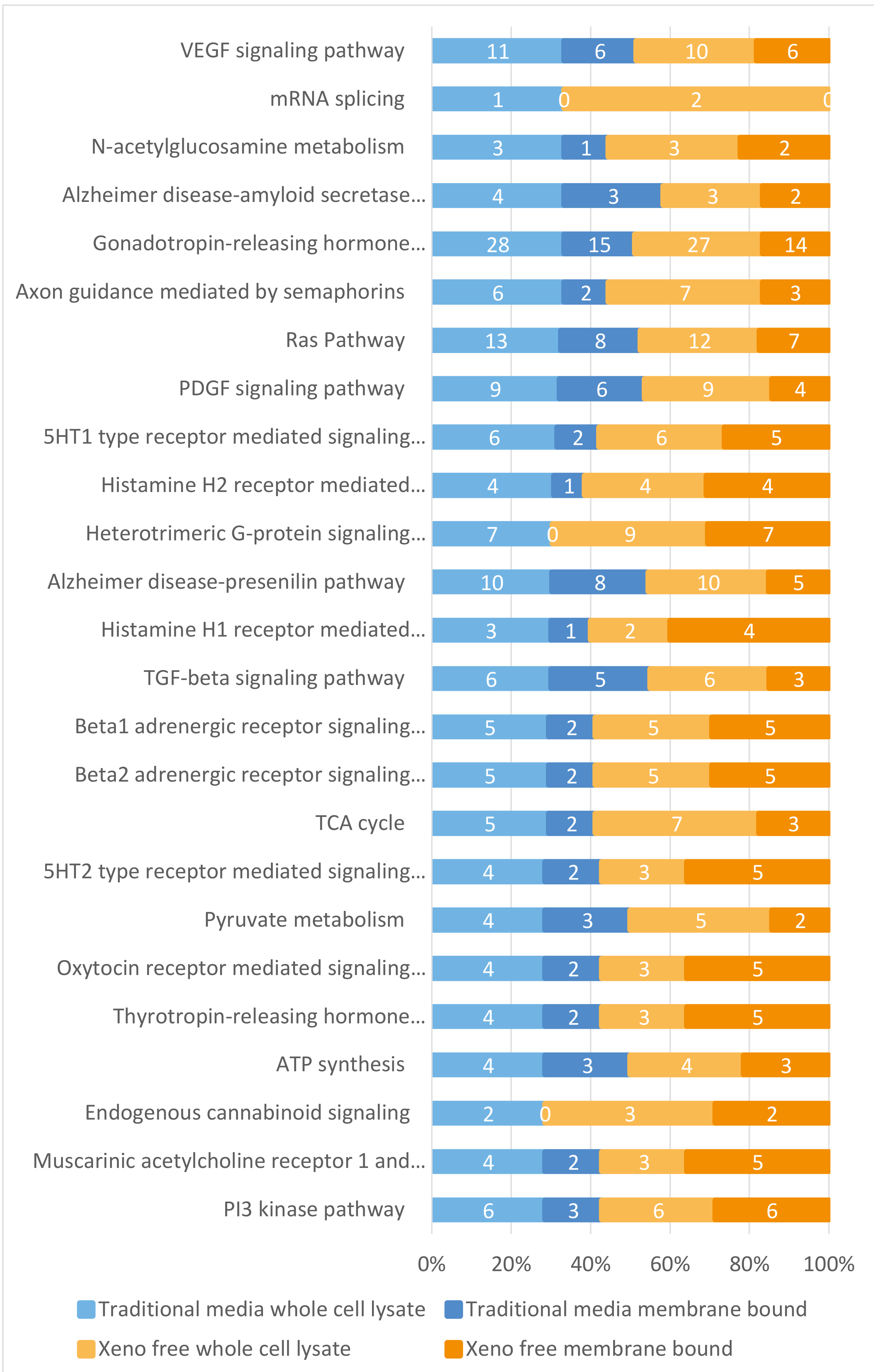
Methionine biosynthesis
Methylcitrate cycle
Adenine and hypoxanthine salvage...
S-adenosylmethionine biosynthesis
Xanthine and guanine salvage pathway
Transcription regulation by bZIP...
p53 pathway by glucose deprivation
Mannose metabolism
Pyridoxal phosphate salvage pathway
Hedgehog signaling pathway
Hypoxia response via HIF activation
Valine biosynthesis
Vitamin B6 metabolism
Arginine biosynthesis
Metabotropic glutamate receptor...
De novo pyrimidine...
Formyltetrahydroformate biosynthesis
Isoleucine biosynthesis
Leucine biosynthesis
Pyridoxal-5-phosphate biosynthesis
De novo purine biosynthesis
Toll receptor signaling pathway
De novo pyrimidine ribonucleotides...
p53 pathway
Ubiquitin proteasome pathway
Parkinson disease
0%
20%
40%
60%
80%
100%
Traditional media whole cell lysate
Traditional media membrane bound
Xeno free whole cell lysate
Xeno free membrane bound



Enkephalin release
Nicotine pharmacodynamics pathway
Oxidative stress response
FGF signaling pathway
Glycolysis
GABA-B receptor II signaling
Axon guidance mediated by netrin
FAS signaling pathway
EGF receptor signaling pathway
Endothelin signaling pathway
Integrin signalling pathway
Cell cycle
Synaptic vesicle trafficking
Fructose galactose metabolism
Heme biosynthesis
Wnt signaling pathway
Cytoskeletal regulation by Rho GTPase
Dopamine receptor mediated signaling...
Pentose phosphate pathway
Vasopressin synthesis
p53 pathway feedback loops 2
Apoptosis signaling pathway
T cell activation
Asparagine and aspartate biosynthesis
O-antigen biosynthesis
0%
20%
40%
60%
80%
100%
Traditional media whole cell lysate
Traditional media membrane bound
Xeno free whole cell lysate
Xeno free membrane bound



CCKR signaling map
Opioid prodynorphin pathway
Muscarinic acetylcholine receptor 2 and...
Nicotinic acetylcholine receptor...
Axon guidance mediated by Slit/Robo
5-Hydroxytryptamine degredation
p38 MAPK pathway
Insulin/IGF pathway-mitogen activated...
Angiogenesis
Cadherin signaling pathway
Inflammation mediated by chemokine...
Metabotropic glutamate receptor...
Heterotrimeric G-protein signaling...
B cell activation
Blood coagulation
Purine metabolism
Pyrimidine Metabolism
Angiotensin II-stimulated signaling...
Serine glycine biosynthesis
Huntington disease
Insulin/IGF pathway-protein kinase B...
Alanine biosynthesis
Ionotropic glutamate receptor pathway
JAK/STAT signaling pathway
Metabotropic glutamate receptor...
Glutamine glutamate conversion
0%
20%
40%
60%
80%
100%
Traditional media whole cell lysate
Traditional media membrane bound
Xeno free whole cell lysate
Xeno free membrane bound



VEGF signaling pathway
11
6
10
6
mRNA splicing
1
0
2
0
N-acetylglucosamine metabolism
3
1
3
2
Alzheimer disease-amyloid secretase...
4
3
3
2
Gonadotropin-releasing hormone...
28
15
27
14
Axon guidance mediated by semaphorins
6
2
7
3
Ras Pathway
13
8
12
7
PDGF signaling pathway
9
6
9
4
5HT1 type receptor mediated signaling...
6
2
6
5
Histamine H2 receptor mediated...
4
1
4
4
Heterotrimeric G-protein signaling...
7
0
9
7
Alzheimer disease-presenilin pathway
10
8
10
5
Histamine H1 receptor mediated...
3
1
2
4
TGF-beta signaling pathway
6
5
6
3
Beta1 adrenergic receptor signaling...
5
2
5
5
Beta2 adrenergic receptor signaling...
5
2
5
5
TCA cycle
5
2
7
3
5HT2 type receptor mediated signaling...
4
2
3
5
Pyruvate metabolism
4
3
5
2
Oxytocin receptor mediated signaling...
4
2
3
5
Thyrotropin-releasing hormone...
4
2
3
5
ATP synthesis
4
3
4
3
Endogenous cannabinoid signaling
2
0
3
2
Muscarinic acetylcholine receptor 1 and...
4
2
3
5
PI3 kinase pathway
6
3
6
6
0%
20%
40%
60%
80%
100%
Traditional media whole cell lysate
Traditional media membrane bound
Xeno free whole cell lysate
Xeno free membrane bound

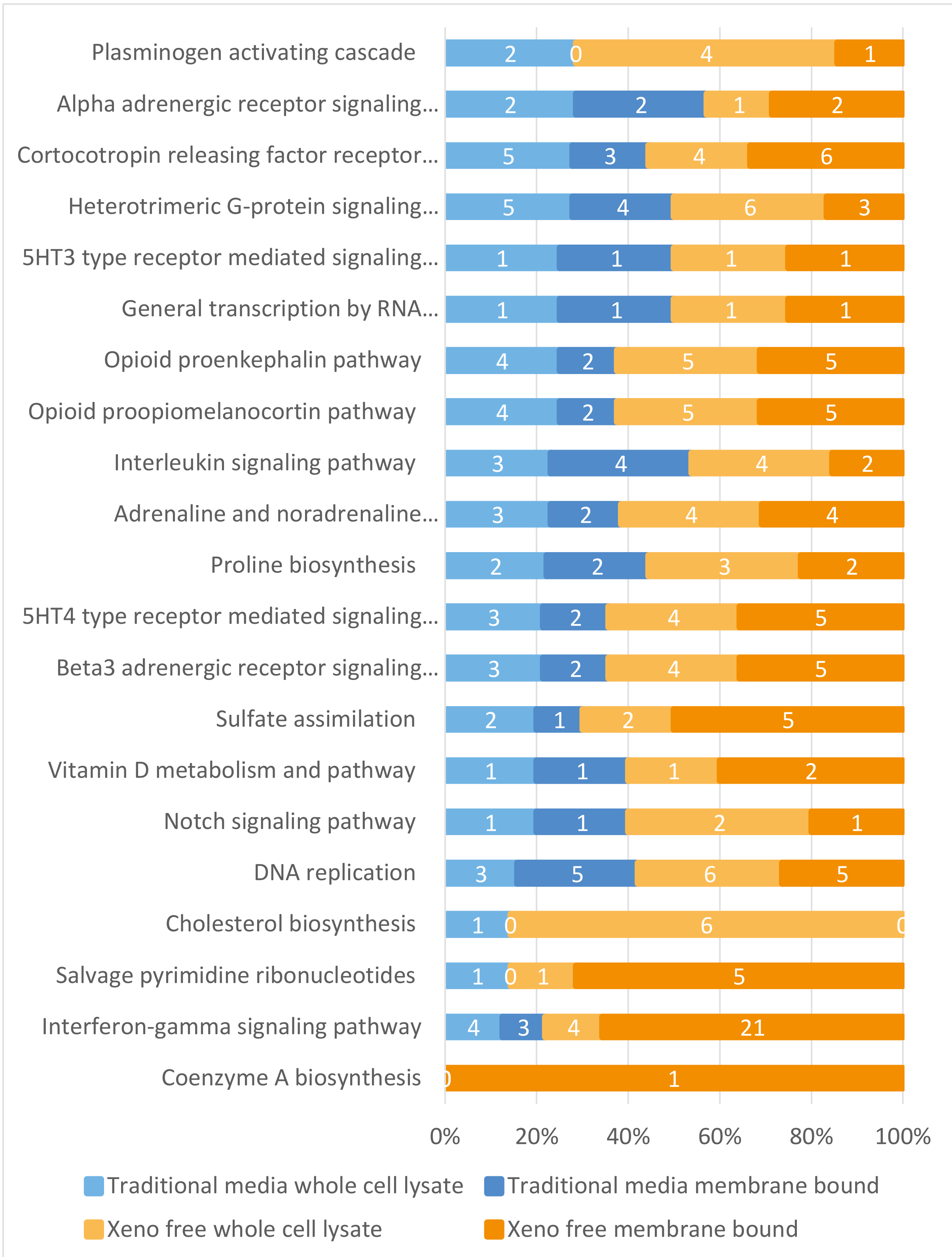


Figure 6: ASC proteins from the traditional media whole cell lysate, traditional media membrane bound fraction, xeno free whole cell lysate and xeno free membrane bound fraction, sorted by pathways.

The PANTHER Classification System detected proteins from 122 different categories of protein pathways, with the majority of detected proteins in the category of integrin signalling pathway. Interestingly, the xeno free membrane bound fraction has 21 proteins in the IFN-γ signalling pathway.

Proteins that were present in a single fraction and not in another were analysed using STRING. This allowed for identification of network interactions and pathways of interest to shed light on what interactions were creating a unique proteomic phenotype. STRING is a useful tool to visualise the proteins of interest which are shown as nodes, and their interactions which are shown as edges. The thicker the edge the more evidence there is for the interaction, and nodes are highlighted based on the most commonly occurring functional enrichments.

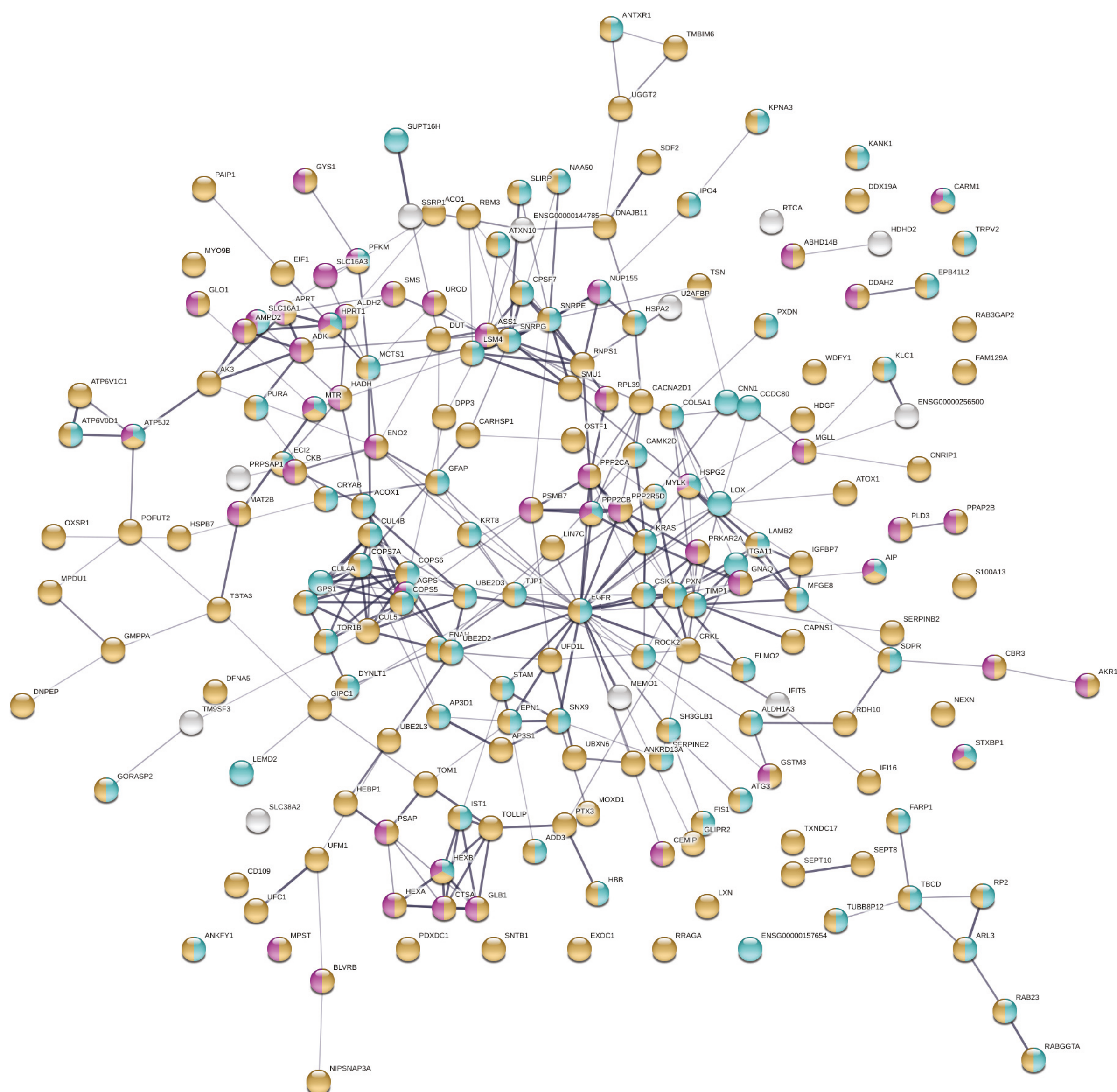


Figure 7: STRING network of detected proteins unique to traditional media whole cell lysate.

STRING detected 204 nodes and 334 edges with an average local clustering coefficient of 0.432. The biological process category cellular component organisation is shown in blue (90 with a false discovery rate of 2.86e-05), the cellular component category cytoplasm is shown in orange (182 with a false discovery rate of 1.73e-20) and the reactome pathway category metabolism is shown in pink (48 with a false discovery rate of 5.09e-05).

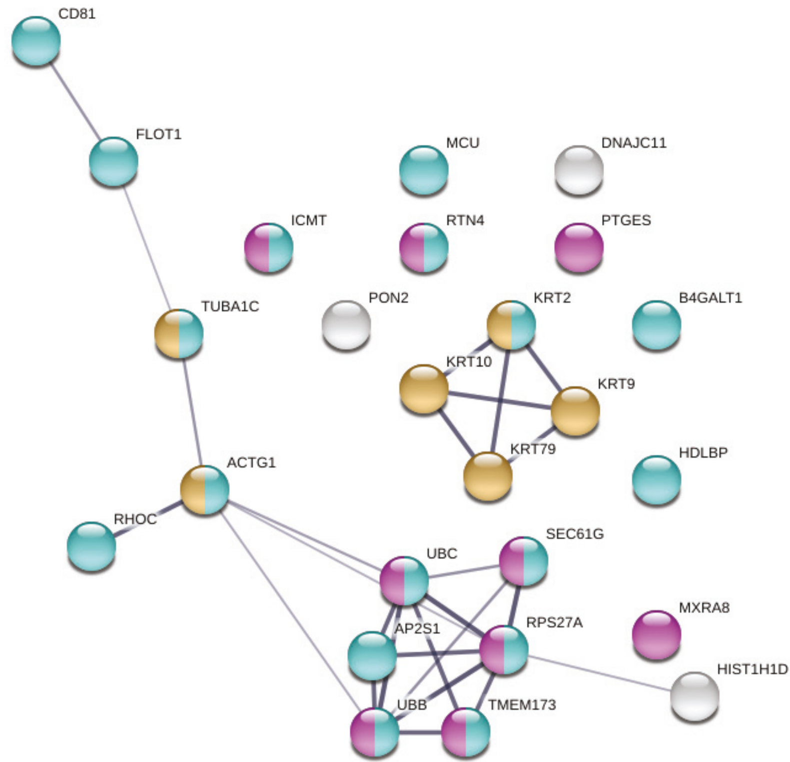


Figure 8: STRING network of detected proteins unique to traditional media membrane bound fraction.

STRING detected 25 nodes and 26 edges and with an average local clustering coefficient of 0.477. The biological process category localisation is shown in blue (17 with a false discovery rate of 1.4e-03), the molecular function category structural molecule activity is shown in orange (6 with a false discovery rate of 1.4e-03) and the cellular component category endoplasmic reticulum part is shown in pink (9 with a false discovery rate of 8.5e-04).

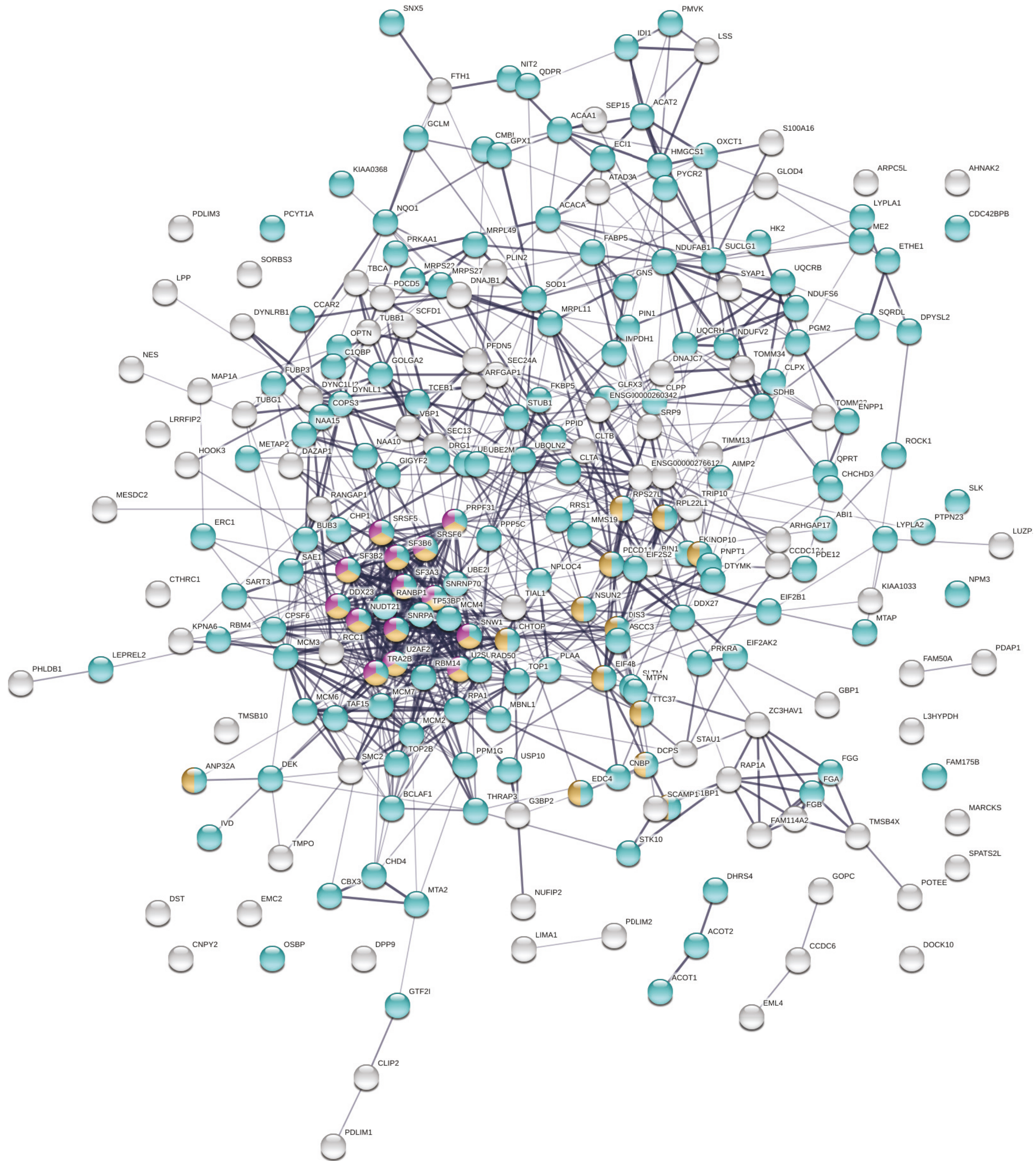


Figure 9: STRING network of detected proteins unique to xeno free whole cell lysate.

STRING detected 247 nodes and 812 edges with an average local clustering coefficient of 0.377. The biological process category cellular metabolic process is shown in blue (163 with a false discovery rate of 3.46e-08), the reactome pathway category metabolism of RNA is shown in orange (27 with a false discovery rate of 7.10e-05), and the reactome pathway category mRNA splicing – major pathway is shown in pink (14 with a false discovery rate of 7.10e-05)

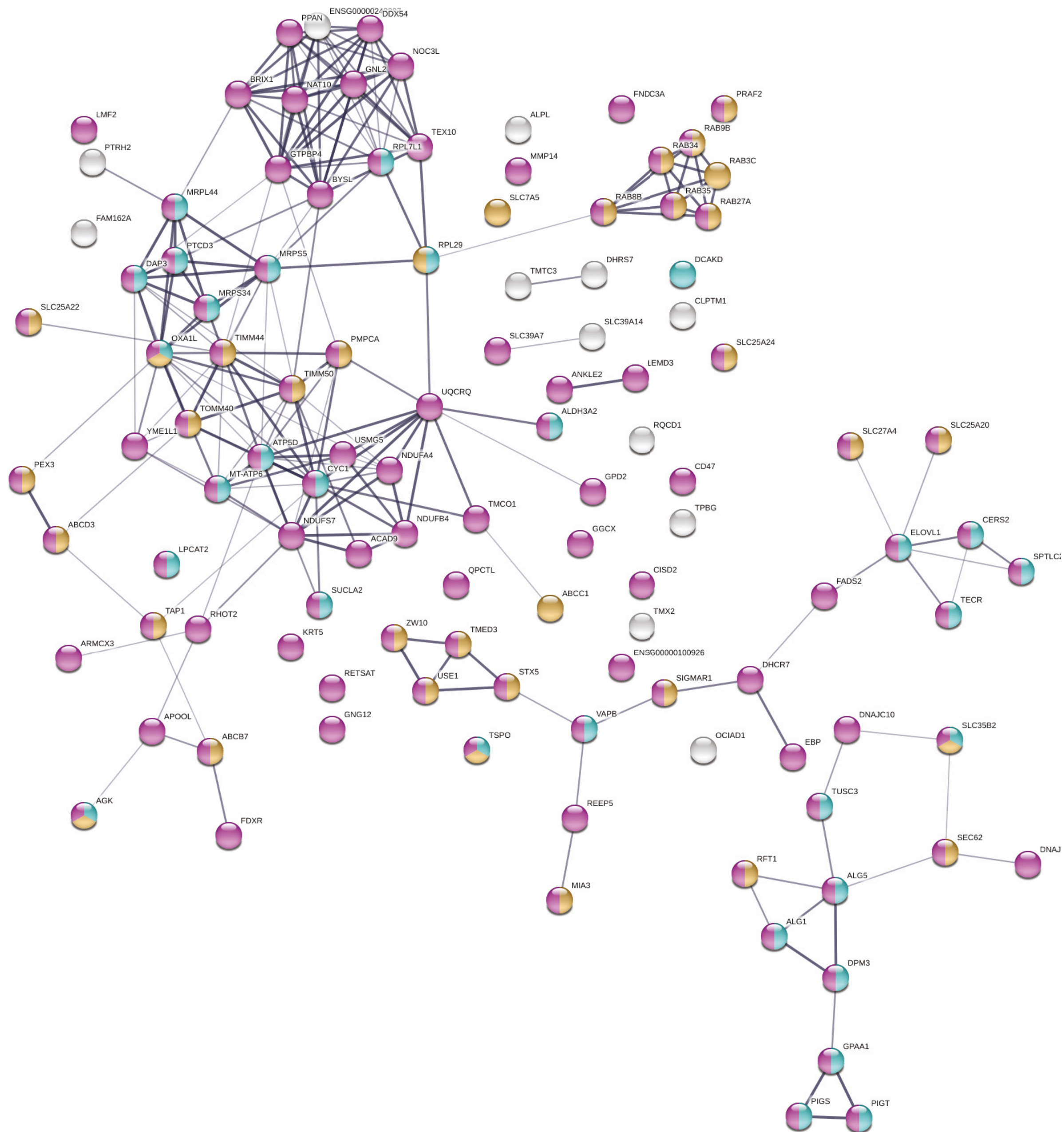


Figure 10: STRING network of detected proteins unique to xeno free membrane bound fraction.

STRING detected 113 nodes and 216 edges with an average local clustering coefficient of 0.484. The biological process category organonitrogen compound biosynthetic process is shown in blue (30 with a false discovery rate of 3.08e-07), the biological process category organic substance transport is shown in orange (34 with a false discovery rate of 2.79e-06) and the cellular component category intracellular organelle part is shown in pink (96 with a false discovery rate of 1.08e-16).

Proteins that were detected in two directly comparable fractions had their abundances compared using a clustered heatmap. Figure 11 compares the whole cell lysate of cells grown in traditional media and xeno free media. Proteins are shown on the y-axis, and patients along the x-axis. The x-axis has formed two distinct clusters, grouping the patient samples from the same media together following the unsupervised clustering. This independent clustering reinforces the reproducibility of the analysis while still demonstrating patient heterogeneity. The y-axis forms 5 clusters, and these are generated based on trends in Z scores. The Z score is a statistical measurement of variation from the mean: 0 indicates the value is the same as a mean, a positive score indicates a value above the mean, and a negative indicates a value bellow the mean. It would be expected that proteins from cells grown in the same media would have similar Z-scores, but that patient-to-patient variation would also observed. The correlation plots for the grouped technical replicate LC-MS/MS injections are shown in Supplementary Figure 1.

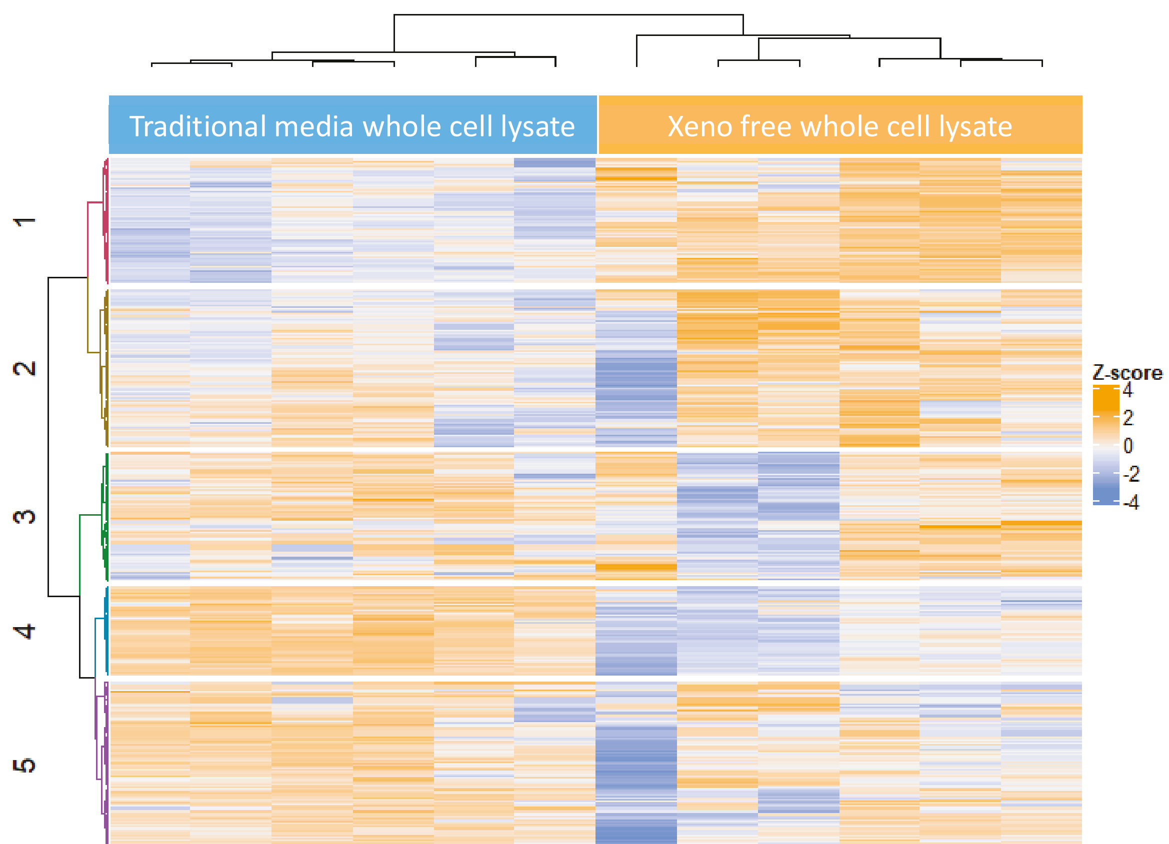


Figure 11: Clustered heatmap of proteins present in both traditional media and xeno free ASC whole cell lysate.

ASC whole cell lysates formed two different patient clusters, traditional media and xeno free. Each patient sample is represented in a column and each row is a protein, of which there are 5 protein clusters. The proteins present in Cluster 1 have predominantly negative Z scores in the traditional media and predominantly positive Z scores in xeno free media. Cluster 2 has less distinct trends but overall has more neutral Z scores in the traditional media and more positive Z scores in the xeno free media. Cluster 3 does not have particularly distinct trends, however the traditional media overall has more positive Z scores while the xeno free media has both positive and negative Z scores. Cluster 4 has predominantly positive Z scores for proteins from cells maintained in the traditional media and predominantly negative Z scores in the xeno free media. Cluster 5 shows that proteins from cells maintained in traditional media have predominantly positive Z scores while those from xeno free media have a mix of both positive and negative Z scores.

The 5 protein clusters were further investigated in their own individual STRING networks (Figures 12-16) to highlight their interactions and to highlight the most common functional enrichment categories.

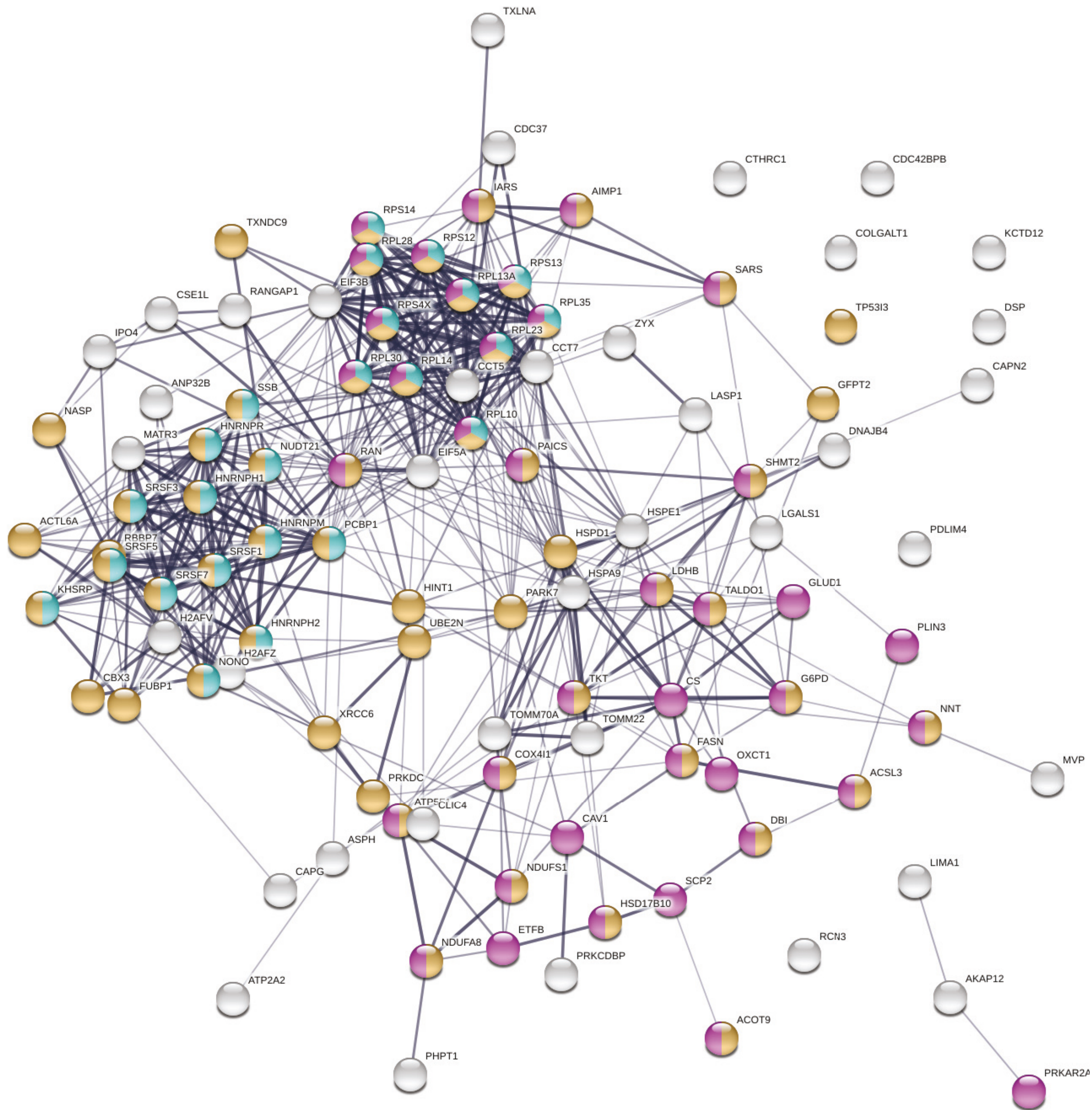


Figure 12: STRING network of proteins detected in Cluster 1 of ASC traditional media and xeno free whole cell lysate.

STRING detected 104 nodes and 521 edges with an average local clustering coefficient of 0.481. The biological process category mRNA metabolic process is shown in blue (24 with a false discovery rate of 1.73e-10), the biological process category heterocycle metabolic process is shown in orange (58 with a false discovery rate of 2.95e-09) and the reactome pathway category metabolism is shown in pink (39 with a false discovery rate of 3.22e-11). Proteins detected in this cluster are more abundant in xeno free media with lower abundance in traditional media.

Figure 13: STRING network of proteins detected in Cluster 2 of ASC traditional media and xeno free whole cell lysate.

STRING detected 118 nodes and 726 edges with an average local clustering coefficient of 0.497. The biological process category translation is shown in blue (18 with a false discovery rate of 1.09e-09). The following reactome pathways categories are highlighted; SRP-dependent cotranslational protein targeting to membrane is shown in orange (17 with a false discovery rate of 7.40e-16), nonsense mediated decay enhanced by the exon junction complex is shown in pink (15 with a false discovery rate of 8.89e-14) and regulation of expression of SLITs and ROBOs is shown in yellow (17 with a false discovery rate of 8.89e-14). Proteins detected in this cluster are more abundant in xeno free media with a comparatively lower abundance in traditional media, however this is variable between patients.

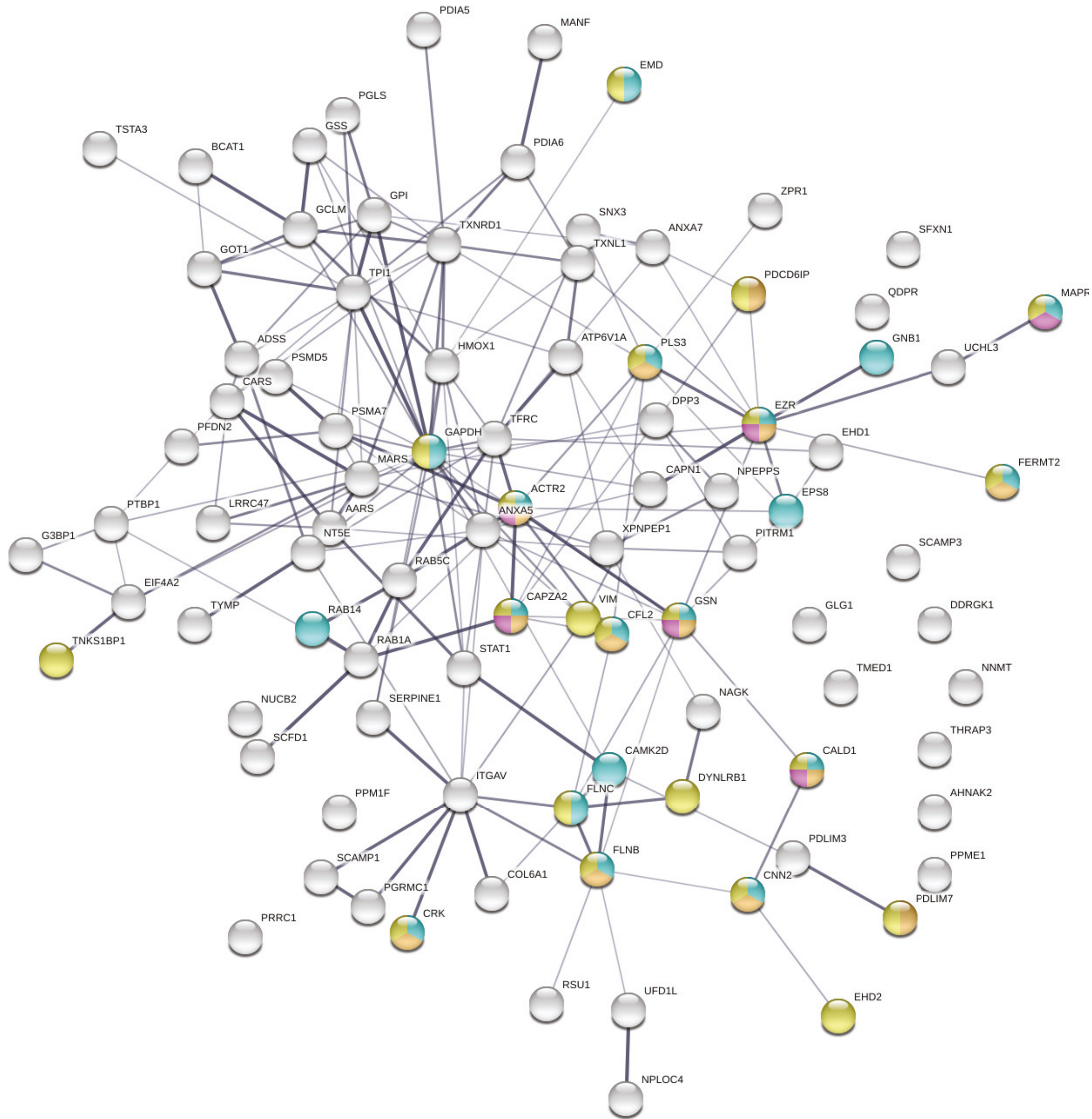


Figure 14: STRING network of proteins detected in Cluster 3 of ASC traditional media and xeno free whole cell lysate.

STRING detected 92 nodes with 177 edges and an average local clustering coefficient of 0.408. The molecular function category cytoskeletal protein binding is shown in blue (19 with a false discovery rate of 1.23e-05), the cellular component category actin cytoskeleton is shown in orange (13 with a false discovery rate of 9.37e-06), the cellular component category cortical cytoskeleton is shown in pink (6 with a false discovery rate of 9.34e-05) and the cellular component category cytoskeleton is shown in yellow (21 with a false discovery rate of 4.1e-03). Proteins detected in this cluster are mostly more abundant in traditional media with variable abundance across patients in xeno free media.

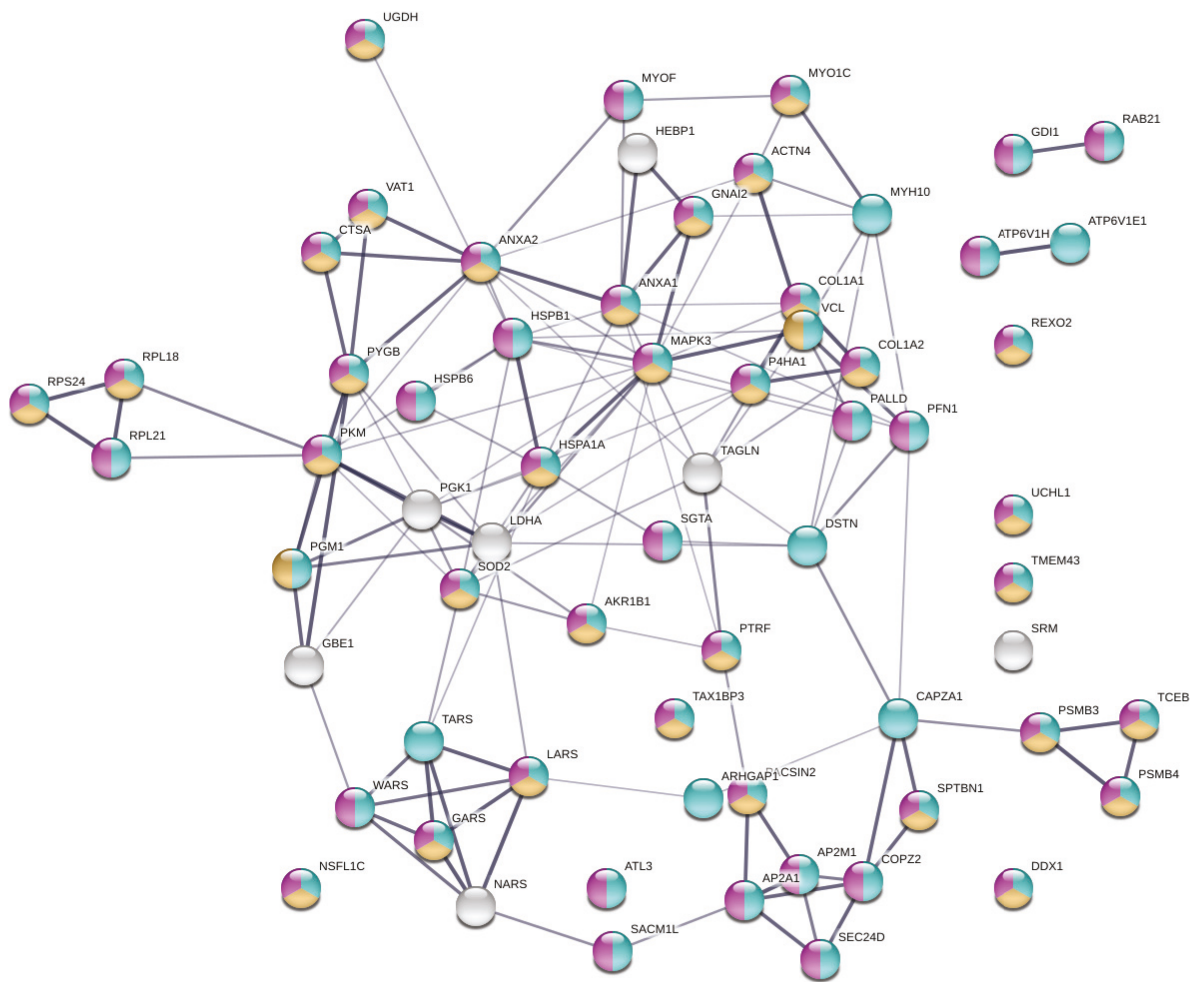


Figure 15: STRING network of proteins detected in Cluster 4 of ASC traditional media and xeno free whole cell lysate.

STRING detected 65 nodes with 134 edges and an average local clustering coefficient of 0.457. The cellular component categories are shown, intracellular organelle is shown in blue (58 with a false discovery rate of 2.41e-05), intracellular organelle lumen shown in orange (35 with a false discovery rate of 4.70e-05) and intracellular membrane bound organelle shown in pink (50 with a false discovery rate of 7.40e-04). Proteins detected in this cluster are predominantly more abundant in traditional media with lower abundance in xeno free media.

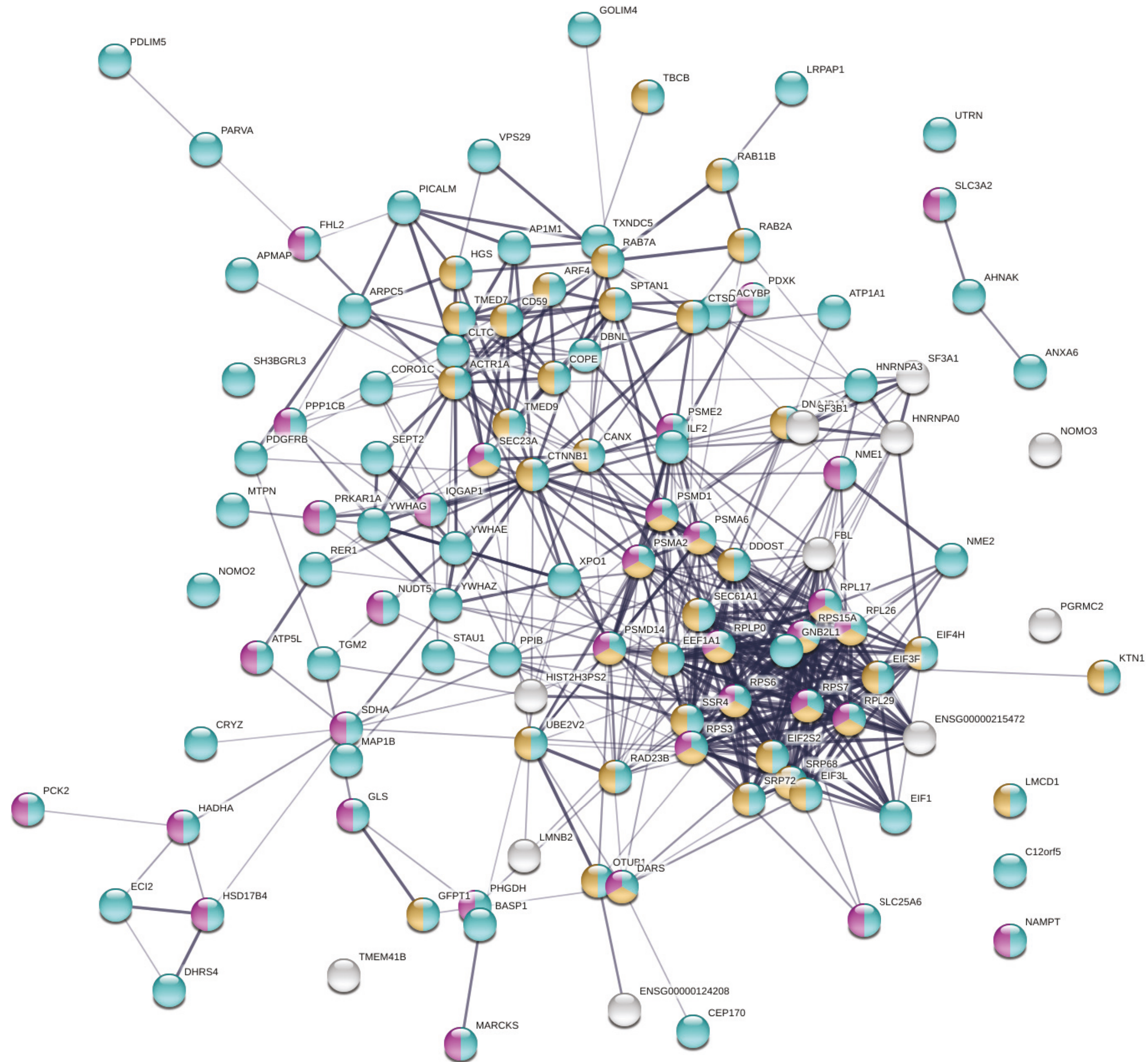


Figure 16: STRING network of proteins detected in Cluster 5 of ASC traditional media and xeno free whole cell lysate.

STRING detected 119 nodes with 514 edges and an average local clustering coefficient of 0.473. The Cellular component category cytoplasm is shown in blue (108 with a false discovery rate of 3.11e-13), the reactome pathway category metabolism of proteins is shown in orange (47 with a false discovery rate of 1.36e-14) and the reactome pathway category metabolism is shown in pink (33 with a false discovery rate of 2.07e-06). Proteins detected in this cluster are mostly more abundant in traditional media with variable abundance in xeno free media.

The heatmap for the membrane bound fraction for cells maintained in either xeno free or traditional media is not displayed, as the patient clusters did not group to reflect the media they were grown in, which was unexpected. One cluster contained 4 xeno free patients, while the second contained all the other patients from both xeno free and traditional media. This could demonstrate patient variation within the membrane bound fractions and requires further investigation. This is reflective of the large number of shared proteins between these two membranes bound fractions.

Select secreted cytokines were also measured from both traditional and xeno free ASC media. These cytokines were quantified using a multiplex immunoassay and are displayed in Figure 17.

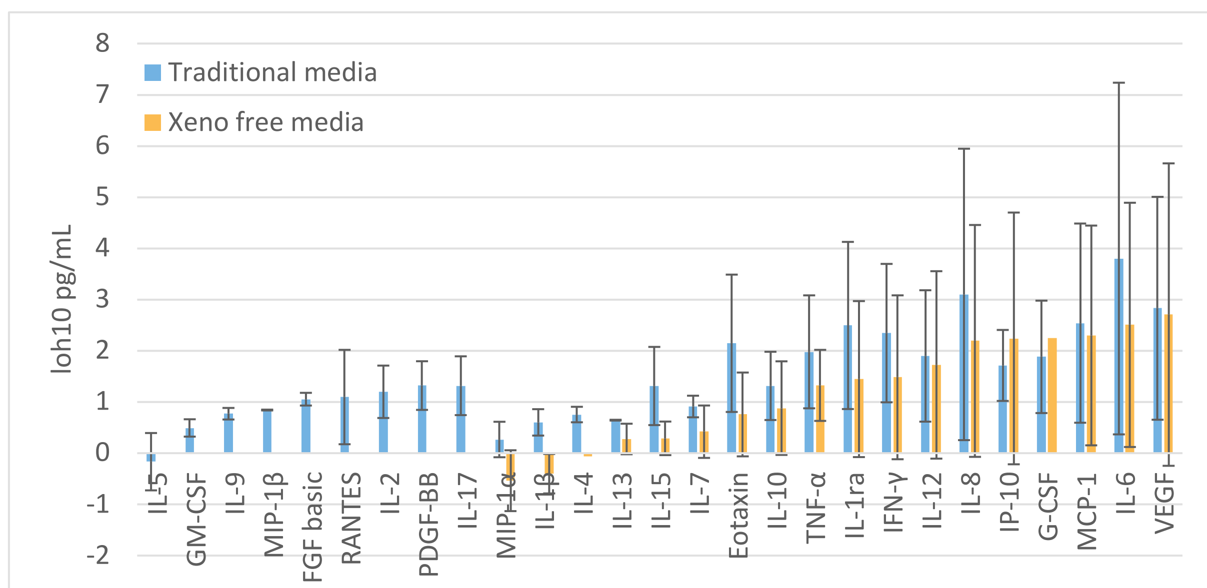


Figure 17: Traditional and xeno free ASC secreted cytokines.

27 cytokines were measured and the standard deviation of the measurements across patients is indicated by the error bars. The standard deviation is large but reflects the expected patient to patient variation. It is important to note that these samples were blanked against the neat media as the media itself contains cytokines. IL-5, GM-CSF, 1L-9, MIP-1β, FGF basic, IL-2, PDGF-BB and IL-17 were not detected in the xeno free ASC media. All other secreted cytokines with the exception of G-CSF are secreted at a lower level in xeno free ASC media.

3.2 Fresh and cryopreserved ASCs

ASCs were isolated and expanded to passage 5 and either maintained in culture (henceforth referred to as 'fresh') or alternatively they were cryopreserved then thawed and maintained and grown to passage 5. The whole cell lysate of these ASCs was analysed using a 'shotgun' LC-MS/MS approach performed with a Q Exactive Plus Mass Spectrometry system. While the fresh samples were run in technical triplicate, the cryopreserved samples were only run as a single replicate. A total of 2326 of were found to be conserved across all patients.

Table 2: Proteins detected from whole cell lysate of fresh and cryopreserved ASCs from 5 patient samples.

	Fresh whole cell lysate	Cryopreserved whole cell lysate
Average per patient	4579	4163
Conserved in all patients	1922	1488

Table 2 demonstrates that while 4579 and 4163 proteins on average were detected for fresh and cryopreserved whole cell lysate ASCs respectively, there was a reduction of 58.03% in the fresh whole cell lysate and a reduction of 64.53% in cryopreserved whole cell lysate when filtering for proteins conserved across all patients.

Proteins that were conserved across all patients are the most accurate representation of ASC proteins, as this removes the variation observed between patients. The proteins that are conserved across all 5 patient samples will be focussed on as they most confidently represent ASCs. As mentioned in chapter 2 and in the previous experiment comparing different medias, it is likely that numerous proteins are present in a majority of patient samples but fall below the limit of reliable detection in a single patient, removing them from further analysis.

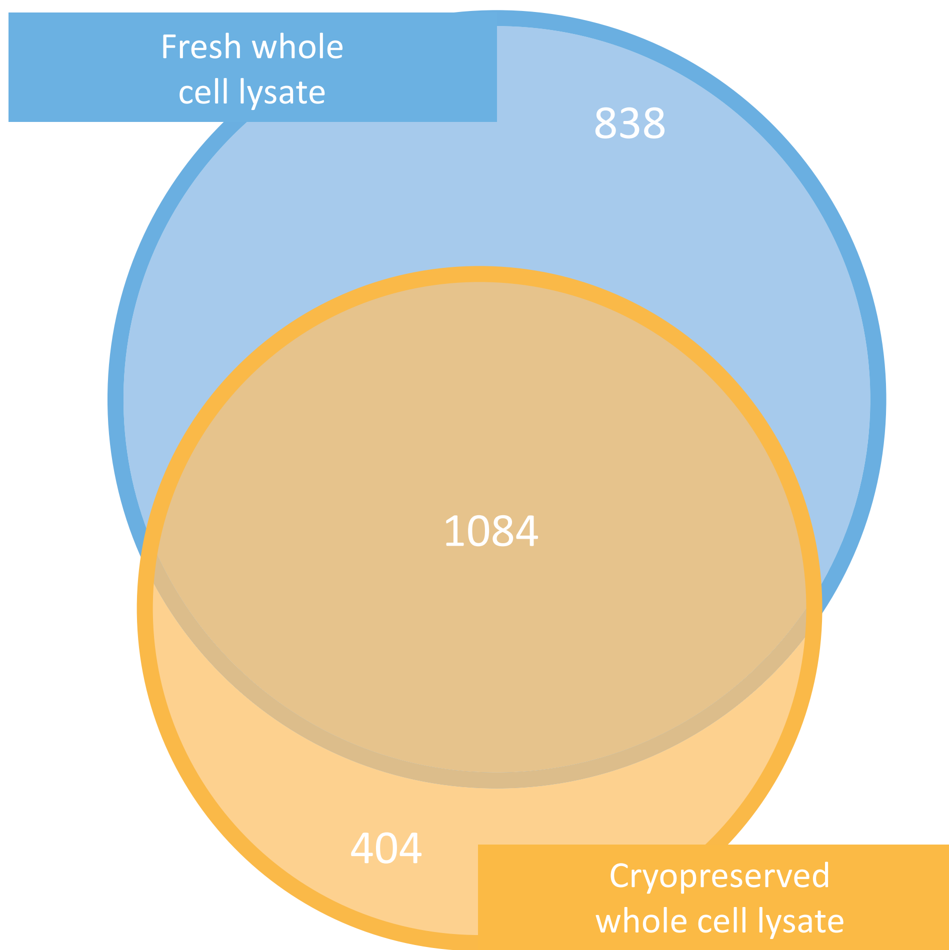


Figure 18: Proportional venn diagram of fresh and cryopreserved ASC whole cell lysate. Figure 18 shows that 1084 proteins were detected in both fresh and cryopreserved ASCs. 838 proteins were only detected in the whole cell lysate of fresh ASCs and 404 proteins were only detected in the whole cell lysate of cryopreserved ASCs.

The proteins from these 2 different fractions were further analysed by the PANTHER Classification System and the detected proteins categorised by molecular function (Figure 19), cellular compartment (Figure 20), biological process (Figure 21), protein class (Figure 22) and protein pathway (Figure 23).

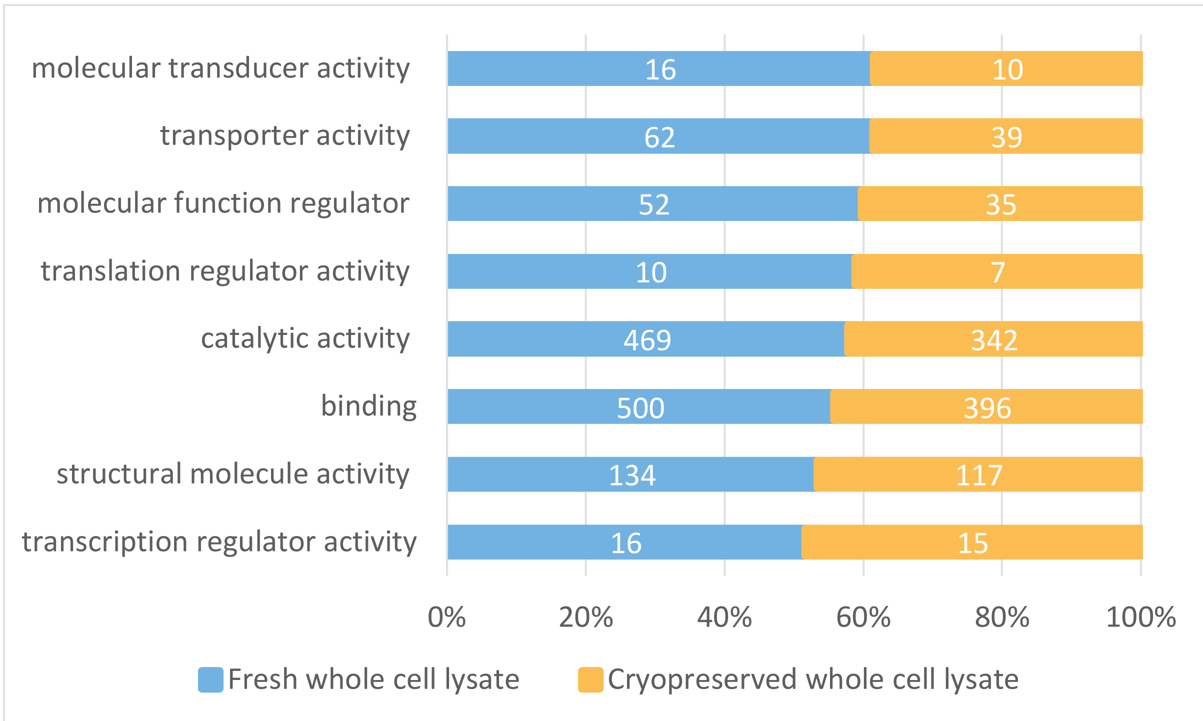


Figure 19: Fresh and cryopreserved ASC proteins sorted by molecular function.

The PANTHER Classification System detected proteins from 8 different categories of molecular functions, with the majority of detected proteins in the categories of binding and catalytic activity.

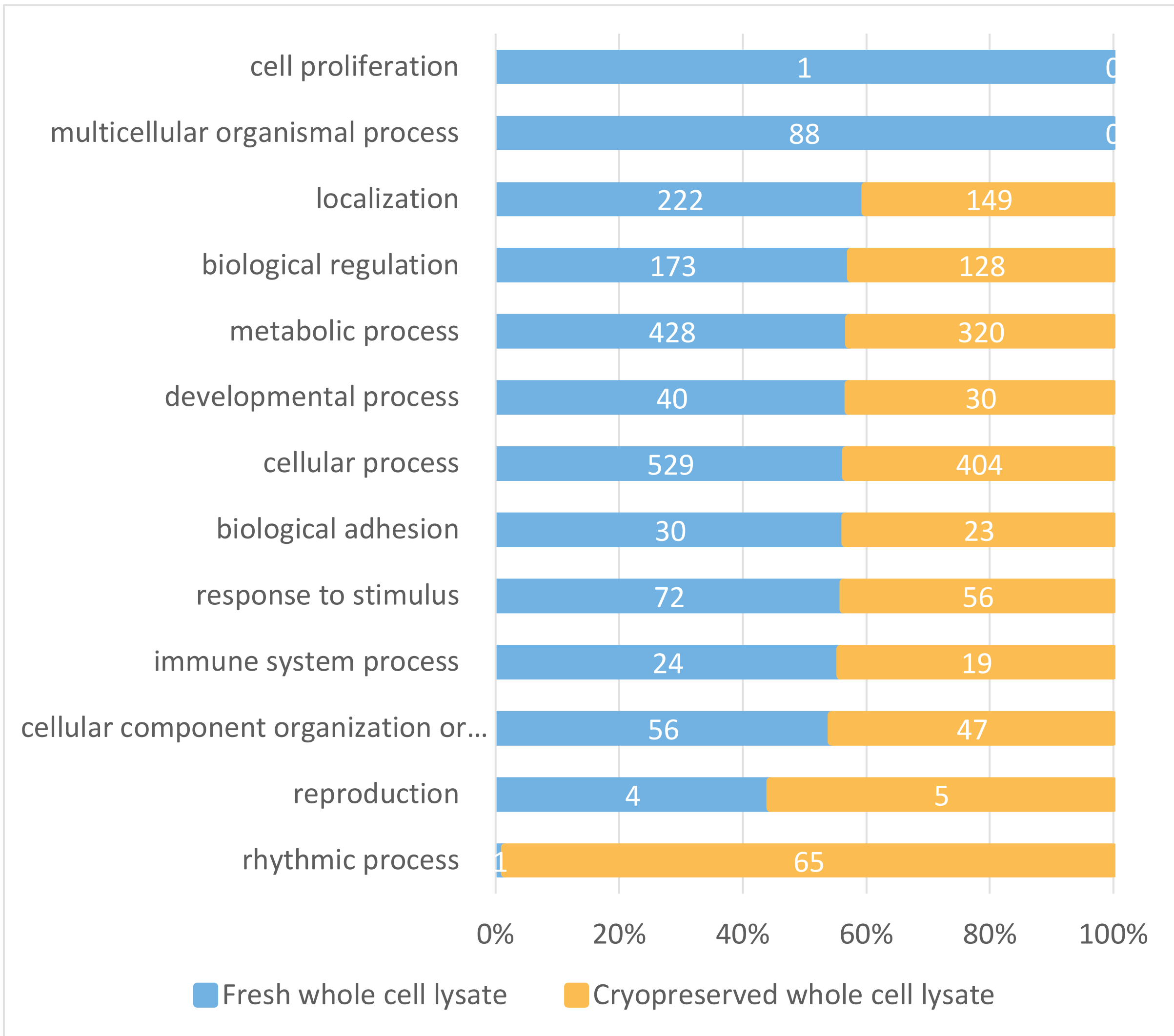


Figure 20: Fresh and cryopreserved ASC proteins sorted biological process.

The PANTHER Classification System detected proteins from 13 different categories of biological processes, with the majority of detected proteins in the category of cellular process. 65 proteins were detected in the category rhythmic purpose.

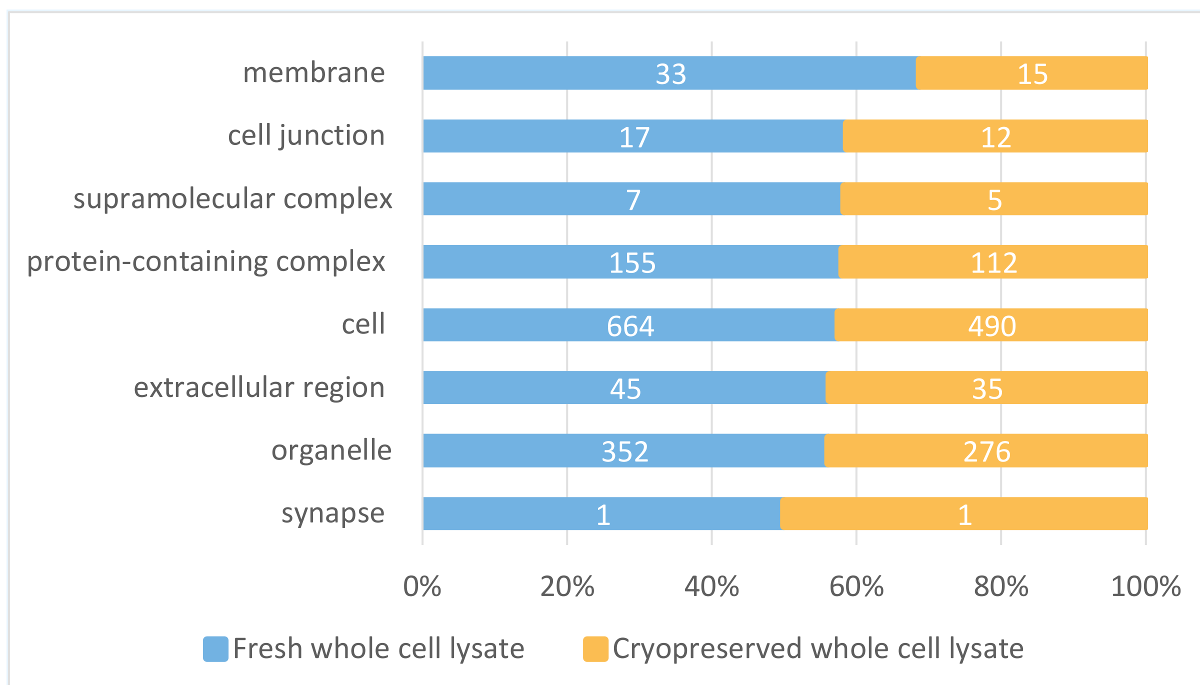


Figure 21: Fresh and cryopreserved ASC proteins sorted cellular compartment.

The PANTHER Classification System detected proteins from 8 different categories of cellular compartments, with the majority of detected proteins the category cell.

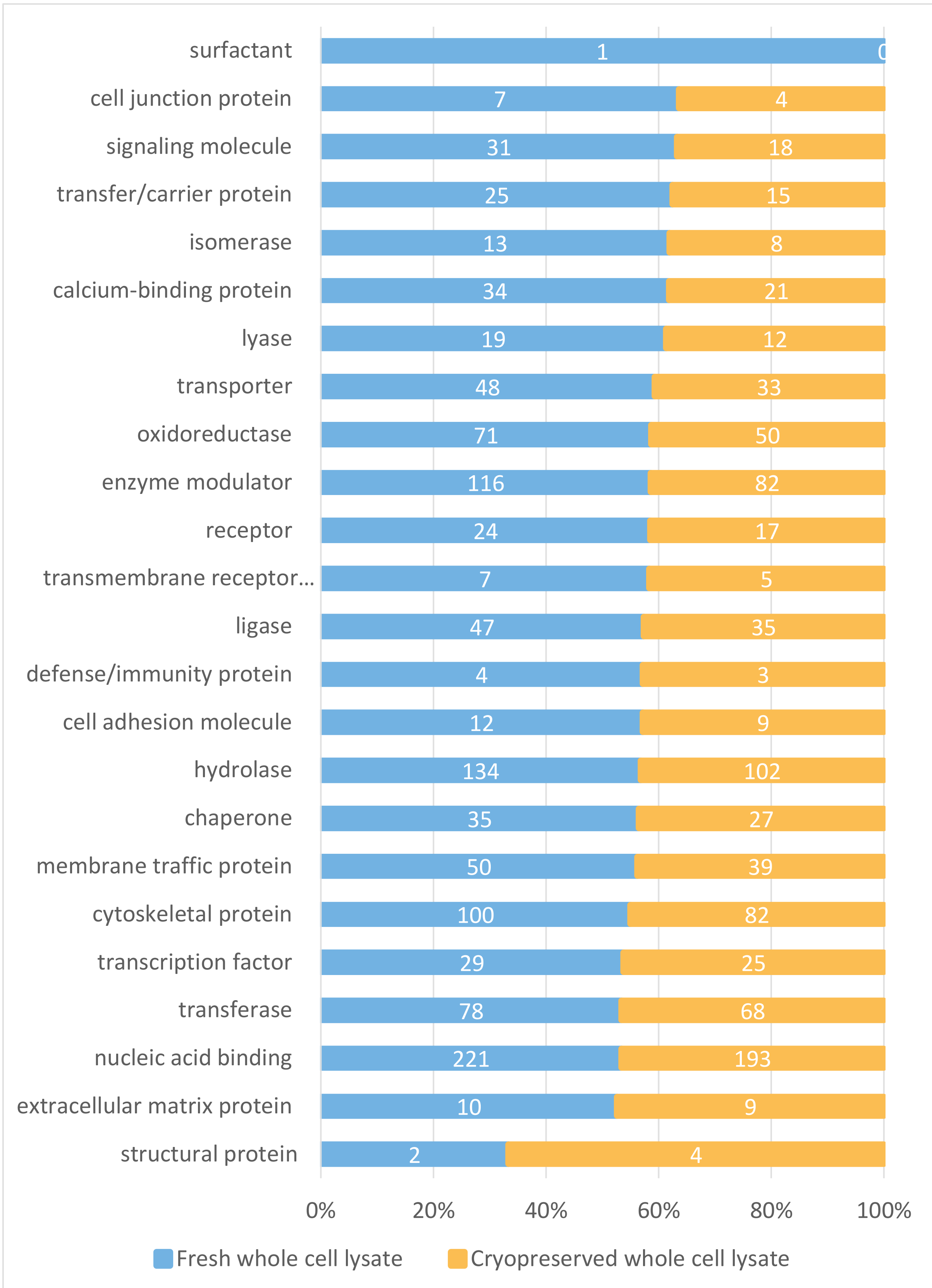
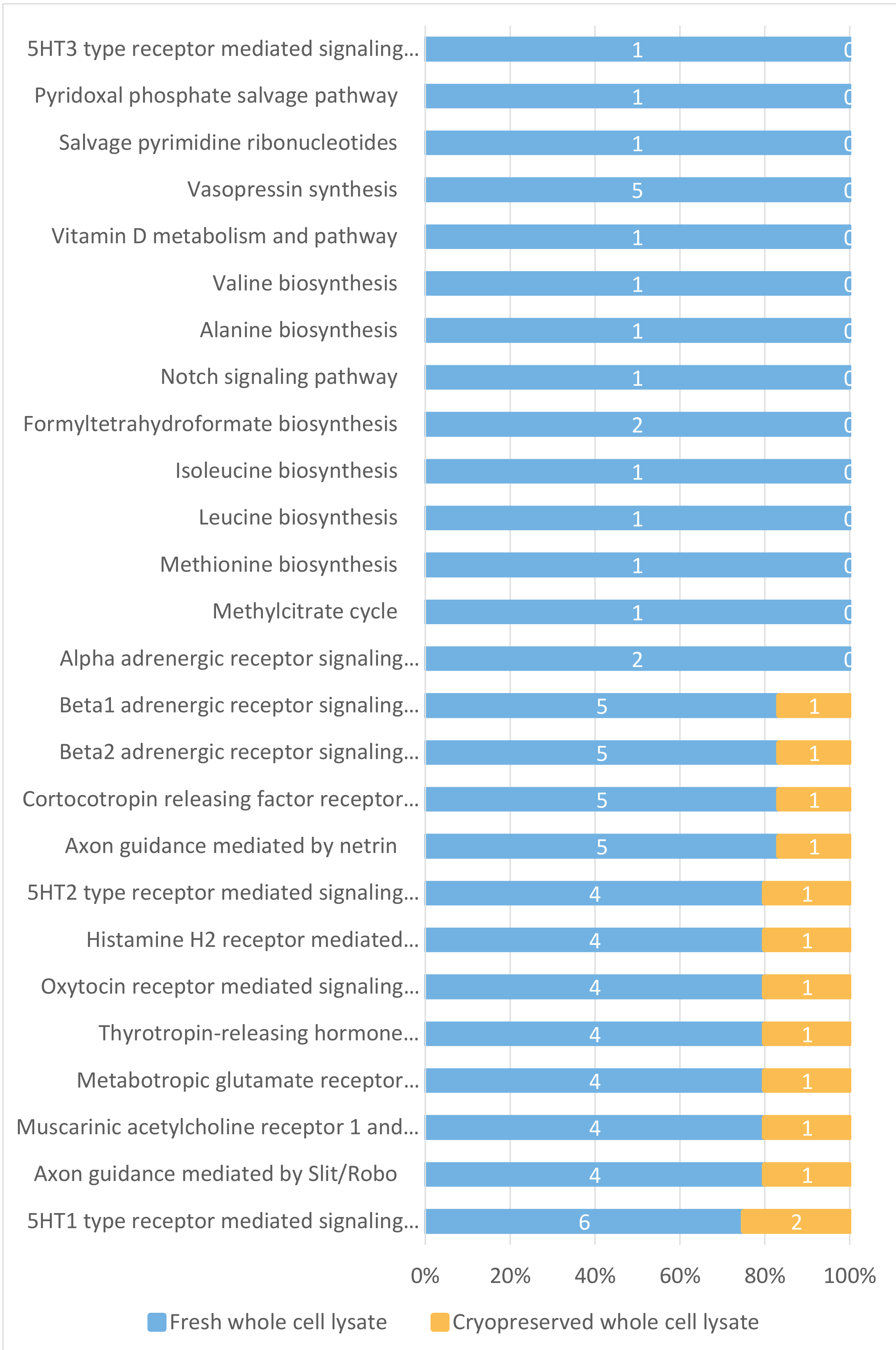


Figure 22: Fresh and cryopreserved ASC proteins sorted protein class.

The PANTHER Classification System detected proteins from 24 different categories of protein classes, with the majority of detected proteins in the category of nucleic acid binding.



5HT3 type receptor mediated signaling...
Pyridoxal phosphate salvage pathway
Salvage pyrimidine ribonucleotides
Vasopressin synthesis
Vitamin D metabolism and pathway
Valine biosynthesis
Alanine biosynthesis
Notch signaling pathway
Formyltetrahydroformate biosynthesis
Isoleucine biosynthesis
Leucine biosynthesis
Methionine biosynthesis
Methylcitrate cycle
Alpha adrenergic receptor signaling...
Beta1 adrenergic receptor signaling...
Beta2 adrenergic receptor signaling...
Cortocotropin releasing factor receptor...
Axon guidance mediated by netrin
5HT2 type receptor mediated signaling...
Histamine H2 receptor mediated...
Oxytocin receptor mediated signaling...
Thyrotropin-releasing hormone...
Metabotropic glutamate receptor...
Muscarinic acetylcholine receptor 1 and...
Axon guidance mediated by Slit/Robo
5HT1 type receptor mediated signaling...
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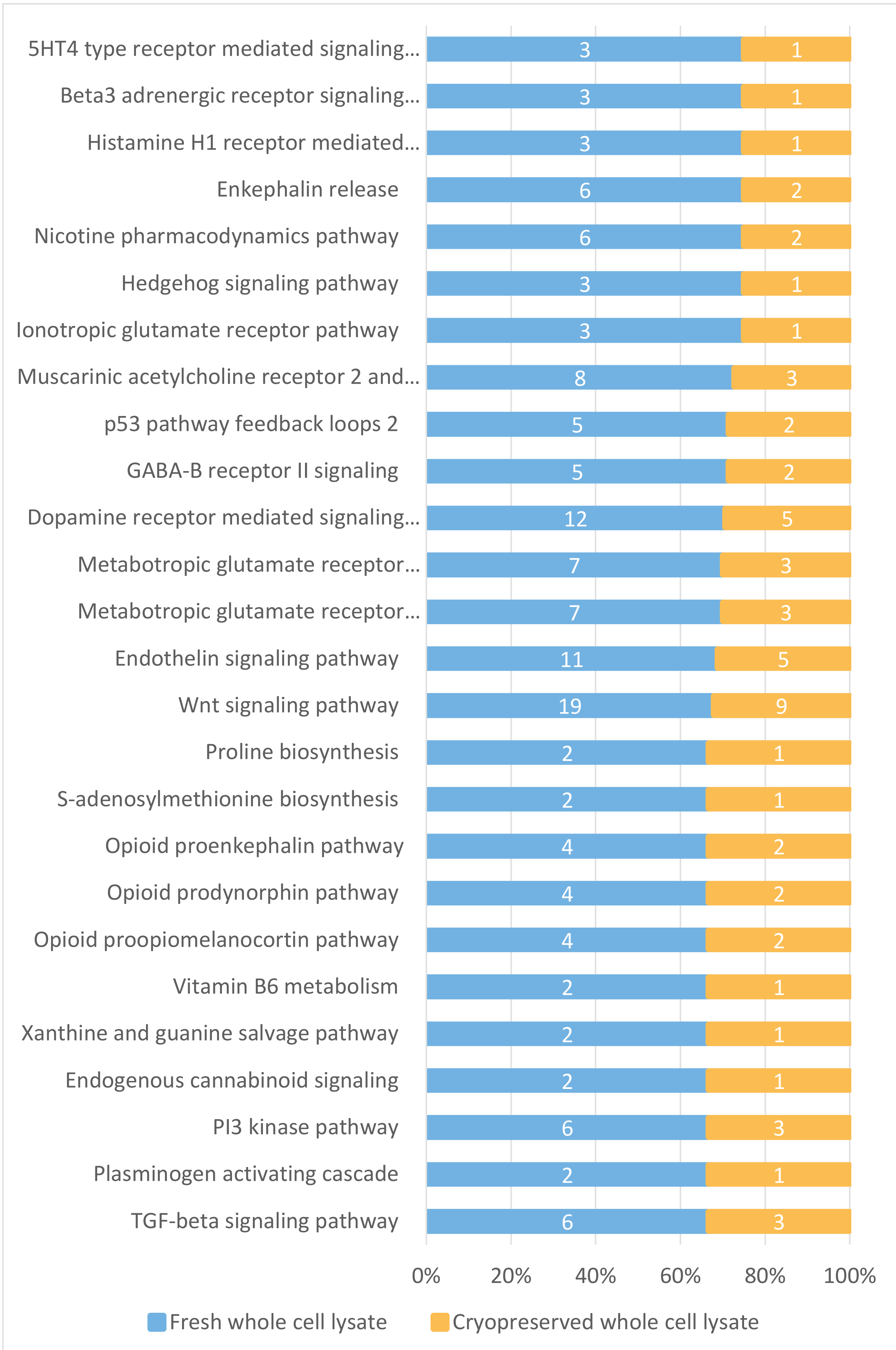
Fresh whole cell lysate
Cryopreserved whole cell lysate


5HT4 type receptor mediated signaling...
Beta3 adrenergic receptor signaling...
Histamine H1 receptor mediated...
Enkephalin release
Nicotine pharmacodynamics pathway
Hedgehog signaling pathway
Ionotropic glutamate receptor pathway
Muscarinic acetylcholine receptor 2 and...
p53 pathway feedback loops 2
GABA-B receptor II signaling
Dopamine receptor mediated signaling...
Metabotropic glutamate receptor...
Metabotropic glutamate receptor...
Endothelin signaling pathway
Wnt signaling pathway
Proline biosynthesis
S-adenosylmethionine biosynthesis
Opioid proenkephalin pathway
Opioid prodynorphin pathway
Opioid proopiomelanocortin pathway
Vitamin B6 metabolism
Xanthine and guanine salvage pathway
Endogenous cannabinoid signaling
PI3 kinase pathway
Plasminogen activating cascade
TGF-beta signaling pathway
3 1
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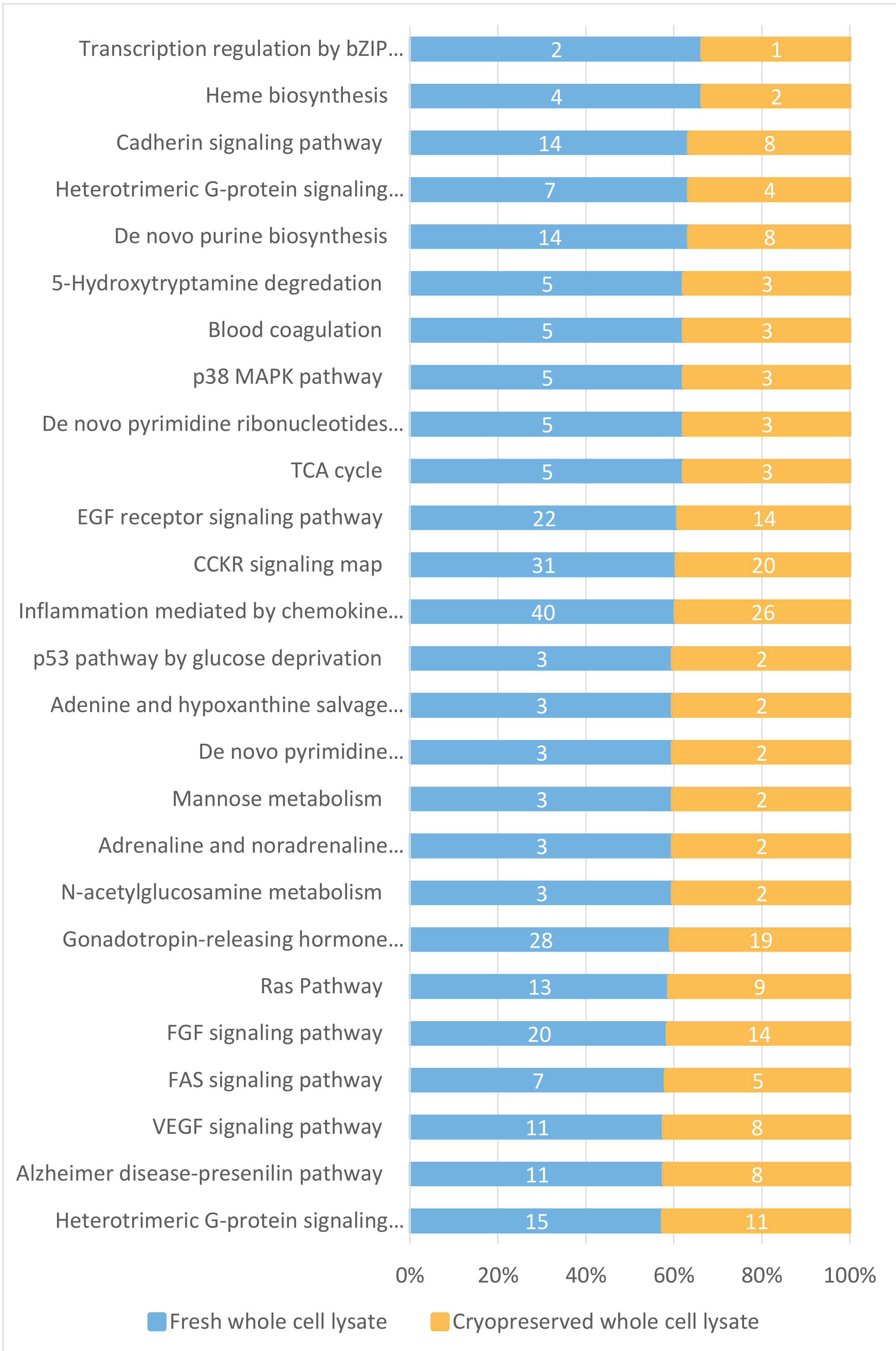
Fresh whole cell lysate
Cryopreserved whole cell lysate



Transcription regulation by bZIP...
2
1
Heme biosynthesis
4
2
Cadherin signaling pathway
14
8
Heterotrimeric G-protein signaling...
7
4
De novo purine biosynthesis
14
8
5-Hydroxytryptamine degredation
5
3
Blood coagulation
5
3
p38 MAPK pathway
5
3
De novo pyrimidine ribonucleotides...
5
3
TCA cycle
5
3
EGF receptor signaling pathway
22
14
CCKR signaling map
31
20
Inflammation mediated by chemokine...
40
26
p53 pathway by glucose deprivation
3
2
Adenine and hypoxanthine salvage...
3
2
De novo pyrimidine...
3
2
Mannose metabolism
3
2
Adrenaline and noradrenaline...
3
2
N-acetylglucosamine metabolism
3
2
Gonadotropin-releasing hormone...
28
19
Ras Pathway
13
9
FGF signaling pathway
20
14
FAS signaling pathway
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VEGF signaling pathway
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Alzheimer disease-presenilin pathway
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Heterotrimeric G-protein signaling...
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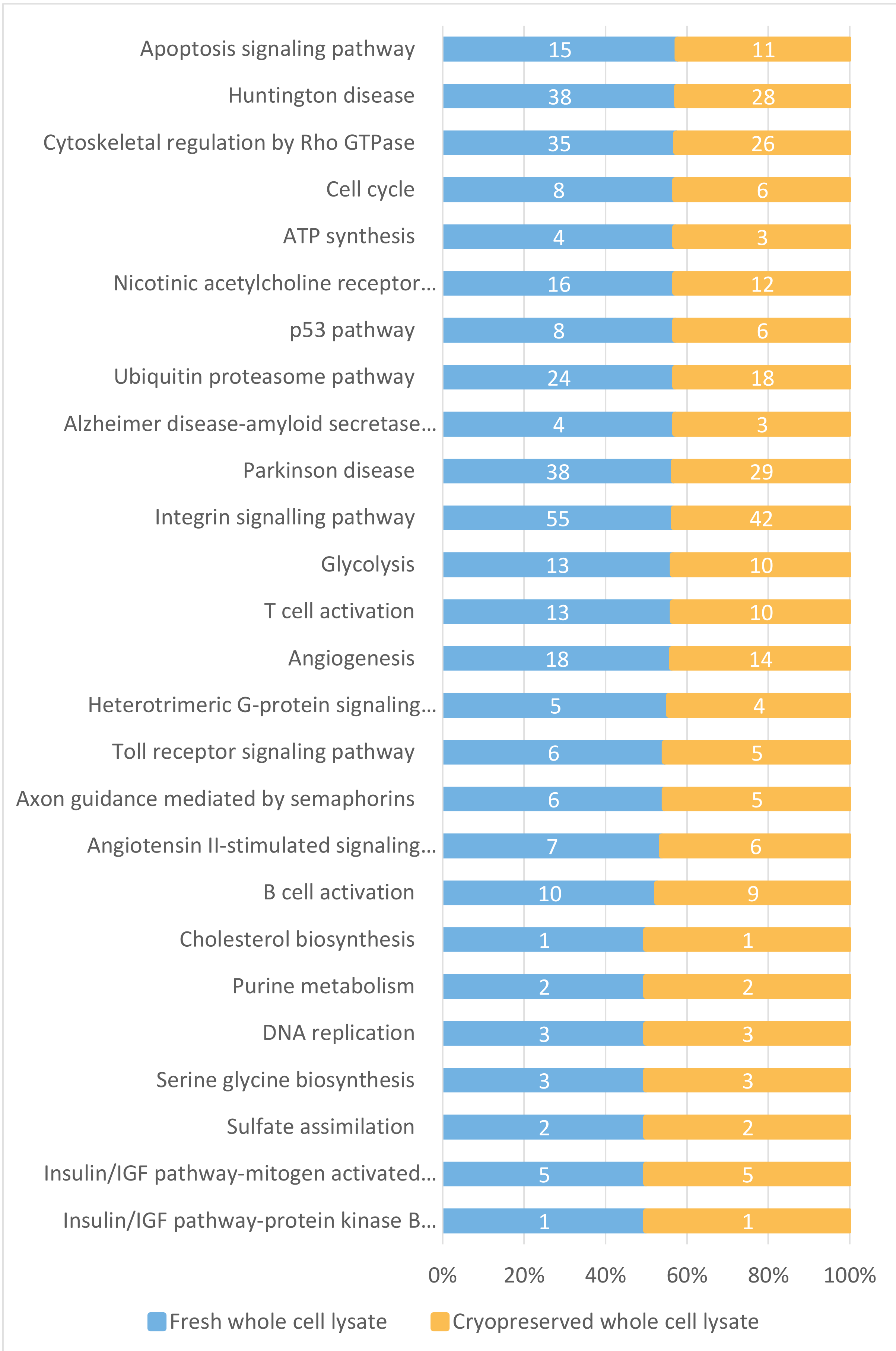
Fresh whole cell lysate
Cryopreserved whole cell lysate


Apoptosis signaling pathway
Huntington disease
Cytoskeletal regulation by Rho GTPase
Cell cycle
ATP synthesis
Nicotinic acetylcholine receptor…
p53 pathway
Ubiquitin proteasome pathway
Alzheimer disease-amyloid secretase…
Parkinson disease
Integrin signalling pathway
Glycolysis
T cell activation
Angiogenesis
Heterotrimeric G-protein signaling…
Toll receptor signaling pathway
Axon guidance mediated by semaphorins
Angiotensin II-stimulated signaling…
B cell activation
Cholesterol biosynthesis
Purine metabolism
DNA replication
Serine glycine biosynthesis
Sulfate assimilation
Insulin/IGF pathway-mitogen activated…
Insulin/IGF pathway-protein kinase B…
15
11
38
28
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Fresh whole cell lysate
Cryopreserved whole cell lysate

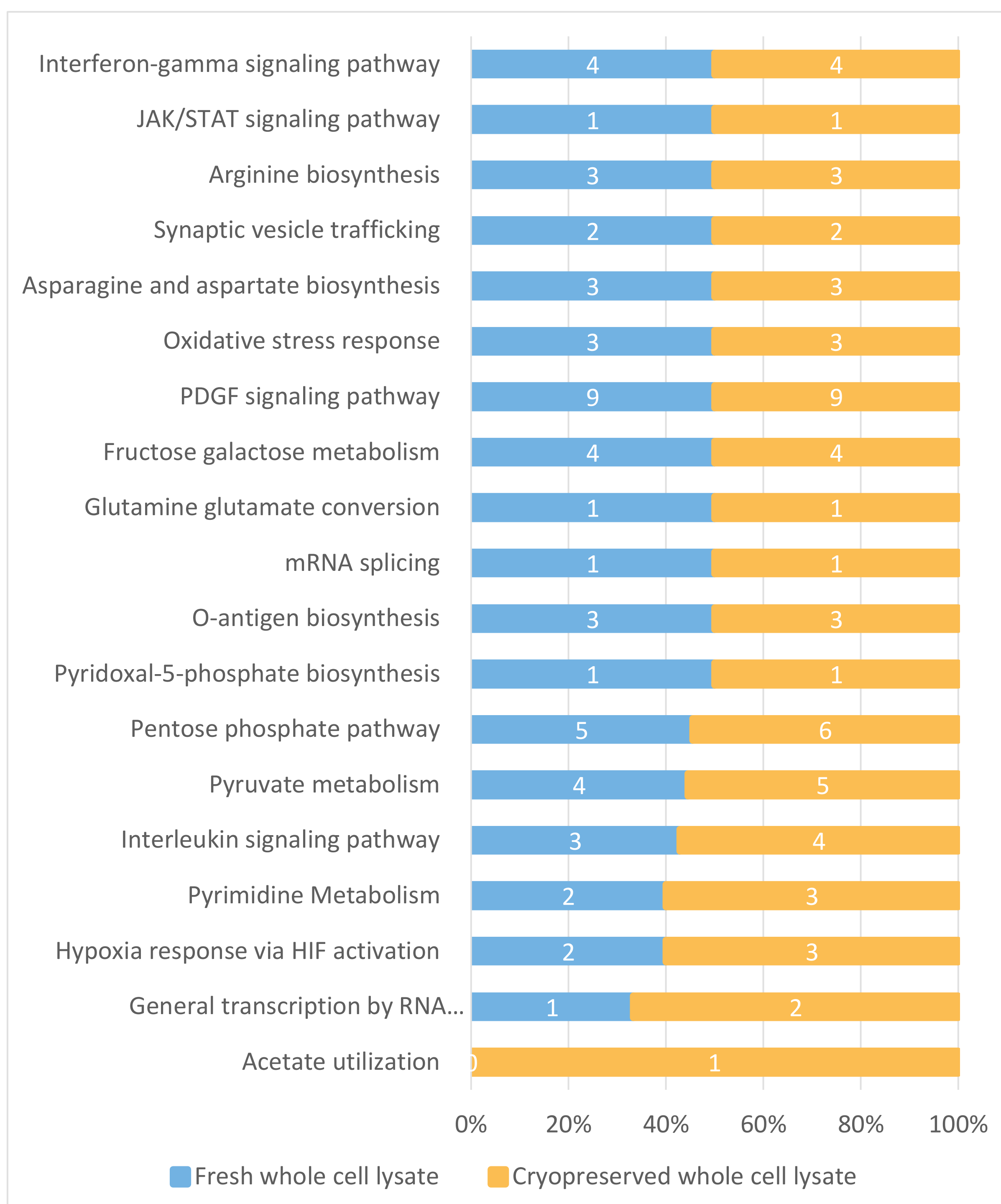

Figure 23: Fresh and cryopreserved ASC proteins sorted pathways.

The PANTHER Classification System detected proteins from 122 different categories of protein pathways, with the majority of detected proteins in the category of integrin signalling pathway.

Proteins that were present in a single fraction and not in another were analysed using STRING. This allowed for identification of network interactions and pathways of interest to shed light

on what interactions were creating a unique proteomic phenotype. STRING is a useful tool to visualise the proteins of interest which are shown as nodes, and their interactions which are shown as edges. The thicker the edge the more evidence there is for the interaction, and nodes are highlighted based on the most commonly occurring functional enrichments.

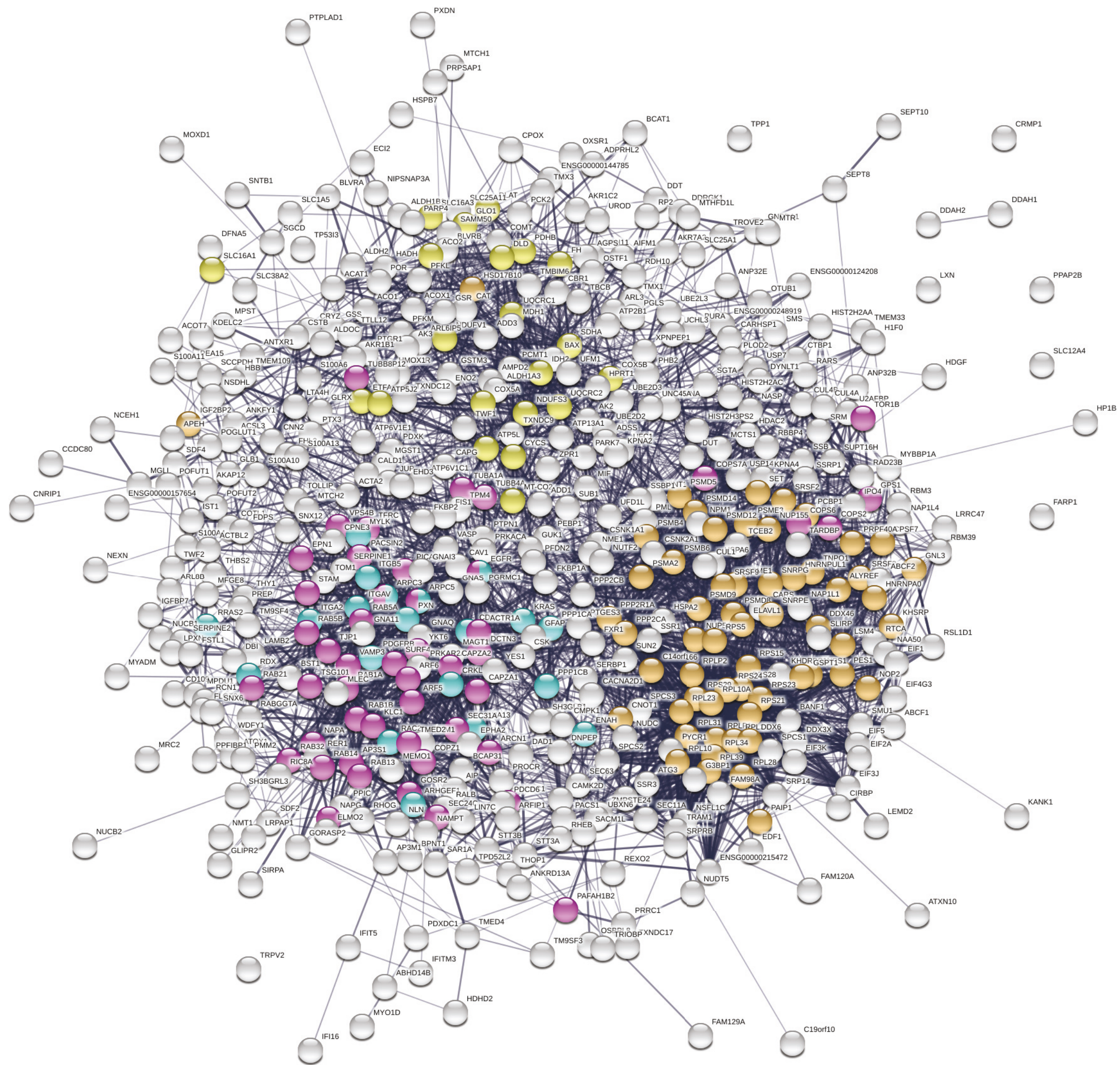

Figure 24: STRING network of detected proteins unique to fresh ASC whole cell lysate. STRING detected 525 nodes and 3841 edges with a local clustering coefficient of 0.323. The KEGG pathway category regulation of actin cytoskeleton is shown in blue (20 with a false discovery rate of 7.06e-05), the reactome pathway category metabolism of RNA is shown in orange (59 with a false discovery rate of 8.29e-13), the reactome pathway category

membrane trafficking is shown in pink (48 with a false discovery rate of 1.09e-08) and the reactome pathway category the citric acid cycle and respiratory electron transport is shown in yellow (21 with a false discovery rate of 1.05e-06).

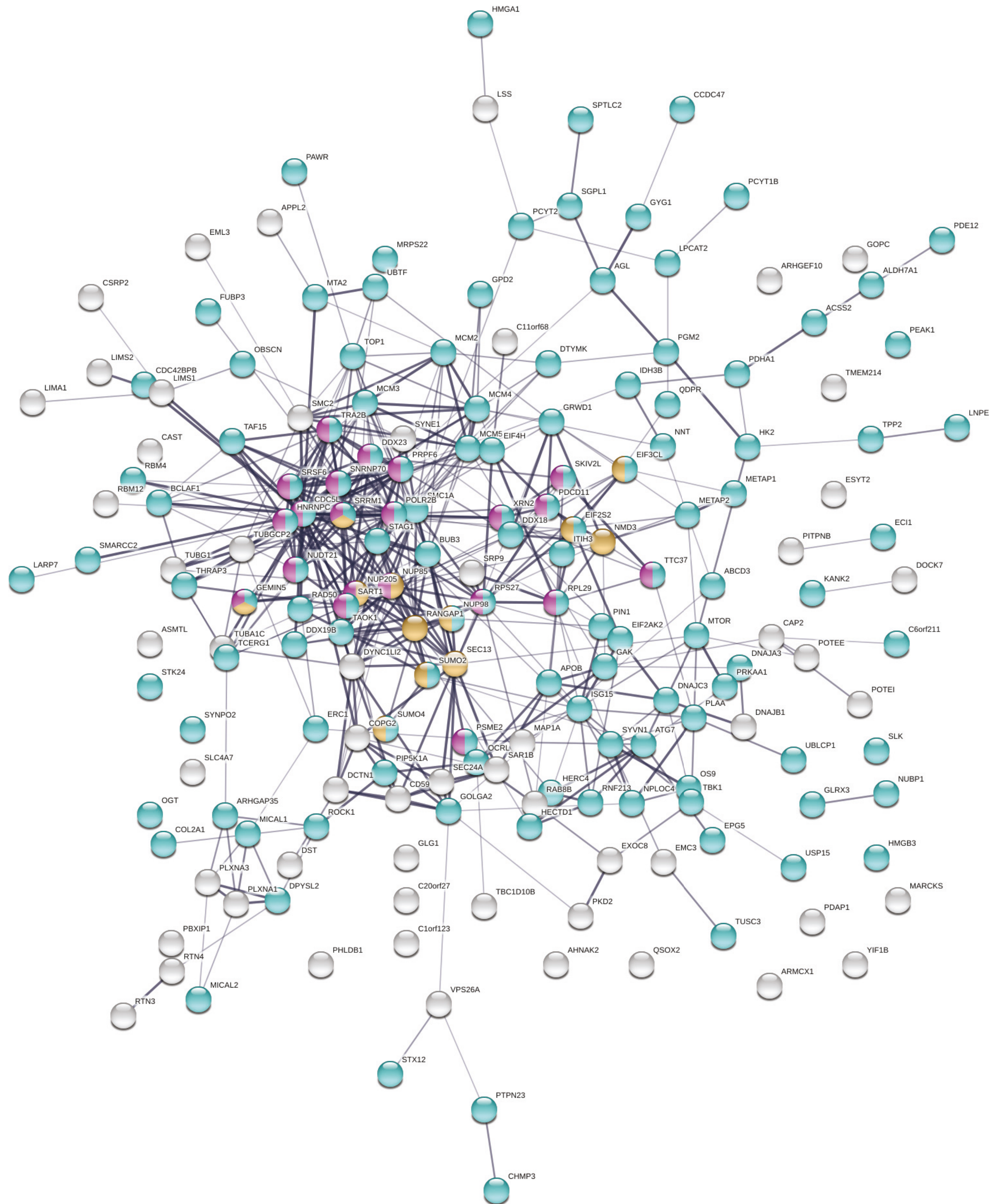

Figure 25: STRING network of detected proteins unique to cryopreserved ASC whole cell lysate.

STRING detected 180 nodes and 465 edges with an average local clustering coefficient of 0.397. The biological process category cellular metabolic process is shown in blue (124 with a false discovery rate of 9.93e-07), the KEGG pathway category RNA transport is shown in orange (12 with a false discovery rate of 1.26e-05) and the reactome pathway category metabolism of RNA is shown in pink (21 with a false discovery rate of 1.5e-4).

Proteins that were commonly found in both the fresh whole cell lysate and cryopreserved whole cell lysate were compared in a clustered heatmap to see how they differ in abundance between treatments. As with the last heatmap, proteins are on the y-axis and patient samples across the x-axis. The x-axis self-assembled into the two respective patient clusters, while the y-axis formed 6 protein clusters based on observed Z scores.

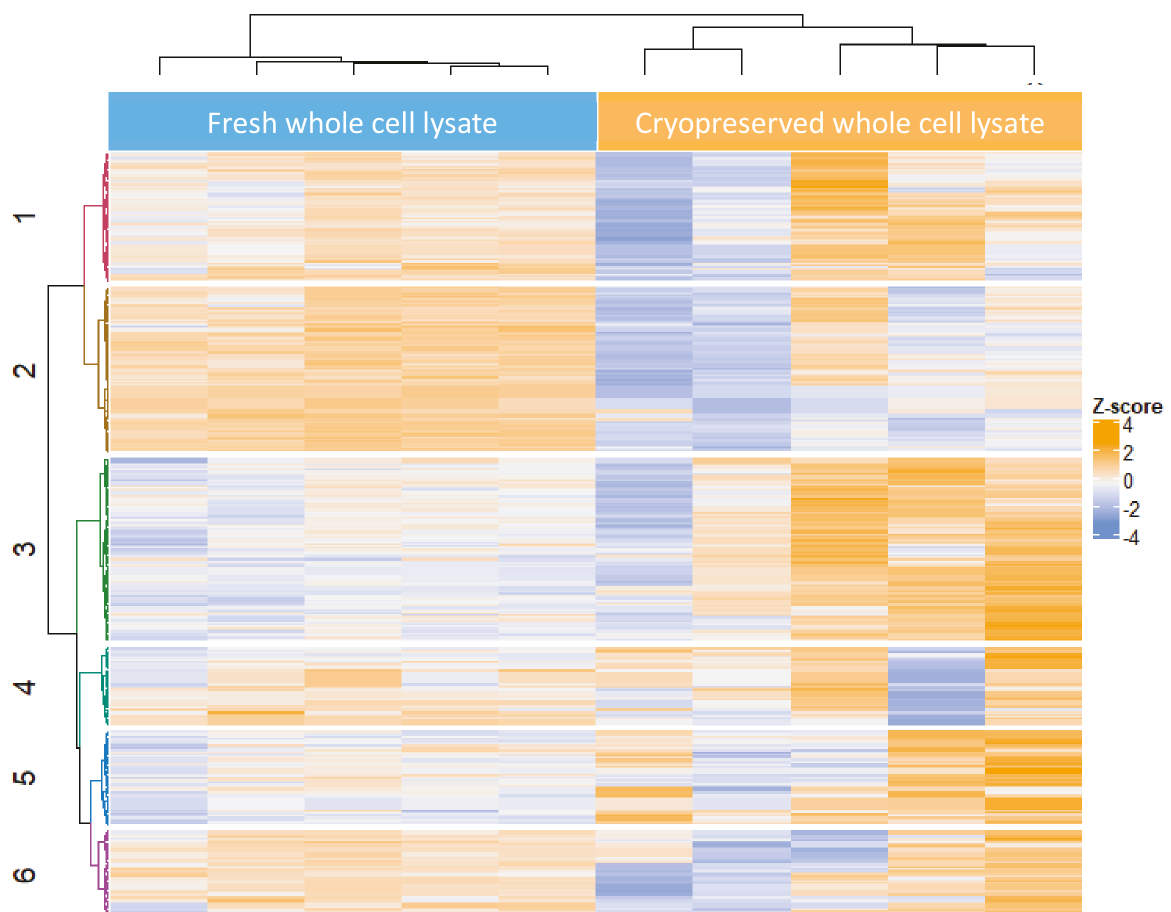

Figure 26: Clustered heatmap of proteins present in both fresh and cryopreserved ASC whole cell lysate.

Fresh and cryopreserved ASC whole cell lysates formed two different clusters on the x-axis, with each patient sample represented in a column. Each row is a protein and there are 6 clusters on the y-axis. Overall, fresh ASC whole cell lysate has a much clearer trend between patients, while cryopreserved ASC whole cell lysate does not have distinct trends, with substantially varied Z score profiles between patients. In Cluster 1, proteins from fresh whole cell lysate have predominantly positive Z scores while proteins from cryopreserved whole cell lysate have both positive and negative Z scores. In Cluster 2, proteins from fresh whole fresh whole cell lysate have predominantly positive Z scores while those from the cryopreserved whole cell lysate have both positive and negative Z scores with a slightly greater number of negative Z scores. In Cluster 3, the proteins of the fresh whole cell lysate have predominantly negative Z scores while those of the cryopreserved whole cell lysate have both positive and negative Z scores, with a slightly higher number of positive Z scores. Cluster 4 has numerous variations in the Z scores for the proteins across both whole cell lysates, however the fresh whole cell lysate has more neutral Z scores than cryopreserved whole cell lysate. In Cluster 5, the proteins of fresh whole cell lysate have predominantly negative Z scores while those of the cryopreserved whole cell lysate have both positive and negative Z scores with mostly positive Z scores. In Cluster 6, the proteins of the fresh whole fresh whole cell lysate have predominantly positive Z scores while those of the cryopreserved whole cell lysate have both positive and negative Z scores.

STRING networks were utilised to investigate the proteins in each of these clusters (Figure 27-32) to showcase their interactions and highlight particular categories of functional enrichment.

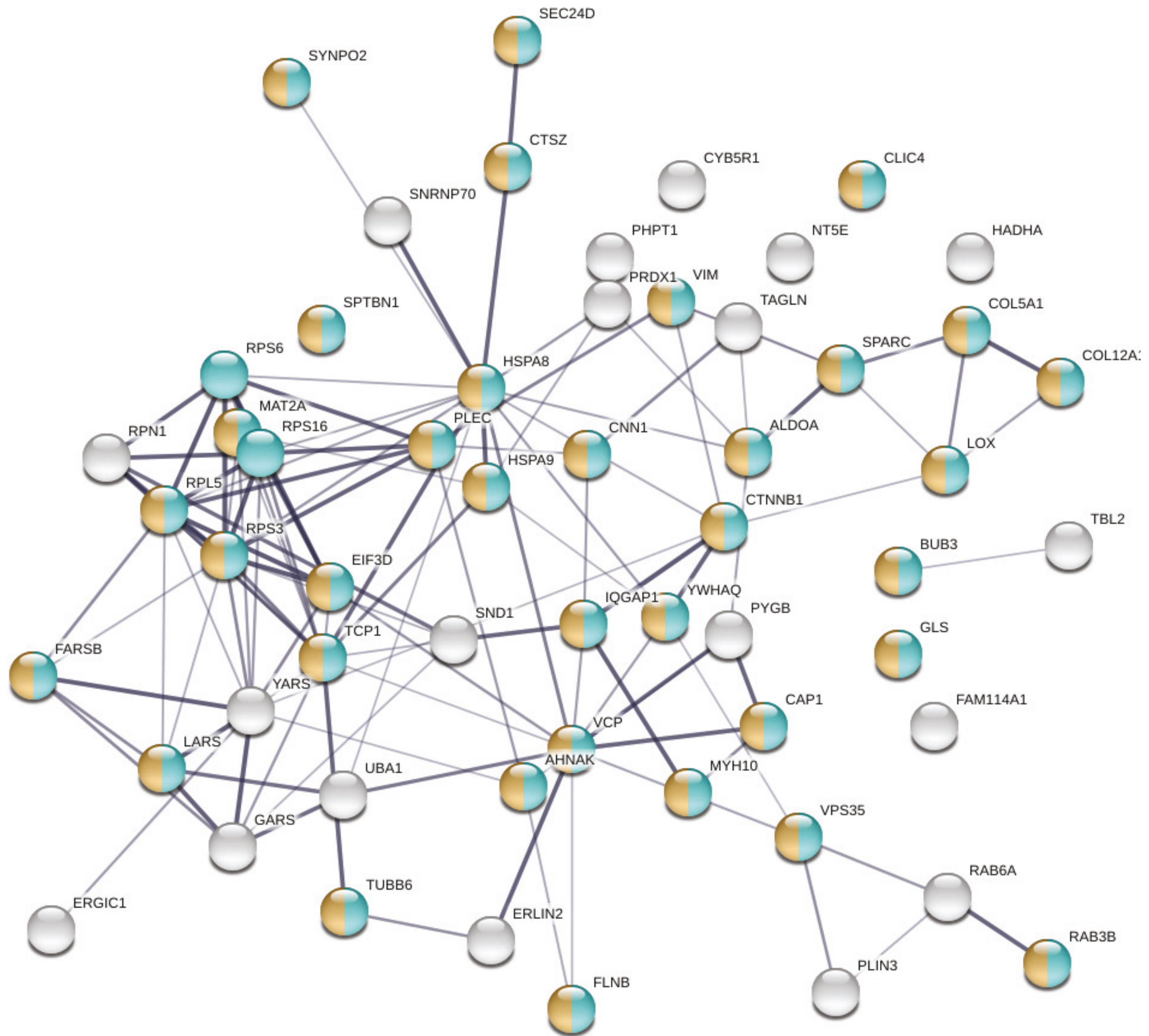

Figure 27: STRING network of proteins detected in Cluster 1 of fresh and cryopreserved ASC whole cell lysate.

STRING detected 56 nodes and 117 edges with an average clustering coefficient of 0.394. The following biological processes categories are highlighted; cellular component organisation or biogenesis is shown in blue (37 with a false discovery rate of 3.93e-06) and cellular component organisation is shown in orange (35 with a false discovery rate of 1.78e-05). Proteins detected in this cluster are of higher abundance in fresh ASCs and exhibit patient-to-patient variation in abundance in cryopreserved ASCs.

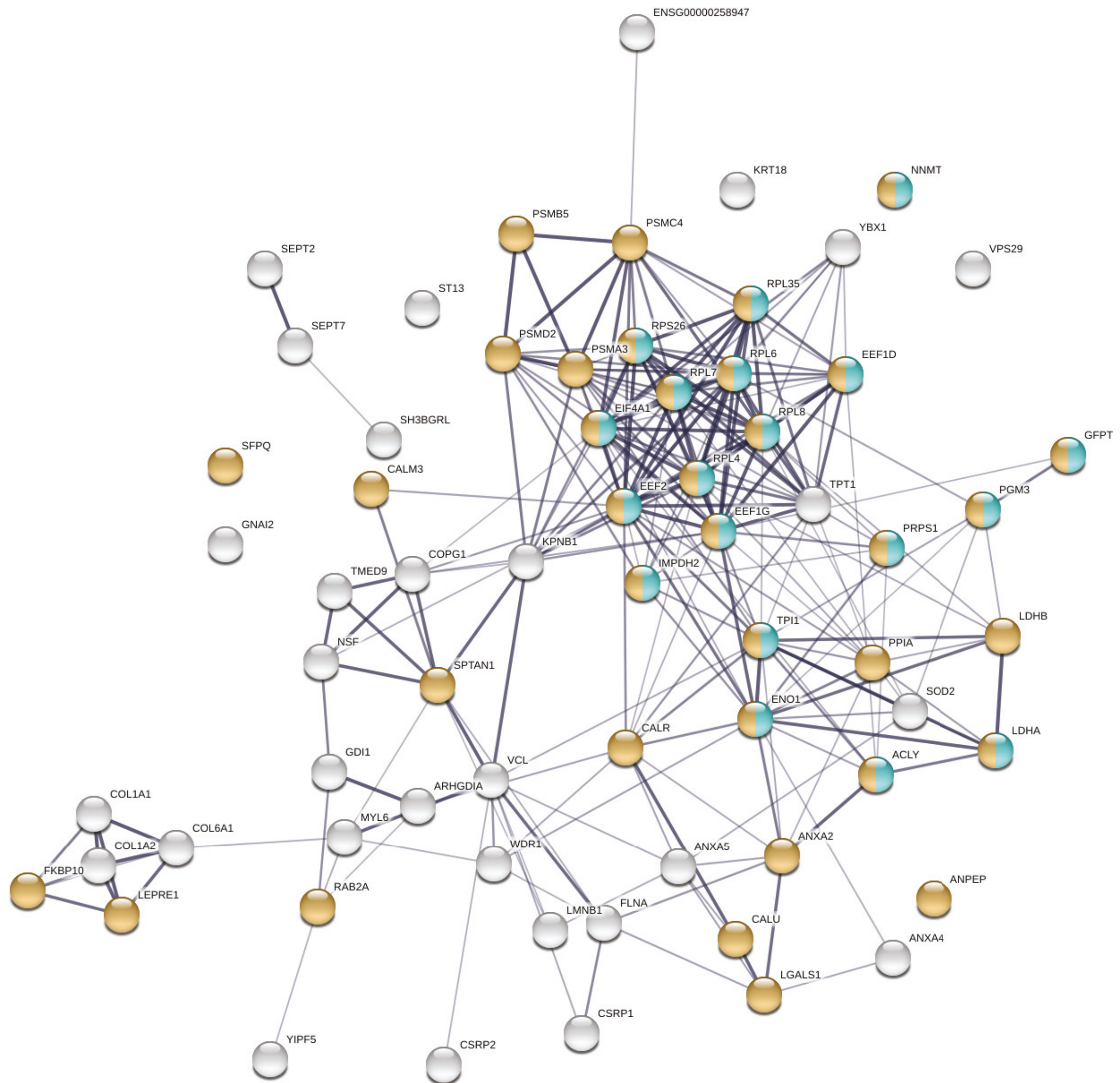

Figure 28: STRING network of proteins detected in Cluster 2 of fresh and cryopreserved ASC whole cell lysate.

STRING detected 66 clusters and 234 edges with an average local clustering coefficient of 0.507. The following biological process categories are highlighted; organonitrogen compound biosynthetic process is shown in blue (19 with a false discovery rate of 4.50e-05) and organonitrogen compound metabolic process is shown in orange (36 with a false discovery rate of 1.90e-04). Proteins detected in this cluster are more abundant in fresh ASCs with patient-to-patient variation in cryopreserved ASCs, but with an overall slightly lower observed abundance.

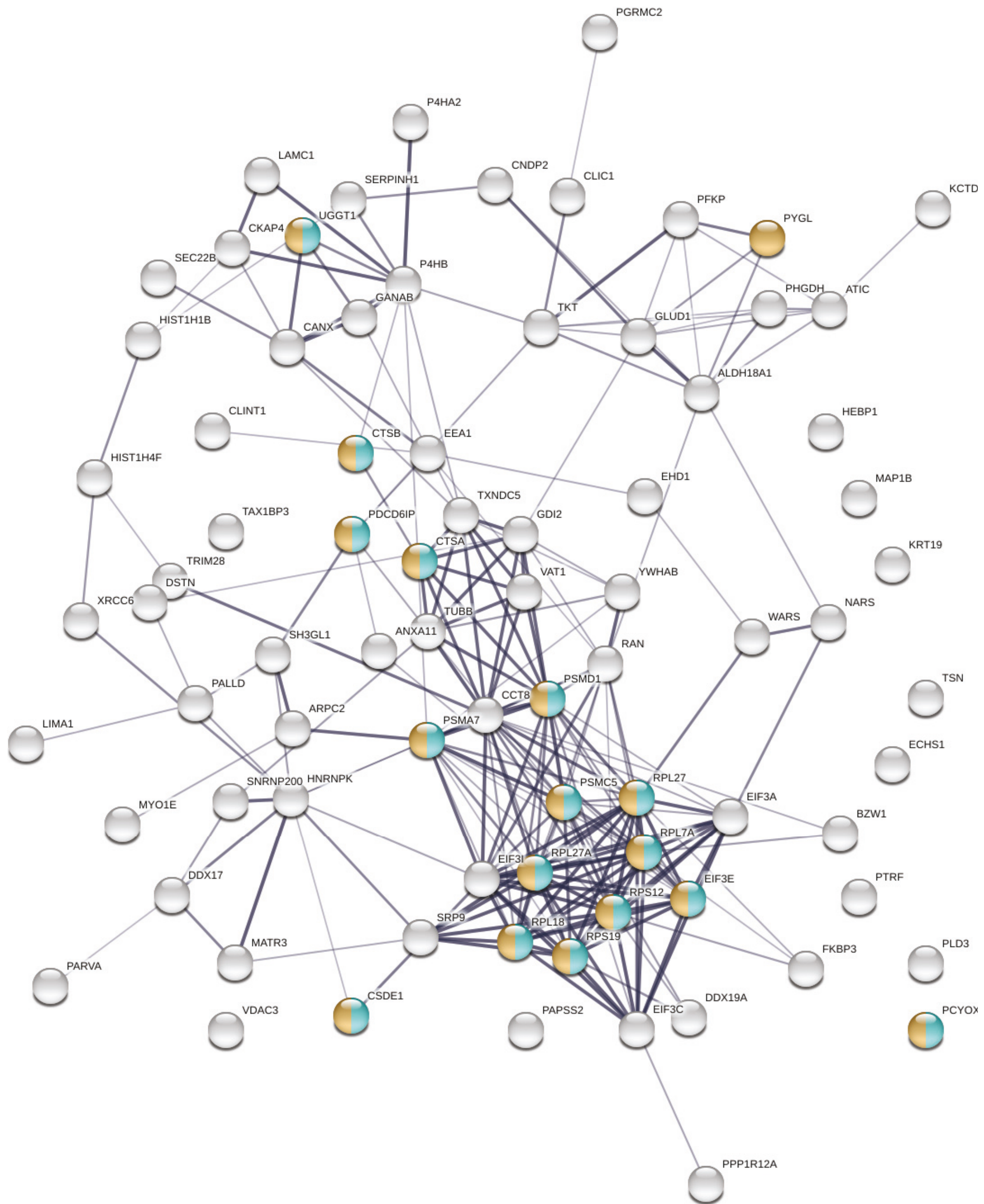

Figure 29: STRING network of proteins detected in Cluster 3 of fresh and cryopreserved ASC whole cell lysate.

STRING detected 81 nodes and 228 edges with an average local clustering coefficient of 0.466. The following biological processes categories are highlighted; cellular macromolecule catabolic process is shown in blue (16 with a false discovery rate of 6.16e-05) and macromolecule catabolic process is shown in orange (17 with a false discovery rate of 6.37e-

05). Proteins detected in this cluster have low abundance in fresh ASCs with slightly higher abundance in cryopreserved ASCs, however the cryopreserved ASCs also exhibit greater patient-to-patient variation.

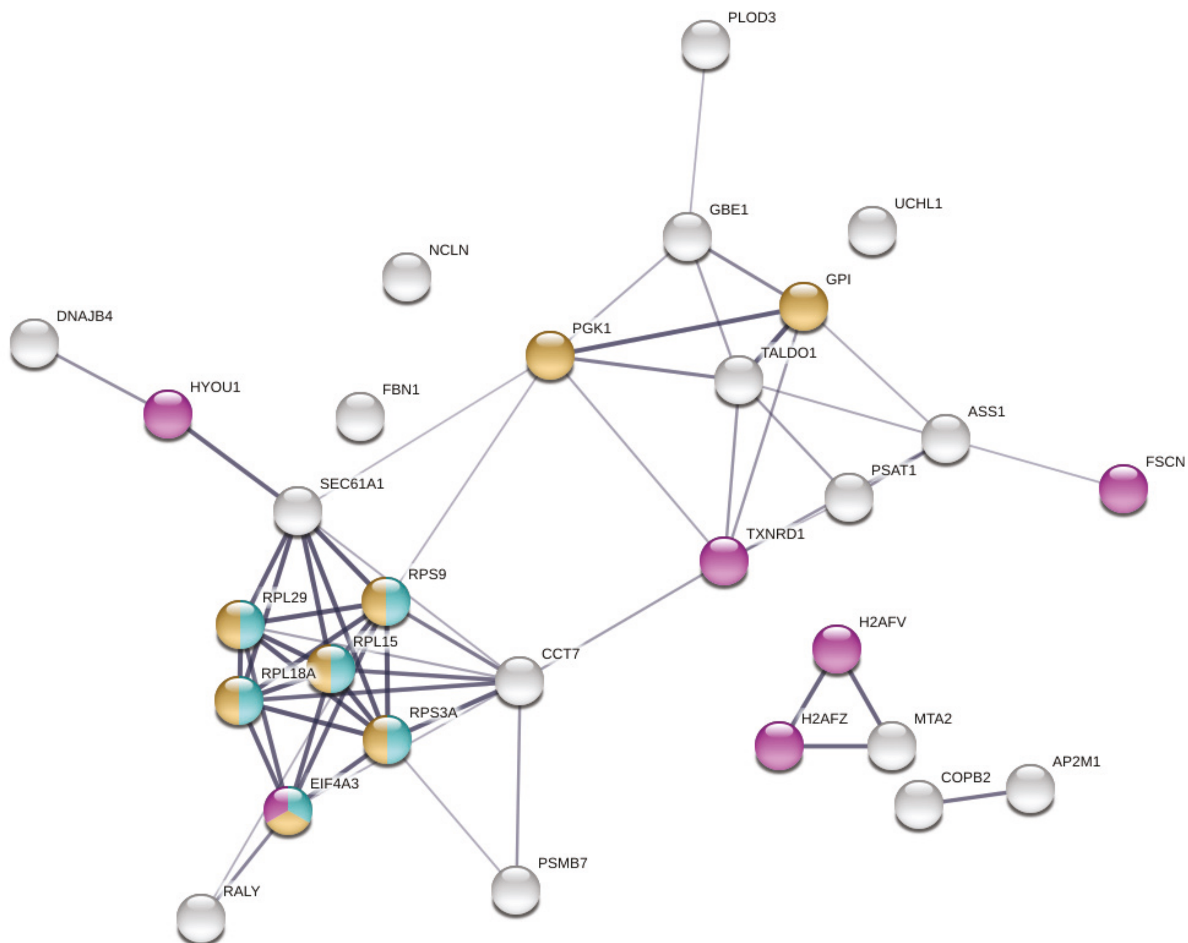

Figure 30: STRING network of proteins detected in Cluster 4 of fresh and cryopreserved ASC whole cell lysate.

STRING detected 29 nodes and 57 edges with an average local clustering coefficient of 0.7. The biological process category nuclear-transcribed mRNA catabolic process, nonsense-mediated decay is shown in blue (6 with a false discovery rate of 6.36e-06), the biological process category nucleobase-containing compound catabolic process is shown in orange (8 with a false discovery rate of 1.18e-05) and the proteins of the reference publication by Zieger *et al.*[317] are shown in pink (6 with a false discovery rate of 8.4e-04). Proteins detected in this cluster have differing abundance in both fresh and cryopreserved ASCs with greater patient-to-patient variation observed in cryopreserved ASCs.

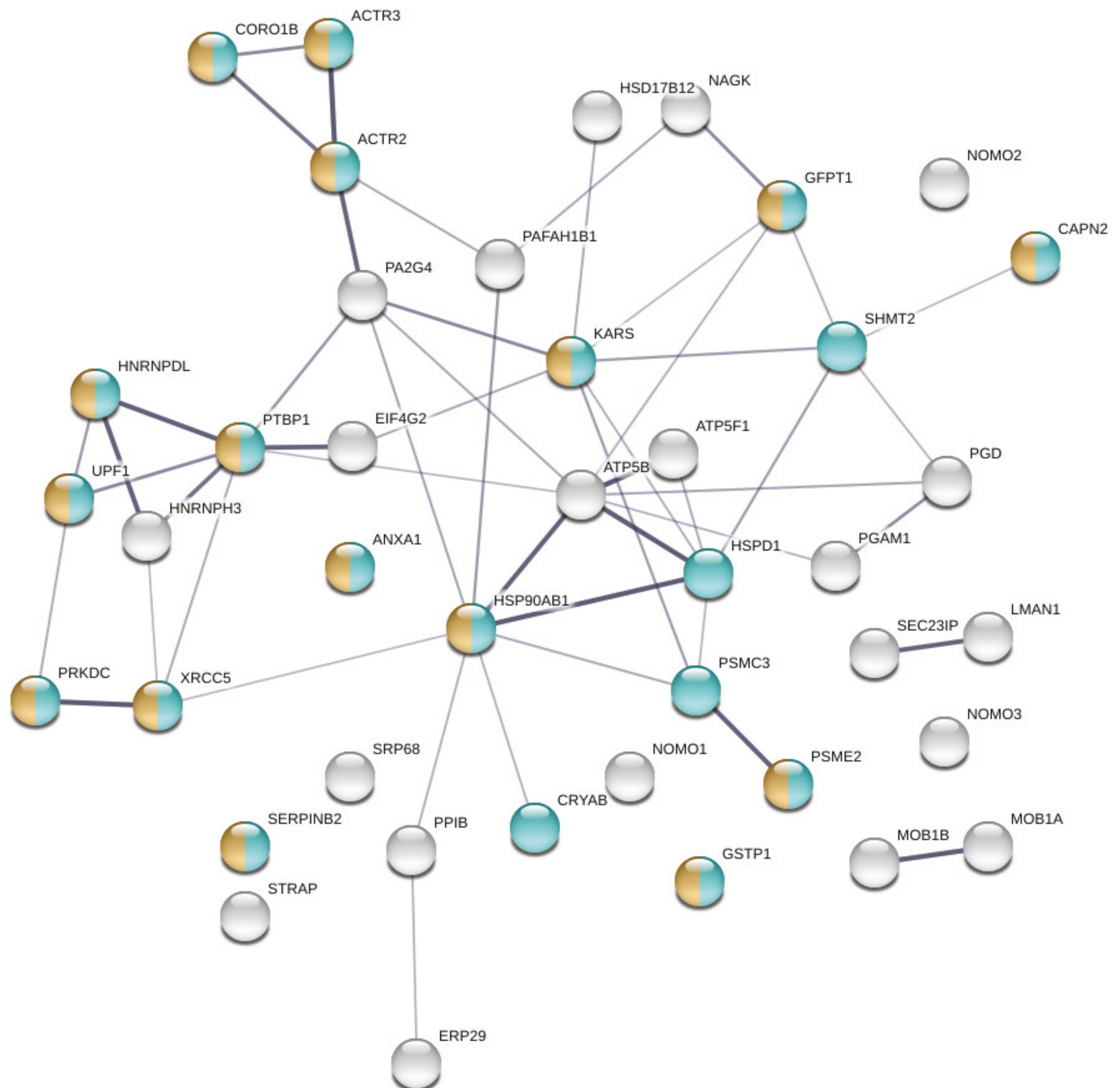

Figure 31: STRING network of proteins detected in Cluster 5 of fresh and cryopreserved ASC whole cell lysate.

STRING detected 41 nodes and 51 edges with an average local clustering coefficient of 0.419. The following biological process categories are highlighted; response to organic substance is shown in blue (20 with a false discovery rate of 2.8e-04) and cellular response to organic substance is shown in orange (16 with a false discovery rate of 1.8e-03). Proteins detected in this cluster have low abundance in fresh ASCs with mostly higher abundance in cryopreserved ASCs. The cryopreserved ASCs also exhibit greater patient-to-patient variation.

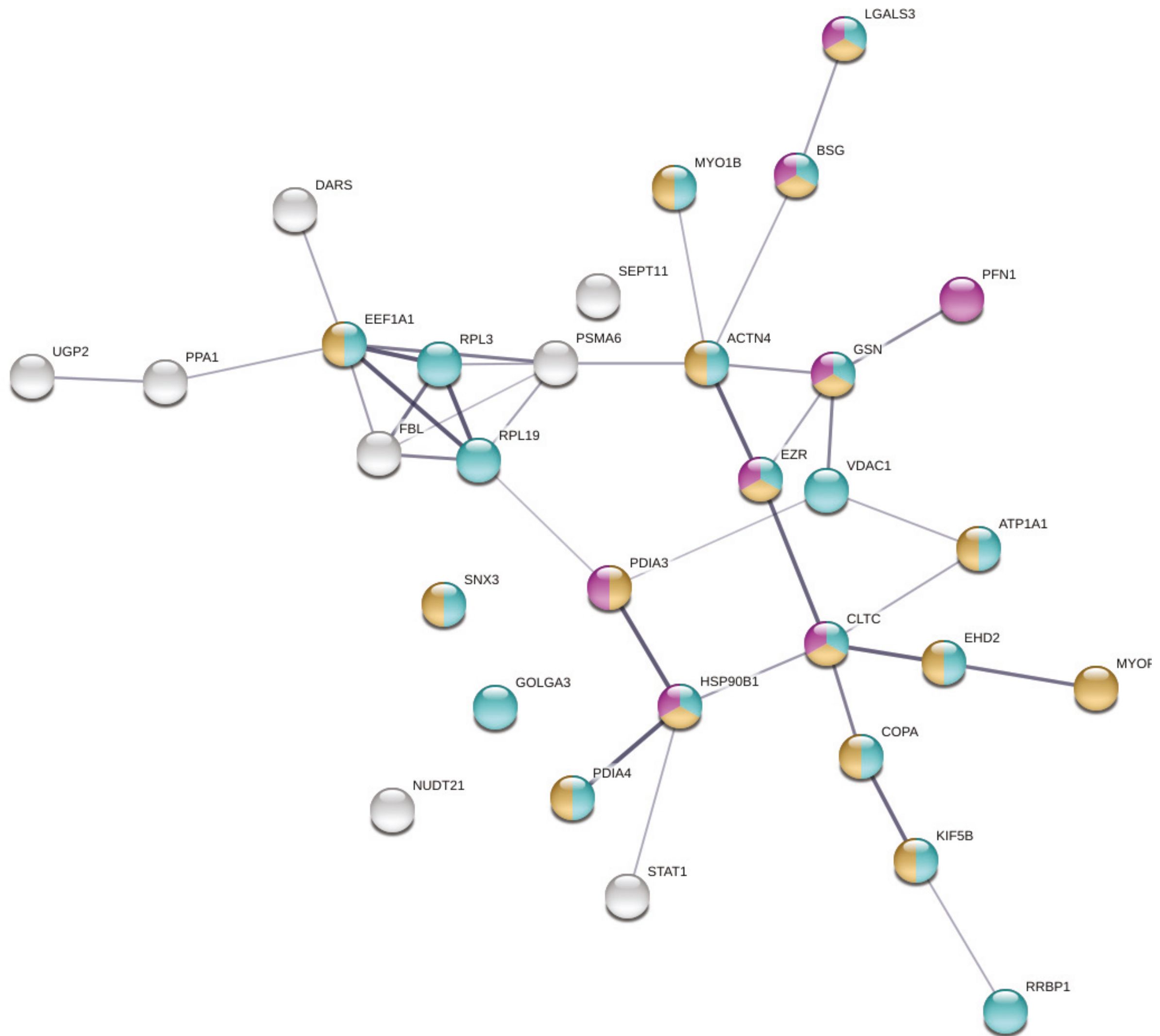

Figure 32: STRING network of proteins detected in Cluster 6 of fresh and cryopreserved ASC whole cell lysate.

STRING detected 31 nodes with 36 edges and an average local clustering coefficient of 0.426. The biological process category transport is shown in blue (20 with a false discovery rate of 3.3e-04), the cellular component category cytoplasmic vesicle is shown in orange (17 with a false discovery rate of 1.65e-06) and the proteins of the reference publication by Taylor *et al*.[318] are shown in pink (8 with a false discovery rate of 4.24e-08). Proteins detected in this cluster are more abundant in fresh ASCs with substantial patient-to-patient variation of abundance in cryopreserved ASCs.

All proteomic data previously reported in this thesis has been analysed in both biological (patients) and technical replicate injections by LC-MS/MS. However, cryopreserved ASCs were

only analysed in a single technical replicate by LC-MS/MS due to time and instrument access constraints. In order to investigate if the variance seen within the cryopreserved ASCs is due to this single technical replicate, the protein group correlation was observed.

The protein groups from PEAKS Studio were exported and are listed in Supplementary Figure 2. The correlation of these protein groups for each patient sample is available in Supplementary Figure 2. When comparing the correlation differences within each treatment (fresh or cryopreserved) across patients, high correlations are observed. This enables the comparison between fresh and cryopreserved samples. Furthermore, the correlation of a single patient sample for both cryopreserved and fresh ASCs is reasonably high. This confirms that the observed variation in the cryopreserved ASC proteome is due to the act of cryopreservation, rather than the single technical replicate that was run.

4. Discussion

4.1 Traditional media and xeno free ASCs

To compare the proteomes of ASCs maintained in either traditional media or xeno free alternatives, ASCs from the liposuction aspirates of 6 patients were isolated and cultured separately in traditional and xeno free media. The cells were expanded to passage 5 and the proteins of the whole cell lysate and membrane bound fractions were extracted. These fractions underwent proteomic analysis using 'shotgun' LC-MS/MS, where peptides generated by trypsin digestion of the proteins were separated by nanoflow reversed phase chromatography and analysed by electrospray tandem mass spectrometry using a benchtop Orbitrap system (Thermo Q Exactive Plus). This type of shotgun LC-MS/MS experiment allows the identification and quantification of the products of open reading frames, hence referred to as 'proteins' rather than intact proteoforms. Media was also collected for analysis using a Bioplex multiplex immunoassay to quantify specific proteins that are below the limit of detection in the LC-MS/MS experiment performed.

2465 unique proteins were conserved across all patients. This is shown in Table 1 where the average number of proteins per patient in the whole cell lysate of ASCs cultured in traditional media was 4634, which was reduced by 59.08% to 1896 when restricting to proteins that are conserved across all 6 patients. The membrane bound fraction of ASCs cultured in traditional media had a smaller average number of proteins per patient of 1860 which was reduced by 62.36% to 700 that were conserved across all patients. The whole cell lysate of cells cultured in xeno free media had an average of 4523 proteins per patient which was reduced by 60.20% to 1800 proteins that were conserved across all 6 patients, and finally the membrane bound fraction of cells cultured in xeno free media had an average of 2772 proteins per patient which was reduced by 68.21% to 881 proteins conserved across all 6 patients.

The distribution of these proteins was visualised in a Venn diagram shown in Figure 1. The membrane bound fraction of cells cultured in traditional media contained 146 proteins that were not detected in the corresponding whole cell lysate and the membrane bound fraction

of cells cultured in xeno free media contained 340 proteins that were not detected in the corresponding whole cell lysate. This demonstrates once again that analysis of both the whole cell lysate and membrane bound fractions increases the number of proteins detected and therefore increases the proteome depth and expands our understanding of the differences in ASCs that have been isolated and cultured in these two mediums. 1538 proteins were commonly found in ASCs cultured in both xeno free and traditional media, and this large overlap indicates common ASC proteins, however this simple analysis does not reveal quantitative changes in abundance of the open reading frame products, which is discussed in the next section. The value of this type of analysis is to highlight the presence or absence of an open reading frame product for which a fold-change cannot be calculated because there is not a value in the other sample. Furthermore, presence of a protein infers biological significance as it is present at a high enough abundance to be detected. This difference between the samples is shown by the 505 proteins that are unique to the traditional media ASCs and 602 proteins that are unique to the xeno free ASCs.

Proteins were identified in traditional media and xeno free ASCs which correlate with what is expected for ASCs, including surface markers that have previously been characterised to positively identify ASCs, such as CD44, CD166 and CD59 [222]. In addition, negative cell surface markers, such as CD31, CD56, CD146, CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR, were not detected in any of our fractions [222,223]. Proteins involved in the self-renewal of stem cells through growth factor pathways were detected in both the xeno free and traditional media ASCs, such as PDGF receptor beta and cation-independent mannose-6-phosphate receptor [224]. Proteins involved in the Wnt signalling pathway that are known to be involved in both self-renewal and differentiation of stem cells were also detected in ASCs from both mediums, such as catenin beta-1, ras-related C3 botulinum toxin substrate 1, serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform, calcineurin B homologous protein 1, and cAMP-dependent protein kinase catalytic subunit alpha [224].

The distribution of proteins of the whole cell lysate and membrane bound fraction of ASCs isolated in traditional and xeno free media is shown in figures based on the PANTHER Classification System. These figures are categorised by molecular function, cellular

compartment, biological process, protein class and protein pathway [202]. The percentage distribution of proteins for individual categories is shown across the x-axis of the figure with the number of detected proteins displayed on the corresponding fraction. In Figure 2, the fractions are sorted by molecular function. The category structural molecule activity (GO:0005198) reflects the distribution of proteins that we would expect, as this is most similar to the distribution of proteins across fractions as seen by the number of proteins from each fraction in Table 1. This distribution can also be seen in Figure 3 in which proteins are sorted by categories of biological process. While proteins belonging to the category cell proliferation (GO:0008283) are only found in traditional media whole cell lysate, proteins from the category biological phase (GO:0044848) are only in xeno free whole cell lysate, and proteins from the category pigmentation (GO:0043473) are only found in the xeno free membrane bound fraction, it is important to recognise that only 1 protein has been detected in each of these processes. This could be because other proteins from this process category weren't present in other fractions, or they were below the limit of detection in other fractions, or alternatively they were simply not annotated into this category in PANTHER. Again, in Figure 4 the distribution of proteins across most of the detected cellular compartments reflects what is expected of this data set. Most of proteins belong to the cellular compartment category cell (GO:0005623) followed by organelle (GO:0043226). Figure 5 is sorted by protein class and the large majority of proteins are in the category nucleic acid binding (PC00171), and these proteins bind DNA or RNA and assist with processing, packing or metabolism.

When examining these figures in isolation, one could assume that ASCs maintained in either traditional or xeno free media have a similar distribution of proteins able to bind nucleic acids. However, this is only reflective of the number of proteins and does not compare the proteins within these fractions. It will be shown over the course of this discussion that isolation in different mediums does alter the ASCs ability to bind nucleic acids. This demonstrates the importance of the heatmaps and STRING networks that were used in this chapter, as they stratify the data allowing for the finer details to be acquired. Sorting the results of the PANTHER Classification System by pathway resulted in 122 different categories of protein pathways as seen in Figure 6. Again, the distribution of proteins is consistent. This is interesting as the Venn diagram (Figure 1) shows a clear proteomic shift. Intriguingly, the membrane bound fraction of the xeno free maintained cells has 21 proteins mapping to the

IFN-γ signalling pathway. The proteins in that fraction alone are shown in Figure 6 to account for 65% of the proteins detected in this pathway when looking at proteins detected from each fraction. IFN-γ has been described as a master checkpoint regulator for many cytokines [319].

STRING was used to create functional protein association networks [203]. The interactions in these networks are reliant on the database that contains information on 24,584,628 proteins [208]. Proteins identified by Peaks Studio when searching the UniProt Human Proteome that are not in this database will not be analysed by STRING and this is a limitation of using this platform. The STRING networks in this chapter have edges set to represent confidence of interaction, and a thicker edge denotes more evidence for a given interaction. Each node represents a protein and they are highlighted by colour with the most common category of functional enrichment. It must be noted that the functional enrichments can introduce bias to the data, as these are manually selected based on which enrichments with a low false discovery rate appear to be of interest. These functional enrichments are categorised by molecular function (based on gene ontology), cellular component (based on gene ontology), reference publications, KEGG pathways, reactome pathways, UniProt keyword, PFAM protein domains, INTERPRO protein domains and features, and SMART protein domains. These sources provide information on the enrichments of interest, but don't necessarily reference the vast amount of literature that would've been utilised to come to these conclusions. For this reason, some information from STRING will not be referenced in this discussion as it was sourced directly through STRING and would make the bibliography longer than the thesis.

The proteins that were unique to a single fraction and not detectable in other fractions were visualised using STRING, and common functional enrichments were demonstrated on the nodes. The STRING network of detected proteins unique to traditional media whole cell lysate is shown in Figure 7. This network has 204 nodes with 334 edges with a modest number of connections, as shown by the average local clustering coefficient of 0.432. The biological process category cellular component organisation (GO:0016043) is shown in blue with 90 nodes. These proteins are found more commonly in the centre of the network, in particular within the dense core. Proteins from this biological process are involved in the assembly or disassembly of cellular components and it is normal to find these proteins in the cytosol of the cell, which is consequently detected in the whole cell lysate fraction. 182 proteins shown

in orange are from the cellular compartment category cytoplasm (GO:0005737) and these make up most of the network as we would expect, as the whole cell lysate is largely comprised of cytosolic proteins. The reactome pathway category metabolism (HSA-1430728) is highlighted in pink, and these 48 proteins are scattered throughout the network. This pathway is utilised for the generation of energy by oxidation of consumed molecules, synthesis of molecules and elimination of toxic molecules. While we would expect to detect these proteins in the traditional media whole cell lysate fraction, we would also expect them in the xeno free whole cell lysate, so their absence is unexpected. It is important to recognise that when the presence and absence of proteins between fractions is discussed, only those proteins that are detectable and conserved across all 6 patient samples are retained after filtering. This means that the protein may not be absent from the ASCs but simply were not present in all patient samples, or alternatively were below the limit of detection. In saying this, it is still interesting that proteins involved in these essential biological processes, cellular components and pathways were not detected in the xeno free ASCs and demonstrates that isolation and expansion in different media is having a significant effect on the detected proteome. Whether or not this effect is preferential or detrimental is difficult to determine, as the traditional media is often perceived as the gold standard, despite the lack of evidence that this is actually the case.

The STRING network for proteins unique to the membrane bound fraction of cells cultured in traditional media only has 25 nodes with 26 edges but has a reasonable average local clustering coefficient of 0.477 (Figure 8). 17 of these proteins are a part of the biological process category localisation (GO:0051179), demonstrating that proteins unique to the traditional media membrane bound fraction are responsible for transporting and tethering of substances within cells. The molecular function category structural molecule activity (GO:0005198) is shown with 6 orange proteins, 4 of which all interact with one another. These proteins contribute to the structural integrity of molecules. The cellular component category endoplasmic reticulum part (GO:0044432) was highlighted in pink; these 9 nodes are part of the membranous endoplasmic reticulum, which is expected of this membrane bound fraction.

Proteins unique to the xeno free whole cell lysate were analysed in a STRING network as shown in Figure 9. This network is not particularly well connected as shown by the average

local clustering coefficient of 0.377. These 247 nodes have 812 edges and 163 of these nodes belong to the biological process category metabolic process (GO:0008152), highlighted in blue. These proteins are scattered throughout the network and are involved in the transformation of chemical substances. The 27 proteins highlighted in orange are found in and around the dense core of the network; these proteins are involved in the reactome pathway category metabolism (HSA-1430728). 14 of these 27 nodes are also a part of the reactome pathway category mRNA splicing – major pathway (HSA-72163). This pathway involves the splicing of pre-mRNA in the splicosome, the resulting mRNA only consisting of joined exons. The detection of these proteins only in the xeno free whole cell lysate indicates they were not detected in the other fractions. This suggests that xeno free isolation impacts metabolism and mRNA splicing.

113 nodes are displayed in the STRING network generated from the xeno free membrane bound fraction in Figure 10. This network has 3 dense zones, but also has many proteins without interactions. Within the network there are 216 edges and an average local clustering coefficient of 0.484. The biological process category organonitrogen compound biosynthetic process (GO:1901566) is shown in blue with 30 nodes dispersed across the network. This process encompasses chemical reactions and pathways that lead to the formation of a compound that contains at least one carbon-nitrogen bond. The biological process category organic substance transport (GO:0071702) is highlighted in orange on 34 proteins, only 3 of which are also highlighted in blue. These orange nodes are scattered across the network but are in the centre of 2 of the 3 dense zones. These proteins are involved in the movement of a substance containing carbon within the cell, out of the cell, or into the cell. The detection of the proteins involved in these two biological processes only in the xeno free membrane bound fraction suggests that xeno free ASC membranes differ to traditional media ASC membranes in their ability to process and transport substances. 96 of the nodes are highlighted in pink to show the cellular component category intracellular organelle part (GO:0044446), reinforcing that the membrane isolation method extracts all membranes of the cell and does not separate different membranes (plasma, nuclear etc) or parts of those membranes. Therefore, in the xeno free ASCs it can be observed that the proteins unique to the membrane bound fraction are those involved in intracellular organelles such as the nucleus, mitochondria, vesicles or ribosomes. This consequently indicates that the different media used for isolation

and expansion of ASCs could cause changes within the membranes of extracellular vesicles. Vesicles were not collected and examined in this work and thus need investigation to confirm this.

As the manufacturer of the xeno free media has provided no list of the ingredients or components, it is difficult to understand why these proteins and processes are increased. This highlights an additional problem with stem cell treatments when trying to maintain xeno free conditions. This is viewed as favourable over traditional media as it could cause potential complications because of products typically of bovine origin, namely the unknown effects on cellular phenotype due to unknown media components. The vast majority of our current knowledge of cellular biology and phenotype is derived from human cells adapted to traditional media and this could be warping our understanding away from reality. The comparison presented in this work demonstrates that a fundamental understanding of the changes that can occur in cellular phenotype due to something as routine as a media change is lacking and that clinical application of autologous stem cell treatments cannot be applied until comprehensive understanding is gained.

The proteins that were detected in multiple ASC fractions were compared in clustered heatmaps. These heatmaps were created using the output from PEAKS which provides quantitative data based on measuring the area under the curve of peptide intensity in the MS1 scan. The heatmap shown in Figure 11 is for the proteins present in the traditional media and xeno free ASC whole cell lysate. Each column represents a patient sample and each row a protein. The patient samples have formed two distinct patient clusters on the x-axis, one for the traditional media and one for the xeno free media. The colours shown are indicative of Z scores, a statistical measurement of variation from the mean. A Z score of 0 indicates the value for the protein in the particular patient sample is the same as the mean, while a positive score indicates a value above the mean, and a negative indicates a value below. There are 5 distinct clusters of proteins on the y-axis, and STRING networks were created for each of these protein clusters. The quantity of individual proteins detected in the ASC traditional media whole cell lysate are more homogenous than those in the ASC xeno free whole cell lysate, and this is visually depicted very clearly in the heatmap by the similar Z scores seen in the traditional media and varying Z scores seen in the xeno free media.

Figure 12 depicts a STRING network of the proteins from Cluster 1 of the ASC traditional media and xeno free whole cell lysate. This network consists of 104 nodes with 521 edges, the network having two dense regions of nodes and an average local clustering coefficient of 0.481. Proteins within this cluster have a higher abundance in xeno free media with lower abundance in traditional media. Indicated in blue, the biological process category mRNA metabolic process (GO:0016071) is highlighted across 24 nodes. These proteins are located in the two dense regions with high connectivity. This biological process involves transcribing DNA to mRNA which is utilised for protein assembly by ribosomes. All of these 24 nodes make up a part of the 58 nodes that are shown in orange to represent the biological process category heterocycle metabolic process (GO:0046483). This encompasses pathways and chemical reactions that involve compounds that have a cyclic molecular structure and at least two different atoms in their ring(s). These proteins are dispersed across the network but are found in the denser regions. 29 of these nodes are also highlighted in pink and make up a portion of the 39 proteins involved in the reactome pathway category metabolism (HSA-1430728). These proteins are concentrated in the densest collection of nodes in the top half of the network as well as the less dense outer region found in the lower right quarter. Collectively this demonstrates that cellular metabolism is affected when cells are isolated and expanded in different mediums. Even when the same proteins are detected, abundance changes of proteins are dependent on the type of media used. In this case the proteins in this cluster are found to have higher abundance in xeno free ASCs and lower abundance in traditional media ASCs.

Cluster 2 of ASC traditional media and xeno free whole cell lysate is shown in Figure 13, made up of 118 nodes with 726 edges. This STRING network has a single dense core and an average local clustering coefficient of 0.497. 4 different functional enrichment categories are shown, and 13 of the highlighted proteins are involved in all 4 of these functional enrichments. Biological process category translation (GO:0006412) is shown in blue with 18 nodes, and translation is the metabolic process of forming a protein from mature mRNA or circRNA. The ribosome facilitates this process and it terminates it by releasing a polypeptide chain. The reactome pathway category signal reaction particle-dependent cotranslational protein targeting to membrane (HSA-1799339) is shown in orange with 17 nodes. This process is

initiated by a nascent polypeptide that contains a hydrophobic signal sequence which is exposed on the surface of the cytosolic complex made up of the ribosome, mRNA and peptide. This signal sequence causes the complex to interact with the cytosolic signal recognition particle, causing translation to stop, and also results in the signal recognition particle receptor docking the complex to the membrane of the endoplasmic reticulum. The ribosome complex is then transported from the signal reaction particle complex to a translocon complex which places the nascent polypeptide into the endoplasmic reticulum lumen. Translation resumes, the signal peptide is cleaved, and elongation continues with the polypeptide chain forming in the endoplasmic reticulum lumen. The reactome pathway category nonsense mediated decay enhanced by the exon junction complex (HSA-975957) is shown in pink with 15 nodes. This pathway is initiated when eRF3 associates with the ribosome but does not interact with PABP as it would in normal translation, but rather interacts with UPF1 [320-324]. The exact degradative pathways are not yet known, but it is known that exon junction complex enhances this nonsense mediated decay and that a key step is the phosphorylation of UPF1 by SMG1 [325]. The reactome pathway category regulation of expression of SLITs and ROBOs (HSA-9010553) is shown in yellow with 17 nodes. The expression of SLITS and ROBOS is regulated during transcription, translation, protein localisation and protein stability. All of the proteins within this cluster have a higher abundance in xeno free media and comparatively lower abundance in traditional media, demonstrating that cellular translation is different between ASCs isolated and cultured in these mediums, with proteins involved in particular aspects of translation detected in higher abundance in the xeno free whole cell lysate ASCs.

The STRING network of proteins detected in Cluster 3 of ASC traditional media and xeno free whole cell lysate is shown in Figure 14. This cluster has 92 nodes and 177 edges. The network is well dispersed without any clear dense areas and it has an average local clustering coefficient of 0.408. The highlighted functional enrichment categories in this cluster focus on the cytoskeleton with 19 proteins for the molecular function category cytoskeletal protein binding (GO:0008092) shown in blue, 13 proteins for the cellular component category actin cytoskeleton (GO:0015629) shown in orange, 6 proteins for the cellular component category cortical cytoskeleton (GO:0030863) shown in pink and 21 proteins for the cellular component category cytoskeleton (GO:0005856) shown in yellow. These proteins are scattered across the

network; 5 of these nodes have 2 colours, 7 have 3 colours and 5 have all 4 colours. The cytoskeleton refers to the internal framework of cells including structures such as microfilaments, microtubules and the microtrabecular lattice. The cytoskeleton is vital for sustaining the shape of the cell but is also important for cellular functions such as movements and division [326]. Cytoskeletal protein binding encompasses any selective or non-covalent interactions with any protein component of any cytoskeleton. Changes to this likely alter the ASCs ability to migrate. The actin cytoskeleton refers to the parts of the cytoskeleton that are composed of actin and includes any associated proteins or complexes [327]. The cortical cytoskeleton is the part that is located beneath the plasma membrane. All of these cytoskeleton related proteins were detected in higher abundance in the traditional media, with both high and low abundance in the xeno free. This variation of detected abundance within the xeno free isolated ASCs is across both proteins and patients. This ultimately indicates that culturing with different media is impacting the levels of cytoskeletal proteins, and within the xeno free ASCs we are seeing substantial differences across patients.

65 nodes with 134 edges make up the STRING network of Cluster 4 of the ASC traditional media and xeno free whole cell lysate. This network in Figure 15 has some regions with slightly more interactions than others, but overall does not have any dense areas and has an average local clustering coefficient of 0.457. Three cellular component categories were highlighted, intracellular organelle (GO:0043229) shown in blue with 58 nodes, intracellular organelle lumen (GO:0070013) shown in orange with 35 nodes and intracellular membrane bound organelle (GO:0043231) shown in pink with 50 nodes. Intracellular organelles are structures within cells that have a distinct morphology as well as function, for example the nucleus, mitochondria, ribosomes and cytoskeleton. These intracellular organelle proteins were detected in high abundance in the traditional media ASCs and in low abundance in the xeno free ASCs, and this difference is not surprising considering previous clusters have already indicated that isolation and expansion with different mediums has affected intracellular organelles.

The final cluster contains proteins which are more abundant in traditional media with varying abundance in xeno free media ASCs. This STRING network is shown in Figure 16 and is made up of 119 nodes with 514 edges. The network has a dense well-connected core in the lower

right quadrant, but also has a lot of proteins without any interactions and an average local clustering coefficient of 0.473. 108 of these proteins are from the cellular compartment category cytoplasm (GO:0005737) as shown in blue. These proteins were detected in higher abundance in the traditional media ASCs, and again with varied abundance in the xeno free media ASCs. The reactome pathway category metabolism of proteins (HSA-392499) is highlighted on 47 proteins shown in orange, and the reactome pathway category metabolism (HSA-1430728) is highlighted on 33 proteins shown in pink. The reactome pathway category metabolism has previously been described in this chapter, however the metabolism of proteins specifically refers to the entire life cycle of a protein ranging from synthesis through to posttranslational modification and degradation [328,329]. The abundance of these proteins involved in cellular metabolism is reasonably consistent in the traditional media ASCs but varies substantially in the xeno free ASCs. This variability in the metabolism of xeno free ASCs across both patients and proteins is not expected, as we don't see the same level of variation in the same patient samples expanded in traditional media.

These differences between the xeno free and traditional media ASCs likely also impact their therapeutic potential. However, a particular exosome with therapeutic potential has been investigated in other stem cells and evidence suggest it's produced in both the xeno free and traditional media ASCs in this project. The exosome of interest is the 20S proteasome and proteins from this were detected in both the traditional and xeno free ASCs: proteasome subunit alpha type-1, proteasome subunit alpha type-2, proteasome subunit alpha type-3, proteasome subunit alpha type-4, proteasome subunit alpha type-5, proteasome subunit alpha type-6, proteasome subunit alpha type-7. The presence of proteasome subunit alpha type 1-7 suggests the presence of the 20S proteasome [191]. Extracellular proteasomes such as this likely play a vital role in degrading soluble peptides in extracellular fluids and preventing protein aggregation [330].

The heatmap for the membrane bound fractions of the traditional and xeno free ASCs has not been displayed or used for analysis, as the clusters on the horizontal axis based on patients did not sort into the relevant clusters. In fact, the first cluster consisted of 4 xeno free patients while the second cluster contained all other patient samples for both mediums. This is

indicative of the substantial differentiation between patients in the membrane fraction but cannot be interpreted in any further detail.

Figure 17 depicts the quantification of 27 secreted cytokines using a Bioplex assay. The standard deviation of the measurements across patients is indicated by the error bars, and this large standard deviation reflects the expected variation seen between patients. It is important to note that these samples were blanked against the neat media as the media itself contains cytokines, so the negative values are indicative of the cells consuming cytokines from the media rather than producing and secreting them. IL-5, GM-CSF, 1L-9, MIP-1β, FGF basic, IL-2, PDGF-BB and IL-17 were not detected in the xeno free ASC media. All other secreted cytokines with the exception of G-CSF were secreted at a lower level in xeno free ASC media when compared to the traditional ASC media. G-CSF is a proinflammatory cytokine [250]. Other proinflammatory cytokines like IL-6, IL-7,IL-11 are all secreted by ASCs but are detected in lower quantities in xeno free media than traditional media [250]. VEGF signalling pathway and TGF-β signalling pathway were shown in Figure 6 to have similar distributions of proteins between the fractions. These proangiogenic cytokines [267] were also secreted in lower quantity by the xeno free ASCs and it is difficult to determine from these differences in secreted cytokines which medium is optimal for ASC expansion. While the lack of cytokine secretions in the xeno free ASCs could indicate a lack of cell function, conversely most cells are grown in the presence of FBS, and this could potentially be negatively impacting the ASCs, causing them to secrete proinflammatory cytokines in response.

PDGF-BB was not secreted from xeno free ASCs but was from traditional media ASCs. However, proteins involved in PDGF signalling pathway were detected by LC-MS/MS in ASCs maintained in both mediums in Figure 6. While this might initially suggest that the absence of this cytokine from xeno free ASCs is detrimental, it may be desirable because while isoforms of PDGF such as PDGF-BB assist with growth, survival and motility of stem cells, PDGF-BB has also been shown to enhance the transmigration of ASCs toward malignancy [276]. There is some evidence that PI3-kinase drives stem cell migration to malignancy [276], and proteins from this pathway are present in both xeno free and traditional media ASCs as shown in Figure 6. This warrants further research into this cytokine and these two pathways in both traditional and

xeno free isolated ASCs, as they are already being delivered to patients as both unproven and clinical treatments.

IFN-γ was detected at lower levels in xeno free media than traditional media. It would be safe to assume that the ASCs with higher levels of IFN-γ would also have more detectable proteins in the IFN-γ signalling pathway. This was not the case as, in Figure 6, 65% of the proteins detected in both samples that are part of the IFN-γ signalling pathway were from the xeno free membrane bound fraction. If an assumption is made that the traditional media is the superior choice, then it could be inferred that a reduction in secreted IFN-γ coupled with the higher number of proteins involved in IFN-γ signalling is problematic. However, this assumption cannot be made. While the traditional media is the one that is used more broadly and has significantly more research behind it, it is not actually known if this is preferable, rather it is often favoured simply because it is readily available and well documented. If we remove this assumption and instead look just at the xeno free ASCs, it could be inferred that IFN-γ is being released and is then binding with the IFN-γ receptor and causing particular parts of the IFN-γ signalling pathway to upregulate, in this case the parts associated with membranes. IFN-γ is also known to be packaged and secreted in extracellular vesicles from stem cells, and this could also mean that more IFN-γ is being produced but simply isn't being detected in this experiment as it's being secreted in extracellular vesicles, which were not isolated. IFN-γ has also been described as a master checkpoint regulator for many cytokines, and it does this through activating STAT1 signalling and inducing the internalization of gp130 which is a component of many cytokine receptors [319]. Thus, the changes in this cytokine are likely linked to the absence of so many other secreted cytokines from the xeno free ASCs. Again, it cannot be assumed that the absence of these cytokines is necessarily indicative of poor cell health as there is not yet enough evidence.

Human platelet lysate has been used in other reported work as an alternative to FBS for expansion of ASCs and BMSCs. Both ASCs and BMSCs had the typical fibroblast-like morphology and expressed similar surface markers, however ASCs had a greater proliferative capacity than BMSCs. ASC and BMSCs expanded under these conditions had differences in secreted proteins such as IFN-γ, basic FGF-1 and stem cell-derived factor-1 [331]. FGF basic and basic FGF-1 are both ligands for FGF receptor 1. FGFs and FGF receptors are known to be

important in embryonic stem cells; furthermore, FGFs promote self-renewal and inhibit cellular senescence [332]. The absence of FGF basic in the xeno free cytokine secretions raises concern as this cytokine is known to have a vital role in stem cell function and has been well characterised across a variety of stem cells. This evidence suggests that traditional media may be preferable for maintaining ASCs over xeno free media.

4.2 Cryopreserved and fresh ASCs

The impact of cryopreservation on ASCs was investigated by comparing the proteomes of fresh and cryopreserved ASCs. This involved extracting ASCs from the liposuction aspirates of 5 patients. The ASCs that were not subsequently cryopreserved are referred to as fresh ASCs while those that were cryopreserved, revived and then cultured are referred to as cryopreserved ASCs. Both cell types were expanded to passage 5 and the proteins of the whole cell lysate were examined. This proteomic analysis utilised a 'shotgun' LC-MS/MS approach, where peptides generated by trypsin digestion of the proteins were separated by nanoflow reversed phase chromatography and analysed by electrospray tandem mass spectrometry using a benchtop Orbitrap system (Thermo Q Exactive Plus). As mentioned previously, this type of shotgun LC-MS/MS experiment allows the identification and quantification of the products of open reading frames, hence referred to as 'proteins', rather than intact proteoforms. 2326 proteins were conserved in all 5 patients. The distribution of these proteins is visualised in Table 2. The fresh whole cell lysate saw a 58.03% reduction in number of detected proteins when only looking at those conserved across all 5 patients, while the cryopreserved fraction saw a reduction of 64.53%.

When discussing presence and absence of proteins it is important to recognise that only those proteins that are detectable and conserved across all 5 patient samples are retained after filtering. This means that the protein may not be absent from the ASCs but simply were not present in all patient samples, or alternatively were below the limit of detection in one or more patient samples. Figure 18 visually demonstrates the distribution of these proteins that are conserved in all 5 patient samples in a proportional Venn diagram. 838 proteins were unique to the fresh whole cell lysate, 404 were unique to cryopreserved whole cell lysate and

1084 proteins were detected in both fresh and cryopreserved whole cell lysate. Further insight on the distribution of these proteins was achieved through the use of the PANTHER Classification System which categorises the proteins by molecular function, cellular compartment, biological process, protein class and protein pathway [202].

Figure 19 is sorted by categories of catalytic activity. It can be seen that for all categories of catalytic activity there were more proteins detected in the fresh whole cell lysate rather than in the cryopreserved whole cell lysate. This is reflected in the distribution of proteins seen in the proportional Venn diagram in Figure 18 where 1084 proteins were detected in both fresh and cryopreserved ASCs, 838 proteins were unique to the whole cell lysate of fresh ASCs and 404 proteins were unique to the whole cell lysate of cryopreserved ASCs. The same trend is also seen in Figure 20 which is sorted by categories of cellular process, with the exception of cell proliferation (GO:0008283) and rhythmic process (GO:0048511) where proteins are only found in the fresh whole cell lysate but only a single protein is present in each category. Proteins sorted by categories of compartment (Figure 21) followed the same trend, with more than 50% of proteins coming from the fresh whole cell lysate. In Figure 22, which is sorted by categories of protein class, there is one category that does not follow the previously observed trend. Structural protein (PC00211) has 4 proteins in the cryopreserved whole cell lysate and only 2 in the fresh whole cell lysate. These proteins are extracellular proteins that form a structure. There are 122 categories in Figure 23 which is sorted by categories of pathways. Only 7 of these 122 pathways have greater than 50% of the detected proteins from the cryopreserved whole cell lysate, and all 7 are only by a single protein.

Proteins that were unique to the fresh ASCs or unique to the cryopreserved ASCs were visualised in their own STRING diagrams. The STRING network for proteins unique to fresh ASCs is shown in Figure 24, which has 525 nodes and 3841 edges. This network is crowded with three dense zones indicating that subsets of proteins are highly connected, but overall the network has a relatively low average local clustering coefficient of 0.323. 20 of these proteins highlighted in blue are from the KEGG pathway category regulation of actin cytoskeleton (hsa04810) and these are located in the dense portion of the network on the lower left quartile. The cytoskeleton is made up of proteins that maintain the structural integrity of the cell and play a role in cellular functions like movements and division [326]. The

actin cytoskeleton refers to the parts of the cytoskeleton that are composed of actin and includes any associated proteins or complexes, making up the main component of the cytoskeleton [327]. It is interesting that proteins involved in this pathway that are vital to the structural integrity of the cell were only detected in the fresh ASCs and not in the cryopreserved ASCs, demonstrating that cryopreservation impacts the actin cytoskeleton of ASCs. The reactome pathway category metabolism of RNA (HSA-8953854) is shown in orange with 59 proteins that are predominantly found in the largest dense region of the network located in the lower right quartile. This pathway involves the modification of RNA transcription products and the regulation of this process [333]. This suggests that cryopreservation alters the ASCs ability to metabolise RNA. The reactome pathway category membrane trafficking (HSA-199991) is shown in pink, and these 48 proteins are located in the same region as the KEGG pathway category regulation of actin cytoskeleton, but only 3 proteins are shown to be functionally enriched for both of these pathways. Membrane trafficking occurs within the secretory membrane system. This system is utilised for the transport of molecules that have been recently synthesised within the cell, and out of the cell. Organelles such as the endoplasmic reticulum, golgi complex, plasma membrane, and tubulovesicular transport intermediates are employed to transport the cargo through specific directional routes towards the cell surface [334]. The lack of detection of proteins in this pathway therefore suggests that cryopreserved ASCs have a reduced ability to regulate membrane trafficking. 21 proteins are highlighted in yellow for the reactome pathway category citric acid cycle and respiratory electron transport (HSA-1428517). These proteins are found in the dense region at the top of the network and are involved in the metabolism of pyruvate which generates acetyl-CoA, which is used in the citric acid cycle to produce energy and the reducing equivalent NADH. Following this NADH is re-oxidised to NAD+ in the electron transport chain and protons are exported across the inner mitochondrial membrane, consequently compelling ATP synthesis. This data suggests that this pathway is altered in ASCs after cryopreservation or that the cells are using alternative sources of energy from the same media. To further investigate, a metabolomics experiment targeting the metabolites of these and alternative pathways could be conducted to determine whether alternative energy processing pathways are being utilised.

The proteins unique to cryopreserved ASCs were put into the STRING network as shown in Figure 25. There are 180 nodes with 465 edges, and the network is quite dispersed with a denser core and many proteins without any interactions on the outskirts, the network having an average local clustering coefficient of 0.397. The biological process category cellular metabolic process (GO:0044237) is shown in blue with 124 proteins highlighted. These proteins are scattered across the whole network and are involved in chemical reactions and pathways that allow cells to transform chemical substances. This suggests that the shift in phenotype that we see in ASCs after cryopreservation is predominately a metabolic change, supporting observations made in the previous paragraph. 12 proteins are highlighted in orange for the KEGG pathway RNA transport (hsa03013), and these are found on the outskirts of the dense inner region of the network. This pathway covers transport of RNA from the nucleus to the cytoplasm which is vital for gene expression. Different types of RNA are exported out of the nucleus and have specific receptors that aid their transport. The appearance of proteins within the cryopreserved ASCs that were not detectable in fresh ASCs that are involved in RNA transport suggests that different RNA is being produced that require distinctive specific receptors. This is further supported by the 21 proteins highlighted in pink from the reactome pathway category metabolism of RNA (HSA-8953854). Proteins involved in this pathway are modifying RNA transcription products and regulate this process [333]. In fact, this pathway was highlighted in the STRING network for proteins unique to fresh ASCs, further reinforcing that the difference observed due to cryopreservation specifically impacts the metabolism of RNA. Furthermore, this also demonstrates the impact on transport of RNA within the cell. Seeing unique proteins in the cryopreserved ASCs involved in metabolism of RNA reinforces that cryopreservation is potentially altering the cellular RNA.

Proteins that were detected in both the fresh and cryopreserved ASCs were compared using a clustered heatmap. This was generated using the output from PEAKS which provides quantitative proteomics based on the measurement of the area under the curve for a peptide's intensity in the MS1 scan. In Figure 26 the 5 patient samples shown in individual columns formed two clusters that reflect whether they were fresh or cryopreserved. This hierarchical clustering based on protein abundance values into these two distinct groups reinforces the clear difference in these ASCs, even in the abundance of the common proteins that were detected in both fresh and cryopreserved ASCs. Proteins are shown in each row

and are sorted into 6 clusters which are further interrogated with STRING networks. Each protein is coloured by its Z score, a statistical measurement of variation from the mean. A Z score of 0 indicates the value is the same as a mean, while a positive score indicates a value above the mean, and a negative indicates a value bellow. The Z scores in the fresh ASCs clusters are more homogenous, while the Z scores in the cryopreserved ASCs vary substantially between both proteins and patients.

Cluster 1 was placed into a STRING network that has 56 nodes and 117 edges with an average clustering coefficient of 0.394 as seen in Figure 27. Two biological process categories were highlighted, the cellular component category organisation or biogenesis (GO:0071840) shown in blue on 37 proteins, and of those 35 are also highlighted orange for differing cellular component category organisation (GO:0016043). Cellular component category organisation refers to the assembly or disassembly of the constituent parts of a cell, and biogenesis is referring to the biosynthesis of these cellular constituents. The proteins involved in this pathway had higher abundance in the fresh ASCs with extremely varied abundance detected in the cryopreserved ASCs. This indicates that cryopreservation causes a change in abundance of proteins involved in organisation and biogenesis, and that the specific change we see varies between patients.

66 proteins with 234 edges make up the STRING network for proteins detected in Cluster 2 of fresh and cryopreserved ASC whole cell lysate. This network in Figure 28 has a dense region in the top right quarter and an average local clustering coefficient of 0.507. Two biological process categories were highlighted, in blue organonitrogen compound biosynthetic process (GO:1901566) and in orange organonitrogen compound metabolic process (GO:1901564). It is interesting that in Cluster 1 the cellular component category biosynthesis was highlighted, while in Cluster 2 the proteins are specifically involved in the biosynthesis of organonitrogen compounds. There are 36 proteins highlighted orange, and of those 19 are also blue. For a protein to be classified into the category of organonitrogen compound biosynthetic process it must be reported to participate in chemical reactions and pathways that result in the creation of an organonitrogen compound, while the metabolic process simply refers to proteins that participate in chemical reactions or pathways involving organonitrogen compounds. The proteins involved in these processes were detected in higher abundance in

the fresh ASCs with varying but overall slightly lower abundance in cryopreserved ASCs. This suggests that cryopreservation potentially reduces ASCs ability to biosynthetically process or metabolise organonitrogen compounds, further suggesting a reduction in metabolic fitness.

The STRING network for Cluster 3 of fresh and cryopreserved ASC whole cell lysate are shown in Figure 29. This network has 81 nodes and 228 edges. There are many proteins without interactions and a well-connected region in the lower right quartile, and the network has an average local clustering coefficient of 0.466. Two biological process categories were highlighted, cellular macromolecule catabolic process (GO:0044265) shown in blue and macromolecule catabolic process (GO:0009057) shown in orange. There are 17 orange proteins, 16 of which are also blue, these are mostly located in the dense core of the network. Macromolecule catabolic process encompasses chemical reactions and pathways that breakdown macromolecules, and examples of cellular macromolecules are proteins, nucleic acids, lipids and carbohydrates. Proteins involved in the catabolism of macromolecules were detected in low abundance in fresh ASCs and with varying but overall higher abundance in cryopreserved ASCs. This suggests that cryopreservation leads to an increase in catabolism of macromolecules in ASCs and when taking this information in the context of the functional enrichments of other clusters, it is likely that the macromolecule being catabolised is RNA.

Proteins from Cluster 4 of fresh and cryopreserved ASC whole cell lysate are seen in a STRING network in Figure 30. The network has 29 nodes and 57 edges, and this network is well connected with a high average local clustering coefficient of 0.7. While Cluster 3 alluded to catabolism of RNA, 6 proteins in the dense core of Cluster 4 are highlighted in blue for the biological process category nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (GO:0000184). This process degrades mRNAs that have premature stop codons [335,336]. The proteomic evidence keeps on pointing to cryopreservation impacting RNA and its metabolism. In this case, the 6 proteins are also a part of the 8 proteins highlighted in orange for the biological process category nucleobase-containing compound catabolic process (GO:0034655). This process encompasses chemical reactions and pathways that breakdown nucleobases, nucleosides, nucleotides and nucleic acids. The proteins involved in both of these biological processes were detected at varying levels in both fresh and cryopreserved ASCs, but there was greater similarity between patients in the fresh ASCs. This demonstrates

that cryopreservation changes the ASCs nonsense-mediated decay of mRNA and ability to breakdown compounds that contain nucleobases. Interestingly, the 6 proteins in pink were previously detected in a publication on the proteomic analysis of endothelial cell cold adaption by Zieger *et al.*[317] While these are very different cells, it is interesting that proteins involved in cold adaption in endothelial cells were also detected in our cryopreserved ASCs and implies that the same process is occurring in the cryopreserved cells.

The STRING network of Cluster 5 of fresh and cryopreserved ASC whole cell lysate is shown in Figure 31. This network has an average local clustering coefficient of 0.419 and is made up of 41 nodes and 51 edges. 20 of these proteins are highlighted in blue for the biological process category response to organic substance (GO:0010033) and of these 16 are also highlighted for the biological process category cellular response to organic substance (GO:0071310) as shown in orange. These processes refer to changes in state or activity in response to an organic substance [337]. These proteins were detected in low abundance in fresh ASCs with differing but mostly higher abundance in cryopreserved ASCs. The higher abundance of these proteins in cryopreserved ASCs may be a result of the cryopreservation process which utilises the organic substance, or more specifically the organosulfur compound, dimethyl sulfoxide.

The final STRING network was for the proteins detected in Cluster 6 of fresh and cryopreserved ASC whole cell lysate. Figure 32 has 31 nodes with 36 edges and an average local clustering coefficient of 0.426. 20 proteins highlighted in blue are from the biological process category transport (GO:0006810), and 17 of those are also highlighted in orange for the cellular component category cytoplasmic vesicle (GO:0031410). The biological process category transport is defined as the directed movement of substances of cellular components within a cell, into a cell, out of a cell or between cells. These substances could be transported by cytoplasmic vesicles, so it is not surprising that these proteins are functionally enriched for this biological process and cellular compartment. Proteins detected in this cluster are more abundant in fresh ASCs with differing abundance in cryopreserved ASCs, suggesting that the change from cryopreservation leads to a potential reduction in cellular vesicles and in turn transport within the cell.

While the cryopreserved whole cell lysate has 5 biological replicates, it only has 1 technical replicate. All other samples have 3 technical replicates, and this discrepancy is the result of issues with the mass spectrometer that prevented repeated analysis before this thesis was due for submission. Because the samples were only able to be analysed as a single injection rather than in technical triplicate, the correlation plots allow visualisation of the similarity between biological replicates from different patients, the comparison across patients within each treatment (fresh and cryopreserved) and between treatments validate the comparison because the correlation values are close to 1.

4.3 Future directions

The previous chapter demonstrated the advantages of characterising the proteins from different fractions of the cell. For this reason, the cryopreservation experiment should also investigate membrane bound proteins, extracellular vesicles and secreted cytokines, while the xeno free experiment should also examine the extracellular vesicle proteome. Doing so would also provide some clarity on the function of IFN-γ in the xeno free ASCs and to examine if the cells are packaging IFN-γ into extracellular vesicles under these different culture conditions.

In Figure 10 it was suggested that the different media used for isolation and expansion of ASCs could cause changes within the membranes of extracellular vesicles. Therefore, in the future, collection and examination of xeno free expanded ASC extracellular vesicles, and in particular the investigation of their membranes, should be conducted. As discussed in the previous chapter, the membrane fractionation applied in this research could be applied to extracellular vesicles, or alternate protocols are also available.

There are pathways such as PGBF-BB and PI3-kinase that were highlighted in Figure 6 from traditional and xeno free isolated ASCs. This requires significantly more research as these pathways are linked to malignancy, and ASCs are already being delivered to patients as both unproven and clinical treatments. It will be difficult to study this, as it is exceptionally challenging to create a model that successfully replicates the complexity of the

microenvironment of a tumour [275]. However, it is vital for this research to be conducted. Conversely, there were some positive discoveries regarding the therapeutic application of stem cells such as the detection of the subunits of the 20S proteasome in traditional media and xeno free ASCs. The 20S proteasome is secreted in an exosome with known therapeutic potential and thus warrants isolation from ASCs and further investigation [191].

Both xeno free and cryopreserved ASCs appeared to differ substantially by the abundance of the proteins involved in the alteration of the metabolome. To further investigate this, a metabolomics experiment targeting the metabolites of the particular highlighted pathways and alternative pathways could be conducted to determine whether alternative energy processing pathways are being utilised. Furthermore, broad RNA sequencing and analysis of RNA amplification could elucidate what these specific metabolic changes are.

In Figure 31, it would seem that the cryopreservation process which utilises the organic substance, or more specifically the organosulfur compound, dimethyl sulfoxide, appears to be affecting the cells as the abundance of proteins related to the processing of organic compounds is altered. To further investigate the effect of dimethyl sulfoxide on ASCs, a wider range of concentrations can be tested. Cryopreservation has also affected membrane trafficking in ASCs and isolating and extracting the membrane fraction either with the method used in chapter 2, or more specific methods as discussed in chapter 2 will provide more insight. Additionally, the extracellular vesicles from cryopreserved cells should be isolated and investigated, particularly because proteins involved in membrane trafficking and secretion of molecules are absent from cryopreserved cells as highlighted in the STRING network of detected proteins unique to fresh ASC whole cell lysate.

5. Conclusions

ASCs were isolated from fresh patient lipoaspirates and cultured in either traditional or xeno free media. The traditional media ASCs were either expanded as fresh cells or were cryopreserved and subsequently revived and expanded. The proteome of traditional and xeno free isolations, and fresh and cryopreserved ASCs were compared. This was done to close the gap in the lack of standardisation of procedures regarding ASC isolation and expansion. Xeno free media was investigated as FBS is known to be highly variable and consequently is poorly defined [294], and the use of bovine derivatives can also result in bacterial, viral and prion infections [23,295,296]. Cryopreservation was analysed as both fresh and cryopreserved ASCs are utilised as clinical treatments [311], yet most research is based on cryopreserved ASCs as these are easier to store. As xeno free, traditional media, fresh and cryopreserved ASCs are regularly administered to patients in both proven and unproven stem cell treatments, it is vital to understand the impact these isolation and storage techniques have on ASCs.

The comparison presented in this work demonstrates that a fundamental understanding of the changes that can occur in cellular phenotype due to something as routine as a media change is lacking and that clinical application of autologous stem cell treatments should not be applied until comprehensive understanding is gained. This is not just a problem for stem cell research, but for any research or experiment that requires a change in media, such as moving to serum free media to collect cell secretions. When comparing traditional and xeno free expanded ASCs there is a clear shift in the proteome, though it is difficult to determine which isolation and expansion medium is preferable. Because the bulk of scientific research has been conducted on traditional media, most of our understanding is based on ASCs isolated in this media. However, it cannot be presumed that traditional media is the superior choice, as there is not enough data to support this. The proteome across patients of cells isolated and maintained in traditional media ASCs was much more homogenous than that of the xeno free isolated ASCs. When investigating the shift in the proteome of these ASCs, it was clear that it was largely a metabolic shift. There were measurable differences in proteins involved in transcription, translation, and the synthesis and degradation of proteins.

Furthermore, the cytokine profile is wildly different and the absence of some important stem cell cytokines such as FGF basic suggests that traditional media may be preferential, however as clearly stated in the future directions substantially more research is required to validate this.

Most research is conducted on cryopreserved ASCs, as it is common practice to store these cells. However fresh ASCs are being used in the clinic, and it is therefore very important to understand the impact that cryopreservation has on ASCs as both fresh and cryopreserved ASCs being administered to patients [338]. A shift could be seen in the proteome of ASCs after cryopreservation, though this shift is not as pronounced as the one observed between traditional and xeno free ASCs. The data also indicates a change in the metabolome as the data suggests that particular pathways are being altered in ASCs after cryopreservation or that the cells are using alternative sources of energy from the same media. Ultimately cryopreservation of ASCs appears to be reducing metabolic fitness. However, as stated in the future directions this would require further validation.

What this research has clearly highlighted is that isolating and expanding ASCs in different media, and maintaining them as either fresh or after cryopreservation, causes substantial changes in the proteome and it appears these changes are likely metabolic. These differences require significantly more research as these ASCs are already being used in both registered clinical trials and in unproven stem cell treatments. In order to attain clinical success of ASC treatments researchers, industry, physicians and regulatory agencies will need to work together [339].

6. Supplementary figures

Normalised protein abundance values for each group of technical replicate samples was individually compared to each other group of samples. If the abundance of the same protein from each group is the same, the dot representing that protein will fall on the diagonal line. If all proteins in the compared samples have the same abundance, the correlation value will equal 1. Individual patient samples represent biological replicates and the correlation value for the same sample from each patient should be as close as possible to 1.

Supplementary Figure 1: Correlation plots of normalised protein abundances of traditional and xeno free ASC whole cell lysate

Supplementary Figure 2: Correlation plots of normalised protein abundances of fresh and cryopreserved ASC whole cell lysate.

Chapter Four

Don’t tryp: the kynurenine pathway in human adipose stem cells

1. Introduction

1.1 Kynurenine pathway

Tryptophan is one of 9 essential amino acids that cannot be synthesised by cells and must therefore be acquired elsewhere, typically through dietary intake. Synthesis of tryptophan is not energetically favourable and even organisms that can synthesize tryptophan will favour acquiring it over synthesizing it [340,341]. Once tryptophan has been obtained and absorbed, it travels through the peripheral circulation in its free form or bound to albumin [342]. A competitive non-specific L-type amino acid transporter transfers the free form of tryptophan across the blood brain barrier [343]. Once in the central nervous system, tryptophan is a precursor for a variety of metabolic pathways that can result in the creation of proteins, metabolites involved in the kynurenine pathway (metabolites) and serotonin of which tryptophan is a precursor for [344,345].

The catabolism of tryptophan can occur through a variety of pathways, however, the kynurenine pathway accounts for the metabolism of more than 95% of tryptophan [346]. As shown in Figure 1 indoleamine 2,3-dioxygenase (IDO) is an enzyme that degrades tryptophan whose expression is generally induced by the pro-inflammatory mediator interferon-γ (IFN-γ) [340,341]. IDO-1 expression is generally regulated by inflammation and the capacity of immune cells like T and Natural killer cells to release IFN-γ. Another variant, IDO-2 is expressed in a range of tissues, and like IDO-1, is also capable of metabolising tryptophan [347]. Another enzyme, tryptophan 2,3-dioxygenase (TDO2) is also capable of the same enzymatic reaction, yet the expression of the enzyme tends to be more constitutive and expression levels do not respond greatly to IFN-γ. With these two enzymes, the body has the means to precisely regulate bioavailable tryptophan in homeostasis, however with persistent, chronic

inflammation there is a breakdown of this delicate balancing act, and in general, too much of the potent neurotoxin quinolinic acid is produced for the body to be able to be able to convert it to the essential co-factor NAD+. The enzyme quinolinate phosphoribosyl transferase mediates the first step of quinolinic acid breakdown, however it is easily saturated at levels above 50nM. In circumstances of chronic inflammation, where quinolinic acid levels are likely to rise through activation of the kynurenine pathway, are therefore likely to pose a challenge to susceptible tissues and organs as quinolinate phosphoribosyl transferase will not be able catabolise quinolinic acid fast enough to prevent its accumulation

When the immune system is activated IFN-γ is secreted by leukocytes and activated T cells, which induces the expression of IDO-1 and ultimately leads to an accelerated and sustained depletion of tryptophan. Creation of kynurenines, thereby reducing bioavailable tryptophan for protein synthesis and proliferation are critical in modulation of the immune response [348].

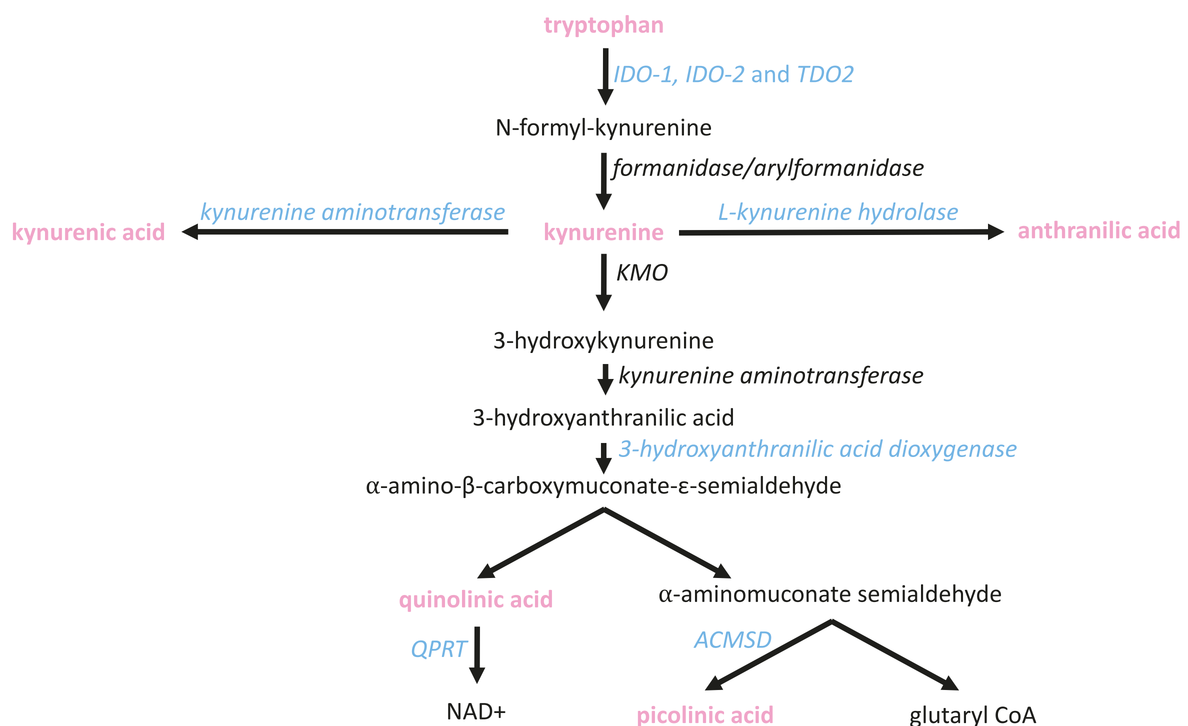

Figure 1: Kynurenine pathway

The kynurenine pathway is shown in Figure 1 with the metabolites investigated in this chapter highlighted in pink and the key enzymes italicised and in blue.

Increased degradation of tryptophan and an increase in kynurenines have been detected in many diseases or disorders including Alzheimer's [349,350], amyotrophic lateral sclerosis [351,352], dementia [353], Huntington's [354,355], multiple sclerosis [356], HIV/AIDS [353], schizophrenia [357] and neoplasia. Depletion of tryptophan is also associated with aging, but substantial depletion of tryptophan is associated with neurodegenerative diseases [358,359]. The kynurenine pathway appears to be dysregulated in multiple sclerosis [360,361]. Tryptophan catabolites and their derivates are utilised as a strategy to treat T helper-1 mediated autoimmune diseases like multiple sclerosis [362].

Because of the role that kynurenine has in these diseases, modulating this pathway has therapeutic potential [360]. Kynurenines that are involved in the suppression of T cell proliferation appear to target immune cells when they are being activated [363], in addition to this they likely work together to exert this effect [364]. Quinolinic acid and the kynurenine pathway are involved in both inflammatory and non-inflammatory neurological disease [365]. The accumulation of quinolinic acid, kynurenic acid and L-kynurenine in the central nervous system appears to be associated with inflammatory diseases. For example, in multiple sclerosis dysregulation of the kynurenine pathway results in high levels of quinolinic acid which is a potent neurotoxin.

The cleavage of the indole ring leads to the oxidisation of tryptophan and this is initiated by TDO2, IDO-1 or IDO-2. TDO2 is typically produced in the liver [366] but has also been detected in the central nervous system [367]. TDO2 is induced by corticosteroids and tryptophan [366]and can be inhibited by several things including IDOs [368]. IDO-1 can be found in macrophages, microglia and neurons and is activated by IFN-γ and has been noted to have immunosuppressive properties on T lymphocytes [369-371]. While IDO-2 has a similar structure to IDO-1 it differs in its expression pattern and signalling pathway [372]. Neurons and activated macrophages and microglia express all the enzymes involved in the kynurenine pathway, while astrocytes lack some enzymes such as kynurenine 3-monooxygenase (KMO) and IDO-1 [356,373]. Cells that express IDO produce kynurenines and are therefore immune privileged, however the kynurenine pathway has also been reported to contribute to cancer cells ability to escape the immune system [374].

Most kynurenines are neuroactive [360]. Kynurenine is capable of immune regulation through its ligand function for Aryl hydrocarbon receptor while kynurenic acid is neuroactive and is a NMDA antagonist [348,375,376]. When in low concentrations kynurenic acid interacts with the glycine modulatory site of the NMDA receptor [366], while at high concentrations it interacts with the glutamate site of the NMDA receptor as well as the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor [377]. Kynurenic acid is also an antagonist to quinolinic acid and has been detected in mammalian brains [378]. While there is insufficient evidence to demonstrate that kynurenic acid is neuroprotective, there is some evidence that suggests it may be. For example, it has been shown to increase proliferation of glial cells while also inhibiting the release of neurotrophic fibroblast growth factor [379]. Kynurenic acid has receptors in the peripheral and nervous tissues where it regulates cell activation and survival [380]. Initial studies determined that increases in kynurenic acid in the brain was had sedative and anticonvulsant activity [381]. While levels of kynurenic acid in the brain are low, the levels at specific sites are likely enough to effect glutamate receptors [382]. Kynurenic acid has been shown in rats to inhibit the release of glutamate [383] and to affect extracellular levels of dopamine [384]. It can also protect against brain ischemia [385]. Kynurenic acid levels in the cerebrospinal fluid have been reported to be elevated in schizophrenic patients [357,386]. Amongst the other products of the pathway, anthranilic acid is important for NAD+ synthesis in neuronal cells but has also been shown to be neurotoxic [387]. 3-hydroxykynurenine is neuroactive [348] while 3-hydroxyanthralic acid, which can be derived from the oxidation of anthranilic acid or from the hydrolysis of 3-hydroxykynurenine [348], is a free-radical generator [341], and can supress T cell proliferation [388]. Quinolinic acid is a heterocyclic amino acid that is capable of selectively activating glutamate receptors of neuronal NMDA [389]. Quinolinic acid is an excitotoxin and N-methyl-D-aspartic acid (NMDA) agonist [389], and can supress T cell proliferation [388], and is involved in the activation of the immune response as it is detected in large quantities after inflammation [390].

During inflammation in the brain, it is macrophages, microglia and dendritic cells that produce most of the elevated levels of quinolinic acid [391-393]. Astrocytes and neurons catabolise quinolinic acid to NAD, however saturation of this system leads to the toxic accumulation of quinolinic acid [358]. Quinolinic acid is a neurotoxin, gliotoxin, proinflammatory mediator, pro-oxidant molecule and has the ability to affect the integrity of the blood brain barrier [394]

quinolinic acid can cause neuronal cell death [395,396], and even with chronic low levels causes ultrastructural abnormalities in neurons reminiscent of those found in dementia [397]. Understandably it is involved in many neurodegenerative disorders [358,359,398]. *Picolinic acid* is a monocarboxylic acid and a neuroprotectant [399]. Picolinic acid blocks quinolinic acid through the attenuation of calcium dependent glutamate release or chelating endogenous zinc [400-402]. Picolinic acid blocks the neurotoxic but not neuroexcitatory properties of quinolinic acid in the brain [403]. Human neurons can produce picolinic acid [404]. Picolinic acid and IFN-γ have an antagonistic effect on macrophage inflammatory protein 1 α / β production [405]. Picolinic acid can reversibly inhibit cell growth [406].

1.2 Kynurenine pathway in stem cells

Stem cell therapies are being investigated for many of the diseases that the kynurenine pathway is involved in, and there has been some investigation into this pathway in stem cells. However, with the exception of bone marrow stem cells (BMSCs), the kynurenine pathway is not well characterised in stem cells. What is known is that Kynurenines and kynurenine enzymes are expressed in human embryonic stem cells (ESCs) [407]. While ESCs supress the survival and proliferation of T cells, there was no detectable change in IDO [408]. In human ESCs, derived mesenchymal stem cells that were stimulated with IFN-γ had significantly higher detectable levels of IDO activity [409]. It was determined that decreased T cell function following hematopoietic stem cell (HSC) treatment is likely due to the production of IDO [410]. Furthermore IFN-γ treatment of CD34+ HSCs resulted in the production of kynurenine. It is suggested that IFN-γ mediated activation of the kynurenine pathway haematopoiesis [410].

Human and mouse neuronal stem cells (NSCs) also express the entire kynurenine pathway [411]. IFN-γ induces high levels of IDO mRNA and protein in human NSCs [412]. Interlukin-1β (IL-1β) also regulated the kynurenine pathway in human hippocampal neurogenesis. Specifically, IL-1β induced upregulation of IDO, kynurenine 3-monooxygenase and kynureninase which are all involved in the neurotoxic aspects of the kynurenine pathway. Furthermore, treatment with IL-1β caused an increase in kynurenine [413]. Mice deficient for TDO showed increased levels of tryptophan, kynurenine and 5-hydroxyindoleacetic acid in plasma, and an increase

in serotonin and 5-hydroxyindoleacetic acid in the hippocampus and midbrain. TDO deficiency induced proliferation of neural progenitors and NSCs in the subventricular zone [414]. And in a 3D NSC model for Alzheimer's disease it was discovered that administering Alzheimer's disease associated amyloid-β42 results in reduction of NSC plasticity through the production of kynurenine acid. This can be restored by the addition of IL-4 which supresses kynurenine aminotransferase which is the enzyme responsible for the production of kynurenic acid [415]. Placental mesenchymal stem cells are both functionally and phenotypically similar to BMSCs and it appears that IDO is an important mediator in their immunosuppressive capabilities [416].

There is a vast amount of literature investigating the kynurenine pathway in BMSCs. Human BMSCs express IDO and when stimulated with IFN-γ these cells exhibit IDO activity [417]. When co-cultured with mixed lymphocytes BMSCs inhibit the allogenic T cell response. In these co-cultures, there is depletion of tryptophan and production of kynurenine. IDO mediated catabolism of tryptophan has an inhibitory effect on T cell mechanism in human BMSCs [417]. In regard to their therapeutic use, modulation of IDO could affect the immunosuppressive properties of BMSCs as IDO mediated T cell inhibition is dependent on the activation of BMSCs [417]. IFN-γ induced the expression of IDO in BMSCs, and this was confirmed by the use of the antagonist 1-methyl-L-tryptophan which restored alloresponsiveness. Furthermore, the addition of kynurenine to mixed lymphocyte reactions resulted in alloproliferation being blocked, suggesting that IDO doesn't exert its effect through the depletion of tryptophan, rather it does so through the local accumulation of bioactive kynurenines [418].

Co-cultures of BMSCs and T lymphocytes with and without cell contact resulted in significant upregulation of IDO and, in cultures with cell contact, a significantly higher level of IL-10 and TGF-β. Cultures without contact had greater inhibitory effects of T cell proliferation and this was correlated the gene expression of IDO [350]. BMSCs are able to supress the proliferation of natural killer cells, CD4+ and CD8+ T lymphocytes but not B lymphocytes. This was not contact dependent, rather it required IFN-γ which was produced by activated T cells and natural killer cells. When IFN-γ was added exogenously, BMSCs were able to also supress B cells because IFN-γ causes BMSCs to produce IDO [419]. There are some contradictory studies, such as an

alloimmune model where BMSCs supressed the survival and proliferation of T cells, however there was no detectable change in IDO [408].

IDO is an important mediator in BMSC induced inhibition of natural killer cells [420]. The production of IDO by BMSCs is induced directly by IFN-γ exposure but also indirectly by autocrine stimulation of cells by prostaglandin E2 [420,421]. Proliferation of naïve and pre-activated T cells can be supressed by soluble mediators in BMSC cell culture supernatant of which IL-10 and IDO play an important role [422]. There are also studies that challenge the notion that IFN-γ induced IDO expression is required for BMSC immunosuppression. Such as the inability of IDO inhibitors to restore peripheral blood mononuclear cells, and human BMSCs with IFN-γ receptor 1 defects inhibited proliferation of peripheral blood mononuclear cells at a similar rate to controls. BMSCs can exercise their immunomodulatory functions even in the absence of IFN-γ receptor 1 signalling and IDO [423]. The immunosuppression of T lymphocytes by BMSCs is mediated IFN-γ [424]. Treatment of BMSCs with IFN-γ resulted in up regulation in the expression of IDO [424]. The kynurenine pathway is important in the differentiation of mouse BMSCs into osteoblasts, and the addition of exogenous IFN-γ accelerates this [425]. Mice that lacked IDO-1 activity were osteopenic meaning their bones were weaker than normal [425]. Kynurenine and other amino acid oxides were able to inhibit BMSC proliferation and the expression of alkaline phosphatase. The expression and activity of the osteogenic markers osteocalcin and Runx2 were also inhibited by BMSCs [426]. IDO-1 is present in BMSCs however compared to osteoclasts, the detectable levels are lower. Kynurenine treated BMSCs in osteogenic culture result in a reduction in the expression of Runx2 [159].

While the kynurenine pathway is evolutionarily conserved there are significant species differences in its expression. Even though IDO-1 can be induced by IFN-γ in mouse BMSCs, it doesn't result in catabolism of tryptophan *in vivo* [427]. Mouse BMSCs do not mediate suppression of T cells through IDO-1 as human BMSCs do [427]. Human and mouse BMSCs express the entire kynurenine pathway including IDO-1 and IDO-2 transcripts [411]. IFN-γ inhibited proliferation of human and mouse BMSCs while also effecting their ability to differentiate via the activation of IDO [411] while mouse BMSCs are able to attenuate liver fibrosis through the suppression of Th 17 cells [428]. In a mouse model of multiple sclerosis

called experimental autoimmune encephalomyelitis, BMSCs were able to ameliorate experimental autoimmune encephalomyelitis via IDO mechanisms. BMSCs treated experimental autoimmune encephalomyelitis mice had increased production of IFN-γ and induction of IDO in CD11c+ dendritic cells [429]. Depletion of Tryptophan reduces the ability of autoreactive T cells to proliferate and is therefore protective in acute relapse phases of experimental autoimmune encephalomyelitis. BMSCs are used to regulate lupus and it was found that CD8+ T cell / IFN-γ / IDO axis mediates the therapeutic effects of BMSCs in lupus patients [430]. BMSCs have *ex vivo* immunosuppressive properties on Crohn's disease mucosal T cells and this is predominantly due to IDO activity [431].

1.3 Kynurenine pathway in adipose stem cells

Adipose stem cells (ASCs) are another source of adult stem cells, however instead of being sourced from bone marrow like BMSCs they are sourced from adipose tissue [24]. ASCs are similar to BMSCs in that there are no significant differences in morphology or phenotype between ASCs and BMSCs [25], however ASCs have been shown to have higher proliferation rates [26]. Adipose tissue is a complex endocrine organ [155], and enzymatic digestion of this tissue yields a population of cells termed the stromal vascular fraction, which contains ASCs [19,20,158-160]. ASCs are capable of mesodermal differentiation and have the capacity to transdifferentiate along endodermal and ectodermal lineages [432] and thus ASCs are utilised in both research and therapeutic contexts, though it is important to recognise they are currently being exploited in unproven stem cell treatments being offered by predatory clinics around the globe.

While it has been established that the kynurenine pathway plays a significant role in BMSCs, there has been some research into this pathway in ASCs which has mainly focussed on immunomodulation in association with immune cell types. It has been shown that kynurenine mediates immunosuppression by ASCs [159]. ASC immunomodulatory properties are mediated by tryptophan catabolism [433]. IFN-γ mediated IDO expression is required for the modulation of lymphocyte proliferation by human ASCs [434]. The IFN-γ/IDO axis has been found to be essential in human ASCs [434] and IFN-γ activated ASCs have an increased ability to inhibit the

proliferation of T lymphocytes [435]. IDO-1 gene expression and the concentration of kynurenine have previously been detected in ASCs and their levels did not change significantly after short term storage [436]. The immunosuppressive properties of ASCs are maintained under hypoxic conditions. Specifically, after IFN-γ and TNF-α stimulation, IDO and programmed death ligand-1 are upregulated. IDO was measured by the accumulation of l-kynurenine [437]. Beyond these studies and limited findings, the function of the kynurenine pathway is ASCs is yet to be characterised.

1.4 Chapter aims

This project aims to investigate the kynurenine pathway in ASCs through the quantification of secreted kynurenines in ASC media of both untreated and IFN-γ treated cells. The addition of IFN-γ leads to the expression of IDO which degrades tryptophan and ultimately induces the kynurenine pathway [340]. Additionally, quinolinic acid toxicity was investigated as it is a known neurotoxin and that is present in the peripheral blood circulation [438]. While a principle method of QUIN-induced cell death is mediated through NMDA receptors, it can also be taken up into cells via amino acid transporters where it can catalyse an increase in oxidative stress, aberrant cytoskeletal rearrangements, mitochondrial dysfunction and Ca^{2+} increases prior to cell death [439], and it's toxicity on ASCs is yet to be explored.

Furthermore, the proteome of ASCs has been established in previous chapters and the metabolome of these cells has been highlighted as an area of interest that requires further investigation. This study provides a small glimpse into the metabolome of ASCs while focussing on a critical pathway that requires examination.

2. Methods

2.1 Tissue culture

The methods are the same as those presented in Chapter 3 cryopreserved method with a few modifications explained bellow. The cryopreserved ASCs had the same seeding density but were in 6 well plates rather than T75s. Cells were then treated in triplicate wells of a 6-well plate with either 500IU/mL IFN-γ (to activate the kynurenine pathway) or with different quinolinic acid concentrations (100nM, 500nM, 1µM, 2µM and 4µM). 72 hours was the chosen timeframe as it is likely to be long enough to invoke transcription of kynurenine pathway genes and metabolism of tryptophan. Culture media was retained, and dead cells removed by centrifugation at 600 x g. The supernatant containing kynurenine pathway metabolites were then frozen at -80°C prior to metabolomic analysis.

2.2 Metabolites

The metabolomic analysis was conducted by Dr Edwin Lim at Macquarie University.

All reagents and kynurenine pathway metabolites were analytical reagent grade and were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise stated. Deuterated internal standards were purchased from Medical Isotopes, Inc (Pelham, NH). Kynurenine pathway metabolites were extracted after protein precipitation using 10% (w/v) trichloroacetic acid and then filtered with a Millex® syringe filter (4mm, 0.22µm) before analysis. Quantification of tryptophan, kynurenine, 3-hydroxykynrenine, 3-hydroxyanthranilic acid, anthranilic acid and kynurenic acid was performed using uHPLC while picolinic acid and quinolinic acid were quantified using GCMS as previously described [440]. The intra- and inter-assay CV was within the acceptable range of 4–8% for uHPLC assays and 7–10% for GCMS assay calculated from the repeated measures of the metabolite's standards incorporated during the sequence run.

2.3 Microscopy

Analysis of microscopy was conducted by Varda Sardesai at St Vincent's Centre for Applied Medical Research.

Phase contrast images were captured on an Olympus CKX41microscope. One image was randomly captured per patient sample per day and the results averaged and graphed, then statistical analysis performed. Confluency of cells in phase contrast images was calculated using the plugin "WEKA segmentation" within FIJI software. Confluency was defined as the calculated threshold area occupied by cells as a percentage of the total image area x 100%. Image analysis was performed by an independent operator blinded to the donor information and culture treatment.

3. Results

Media was collected from ASCs after 72 hours in culture. The ASCs were either untreated or treated with IFN-γ. The following metabolites were quantified in order to provide insight on the kynurenine pathway before and after IFN-γ activation: tryptophan, kynurenine, kynurenic acid, anthranilic acid, quinolinic acid and picolinic acid. The confluency of ASCs was also measured. In addition, the impact of quinolinic acid on cell confluence was also investigated. When interpreting the results, it is also important to understand that some of the kynurenine pathway metabolites naturally present in the FBS are endpoint metabolites, meaning that they tend to accumulate, and cannot be converted to other metabolites lower down in the pathway. Examples of this include kynurenic acid and picolinic acid. Other metabolites, such as kynurenine and anthranilic acid can be converted to metabolites lower in the pathway as long as the enzymes which undertake those conversions are expressed in the cell type being studied.

3.1 Kynurenine pathway metabolites

Metabolites from the kynurenine pathway were measured from untreated ASCs, IFN-γ treated ASCs and the media DMEM/F12 + 10% FBS.

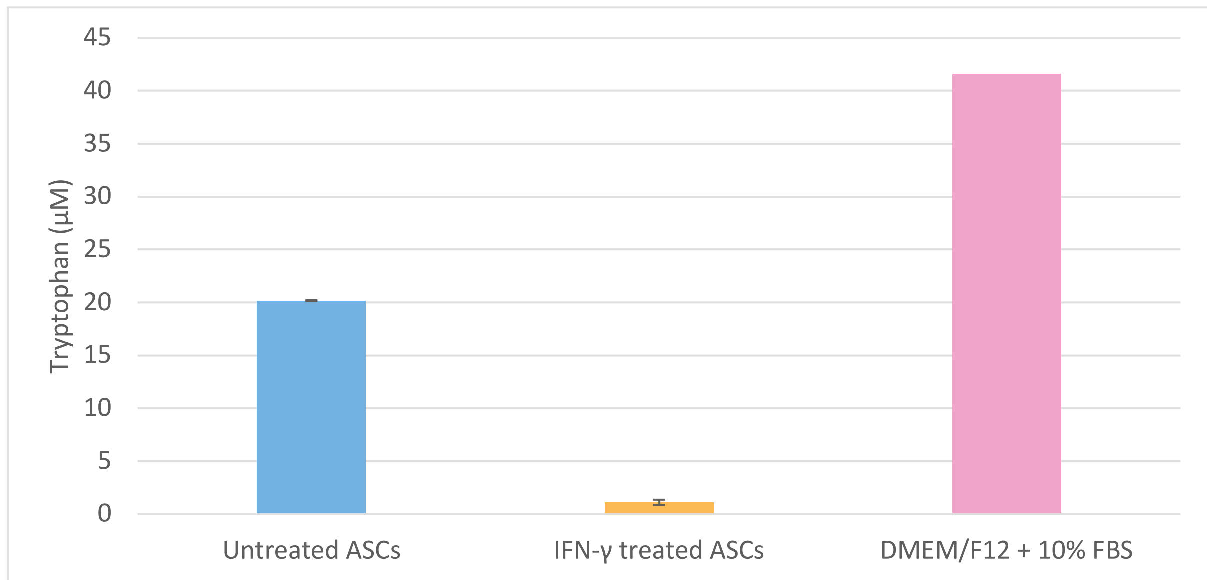

Figure 2: Tryptophan concentration of media for untreated ASCs, IFN-γ treated ASCs and neat media.

The media from untreated ASCs cultured for 72 hours were found to have 20.17µM of tryptophan, however the media from ASCs treated with IFN-γ for 72 hours only had 1.1µM of tryptophan. The media applied to the cells had 41.62µM of tryptophan prior to addition to the cells.

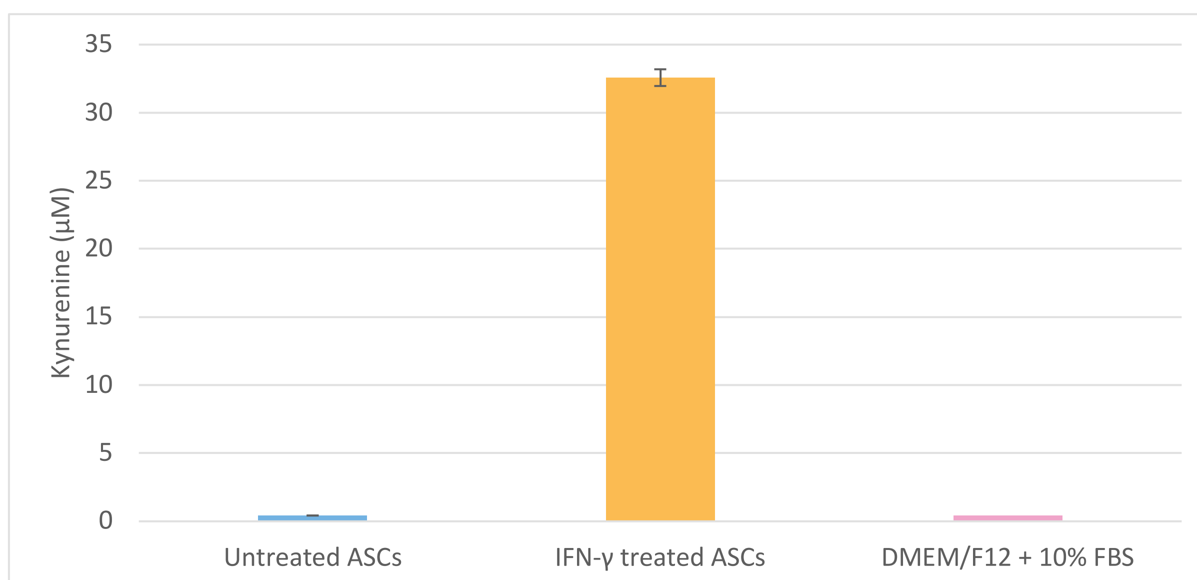

Figure 3: Kynurenine concentration of media for untreated ASCs, IFN-γ treated ASCs and neat media.

The media from untreated ASCs cultured for 72 hours were found to have 0.43µM of kynurenine, however the media from ASCs treated with IFN-γ for 72 hours had 32.58µM of kynurenine. The media applied to the cells had 0.41µM of kynurenine prior to addition to the cells.

Table 1: Kynurenine/tryptophan ratio of untreated and IFN-γ treated ASC media.

	Tryptophan (µM)	Kynurenine (µM)	Kynurenine/ tryptophan ratio
Untreated ASCs	20.17	0.43	0.02
IFN-γ treated ASCs	1.11	32.58	29.22

The concentration of tryptophan and kynurenine were utilised to derive the kynurenine/tryptophan ratio. This ratio is frequently used as a measure of IDO activity in cell culture, however it is important to recognise that this ratio does not provide conclusive evidence of IDO activity [441].

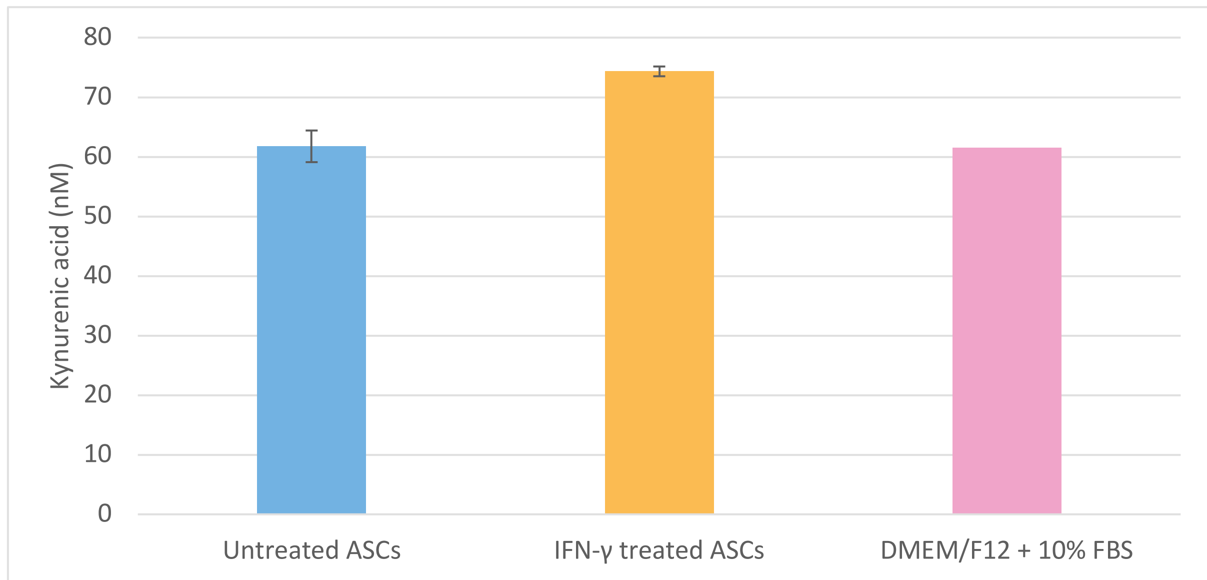

Figure 4: Kynurenic acid concentration of media for untreated ASCs, IFN-γ treated ASCs and neat media.

The media from untreated ASCs cultured for 72 hours were found to have 61.75nM of kynurenic acid, however the media from ASCs treated with IFN-γ for 72 hours had 74.33nM of kynurenic acid. The media applied to the cells had 61.48nM of kynurenic acid prior to its application to the cells.

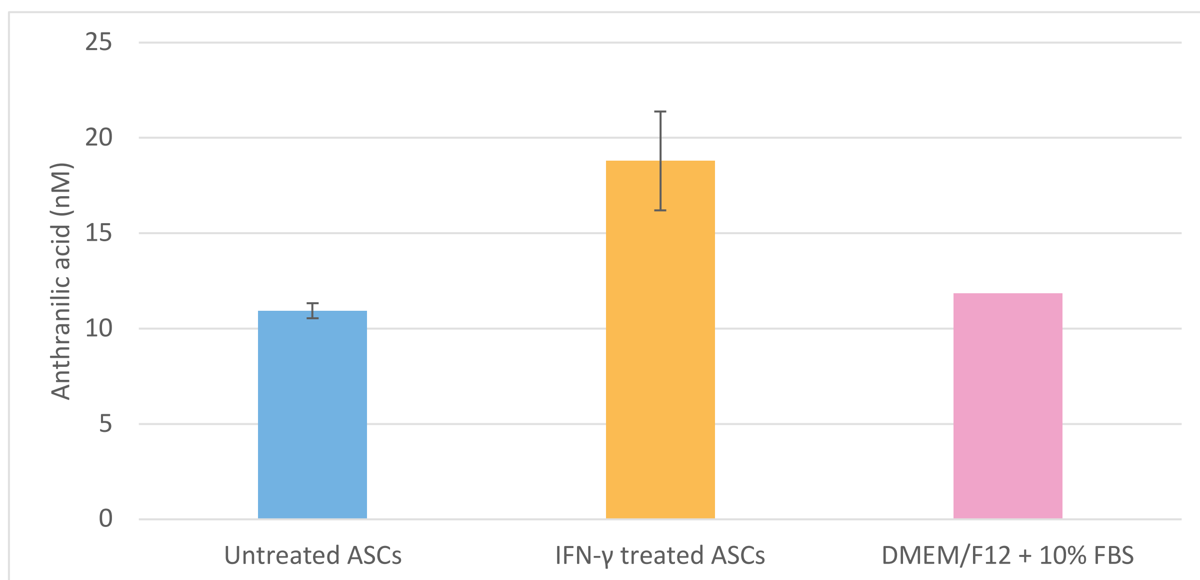

Figure 5: Anthranilic acid concentration of media for untreated ASCs, IFN-γ treated ASCs and neat media.

The media from untreated ASCs cultured for 72 hours were found to have 10.94nM of anthranilic acid, however the media from ASCs treated with IFN-γ for 72 hours had 18.79nM of anthranilic acid. The media applied to the cells had 11.86nM of anthranilic acid prior to its addition to the cells.

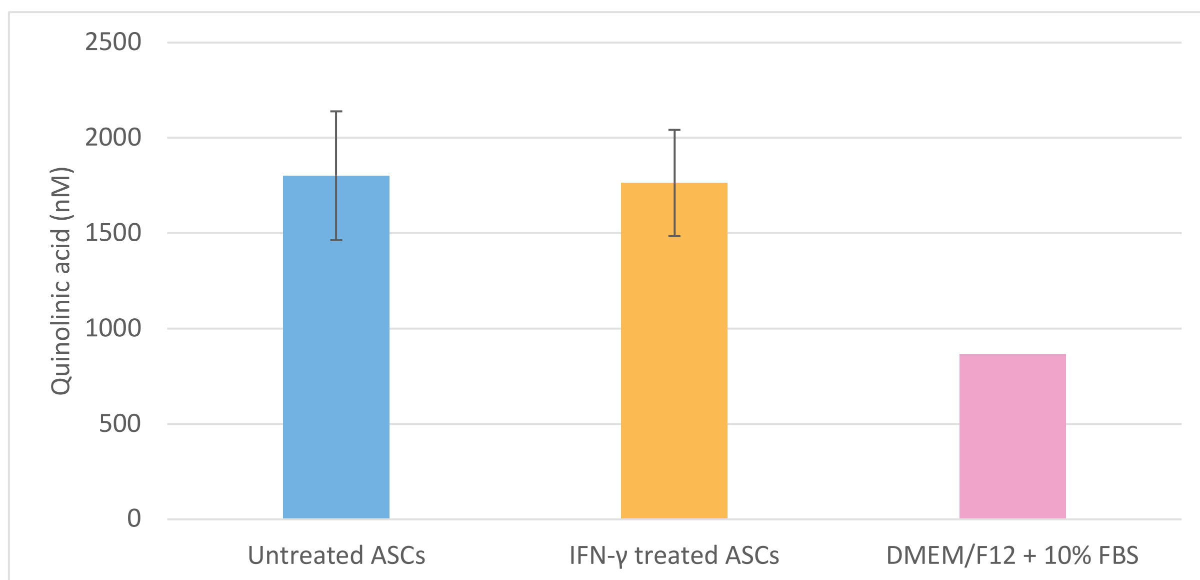

Figure 6: Quinolinic acid concentration of media for untreated ASCs, IFN-γ treated ASCs and neat media.

The media from untreated ASCs cultured for 72 hours were found to have 1801.93nM of quinolinic acid, however the media from ASCs treated with IFN-γ for 72 hours had 1763.839nM of quinolinic acid. The media applied to the cells had 867.05nM of quinolinic acid prior to addition to the cells.

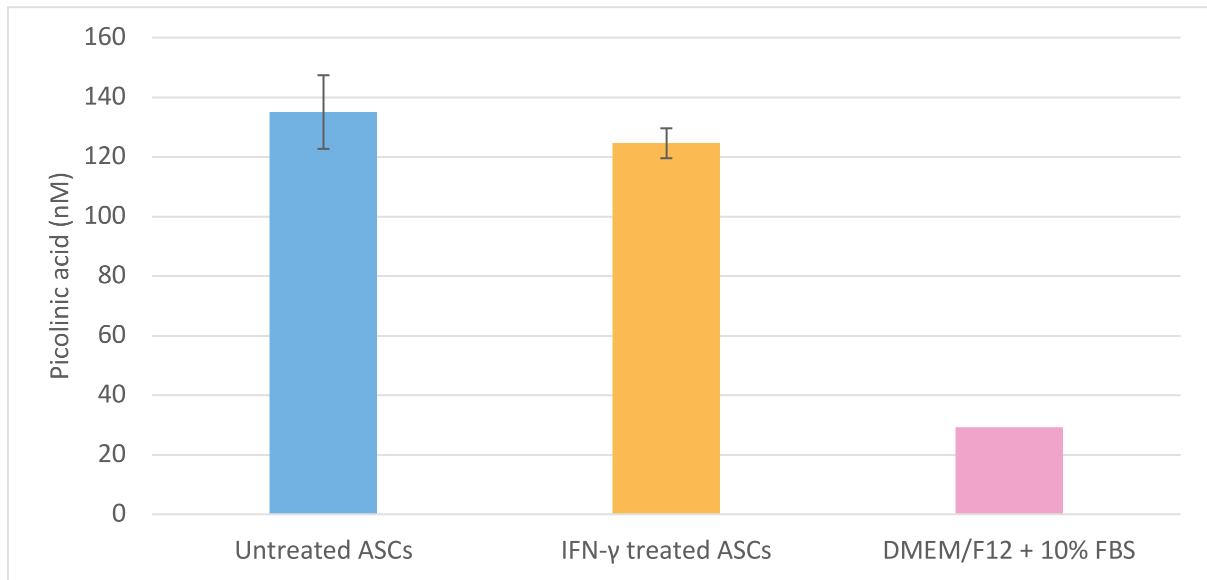

Figure 7: Picolinic acid concentration of media for untreated ASCs, IFN-γ treated ASCs and neat media.

The media from untreated ASCs cultured for 72 hours were found to have 135.00nM of picolinic acid, however the media from ASCs treated with IFN-γ for 72 hours had 124.52nM of picolinic acid. The media applied to the cells had 29.18nM of picolinic acid prior to its application to the cells.

3.2 Adipose stem cell confluence

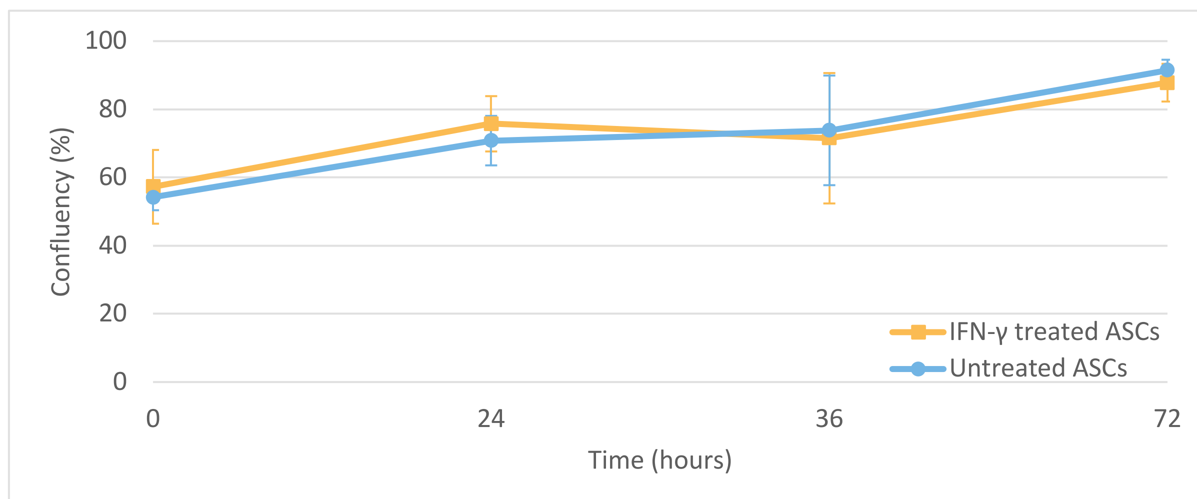

Figure 8: Confluency of untreated and IFN-γ treated ASCs.

The confluency of ASCs that were either untreated or treated with IFN-γ were measured at 0, 24, 36 and 72 hours. When taking to account the standard deviation shown in error bars, the ASCs have an increasing growth trend that is not affected by IFN-γ.

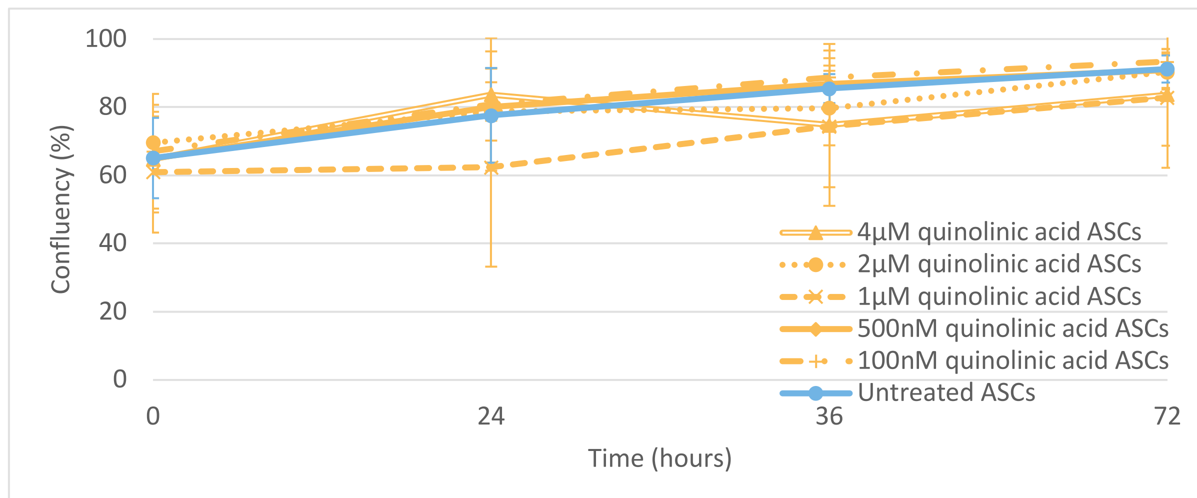

Figure 9: Confluency of untreated and quinolinic acid treated ASCs.

The confluency of ASCs untreated or treated with quinolinic acid were compared. 100nM, 500nM, 1µM, 2µM and 4µM of quinolinic acid were tested. All ASCs have an overall increasing confluency over time (0, 24, 36 and 72 hours). While 1µM, 2µM and 4µM of quinolinic acid did result in a slight decrease in confluency compared to untreated ASCs at some time points, however this was not significant. There are no substantial differences between untreated and treated ASC confluency.

4. Discussion

4.1 Kynurenine pathway in adipose stem cells

Little is known about the kynurenine pathway in ASCs, so this study sought to fill that gap by investigating kynurenines secreted by ASCs. These ASCs were either left untreated or treated with IFN-γ as it leads to the expression of IDO-1 which degrades tryptophan and induces the kynurenine pathway [340]. After 72 hours of tissue culture, the following metabolites were quantified: tryptophan, kynurenine, kynurenic acid, anthranilic acid, quinolinic acid and picolinic acid. This study revealed that untreated ASCs release tryptophan into the media at a concentration of 20.17µM, however once treated with IFN-γ this concentration reduces substantially to only 1.1µM as shown in Figure 2. This demonstrates that IFN-γ causes ASCs to secrete less tryptophan and suggests that this is being utilised within the cell. The media itself is also shown to contain high levels of tryptophan, and as this value is greater than the untreated ASCs it implies that the cells utilise this tryptophan from the media. This consumption of tryptophan suggests that TDO may be active in ASCs, or alternatively the levels IFN-γ that are basally secreted by ASCs as shown in Chapter 2 may be enough to stimulate IDO-1 and consequent tryptophan degradation. However, if this was the case, we would also expect to detect kynurenine in the untreated ASCs. This depletion could be due to incorporation of tryptophan into the cells for synthesis of new proteins, proliferation of the cells in culture and uptake, or depletion by the enzyme TDO2, which is known to be constitutively active and expressed in cells with high metabolism, such as in the liver and brain.

Figure 3 shows the kynurenine concentration of untreated and IFN-γ treated ASCs. It can be seen that untreated ASC media has a low concentration of kynurenine, only 0.43µM. However, after IFN-γ activation the ASCs secrete a substantially higher concentration of kynurenine (32.58µM). The fact that kynurenine concentration was so low in untreated ASCs suggests that the rate limiting enzyme in the kynurenine pathway, IDO-1, is not expressed in ASCs. However, the detection of quinolinic acid (Figure 6) in substantial levels could mean that kynurenine is rapidly converted to other downstream metabolites soon after it's

synthesis. Such substantial levels accumulated in IFN-γ treated ASCs suggests that enzymes lower in the pathway cannot metabolise kynurenine faster than it is produced. The substantial increase in kynurenine levels in the media after IFN-γ treatment suggests that IDO-1 is expressed and causes the subsequent degradation of tryptophan to kynurenine. This is further supported in Table 1 which shows the kynurenine/tryptophan ratio. This ratio commonly utilised as a measure of IDO activity in cell culture, but does not provide conclusive evidence of IDO activity [441]. The kynurenine/tryptophan ratio in untreated ASCs is only 0.02, and this ratio increases to 29.22 in the IFN-γ treated ASCs. This increase in the kynurenine/tryptophan ratio implies that IDO activity is increased by IFN-γ treatment. This data supports the notion that IFN-γ activates the kynurenine pathway in ASCs and degrades tryptophan to produce kynurenine. Kynurenine has previously been detected in with concentration of 25.24uM in unstored and 27.54uM in stored ASCs [436]. This study also detected IDO-1 gene expression, further supporting that the kynurenine pathway can be induced in ASCs [436].

Kynurenic acid and anthranilic acid were measured next, as this would provide insight on the subsequent steps in the kynurenine pathway. As shown in Figure 4, kynurenic acid was secreted at a concentration of 61.75nM by untreated ASCs, and this increased to 74.33nM in IFN-γ treated ASCs. While this increase is small, it does suggest that activation of the kynurenine pathway leads to a slight increase in kynurenic acid production. A similar trend is also seen with anthranilic acid, with a concentration of 10.94nM measured in untreated ASCs increasing to 18.79nM after IFN-γ treatment (Figure 5). This implies that activation of the kynurenine pathway likely causes a small increase in the production of anthranilic acid. This slight increase observed for kynurenic acid and anthranilic acid after IFN-γ treatment both fall outside of the standard deviation as shown with error bars. This provides some insight into the direction of flow of metabolites within the kynurenine pathway in ASCs.

To find out if the enzyme KMO is present in ASCs and to investigate if the kynurenine pathway proceeds down that branch, picolinic acid and quinolinic acid were measured in ASC media. Picolinic acid along with IFN-γ have been shown to regulate nitric-oxide synthase mRNA expression [442]. IL-4 is reported to inhibit the costimulatory activity of IL-2 or picolinic acid but not of lipopolysaccharide in IFN-γ treated macrophages [443]. Picolinic acid has also been shown

to have a dose-dependent osteogenic effect on mouse BMSCs *in vitro* [425]. In this work, Picolinic acid was shown to have a slight decrease (Figure 7) from 135.00nM in untreated cells to 124.52nM after IFN-γ treatment. However once again, when considering the standard deviation error bars, it is unlikely that this reduction was due to the IFN-γ itself. Furthermore, in Chapter 2 of this thesis, it was identified that ASCs basally secrete IL-4 which may be impacting picolinic acid production. Quinolinic acid in untreated ASC media was measured to be 1801.93nM and this value reduced to 1763.839nM after IFN-γ treatment. However as seen in Figure 6, the standard deviation error bars show that this very minor decrease is likely from variation in the secretions observed across the 3 patients. The high levels of quinolinic acid observed in Figure 6 are potent enough to kill oligodendrocytes [444,445], astrocytes [446] as well as to cause chronic subcellular abnormalities in neurons [397].

Confluency of these untreated and IFN-γ treated cells were measured, to ensure the changes observed in metabolite production were not due to cell death. As shown in Figure 8, the ASCs have an increasing growth trend that is not impacted by IFN-γ treatment. Cell confluency was also measured for cells treated with varying concentrations of quinolinic acid and toxicity was investigated as it is a known neurotoxin and it's toxicity on ASCs had yet to be explored [438]. Figure 9 shows the 5 concentrations of quinolinic acid ranging from 100nM to 4µM that were tested. 100nM has previously been shown to cause subcellular abnormalities in neurons [397]. While concentrations about 1µM caused a slight decrease in ASC confluency at 36 hours when compared to untreated ASCs, this appears to recover by 72 hours. Notably there are is large standard deviation as shown with error bars that could also be causing this variation in confluency, which may be indicative of variability in the cultures if there are cytoskeletal effects of quinolinic acid at these high levels. However, this data does demonstrate that quinolinic acid is not toxic to ASCs at concentrations as high as 4µM. Additionally, the secretion of quinolinic acid was not enhanced by IFN-γ, while other metabolites like kynurenine were substantially increased. This may be due to the fact that only a single time point was measured, and as the enzymatic machinery to produce quinolinic acid is clearly present in ASCs it is possible that these kynurenines will over time metabolise into quinolinic acid.

As proteomics was not conducted on the ASCs in this particular experiment, kynurenine pathway related proteins were searched for in data generated from Chapter 2 and 3 of this thesis. It is vital to recognise that these are different experiments, the ASCs in Chapter 2 being fresh isolates and the data measured for the whole cell lysate, membrane bound fraction and extracellular vesicles. The data from Chapter 3 analyses cryopreserved ASCs and the only fraction analysed was the whole cell lysate. The data from Chapter 3 is likely a better representation of the ASCs utilised in this experiment, as these ASCs were cryopreserved. Only three proteins related to the kynurenine pathway were identified.

Tryptophan--tRNA ligase, cytoplasmic (P23381) was detected in the fresh whole cell lysate, fresh extracellular vesicles and cryopreserved whole cell lysate. This protein has been shown to be involved in disease mutation, neurodegeneration and neuropathy. Specifically, a decrease in the activity of this protein is associated with neuronopathy and distal hereditary motor neuropathy [447]. This enzyme is important for the catalytic activity of tryptophan [447,448]. Additionally, aspartate aminotransferase, mitochondrial (P00505) was detected in fresh whole cell lysate, fresh membrane bound fraction, fresh extracellular vesicles and cryopreserved whole cell lysate. This protein is known to catalyse the irreversible transamination of kynurenine to kynurenic acid [449]. The identification of these two proteins in previous ASC data sets that were not treated with IFN-γ is interesting. It supports the cells utilisation of tryptophan from the media that is observed in this work and may even allude to kynurenine being irreversibly turned into kynurenic acid as very little kynurenine is detected in untreated ASCs, but kynurenic acid is still detected at comparable levels to IFN-γ treated ASCs. Lastly, methionine aminopeptidase 2 (P50579) was also detected in cryopreserved whole cell lysate. Anthranilic acid sulphonamides have been shown to inhibit this protein [450], however this is likely not the case in ASCs as anthranilic acid was detectable in untreated ASCs as was this protein. However, the proteomics data drawn on for this comparison is from a different set of experiments and can only be used as an indication of what to expect when analysing the proteome of ASCs from this experiment in the future.

4.2 Future directions

While the data generated from this project provided unique insight into the kynurenine pathway in ASCs it also requires further validation. For example, searching for kynurenine pathway related proteins in ASC data from Chapter 2 and 3 did successfully identify some proteins of interest, however no conclusive deductions can be made from this as the ASCs in those experiments were grown under different conditions and were not treated with IFN-γ. It is necessary to investigate the proteome of ASCs treated with IFN-γ. This will allow for the quantification of proteomic changes in ASCs after IFN-γ treatment. A targeted proteomics approach may allow for identification of proteins that are currently below the detectable limit in untargeted analysis. If the targeted proteomic approach were to focus on components of the pathway it would increase the sensitivity, however this will be at the expense of not investigating other proteomic changes that are related to this pathway in ASCs but not currently annotated as being so. A polymerase chain reaction or western blot could be done on the enzymes involved in producing the metabolites. This study has also shown us that while quantifying metabolites from the kynurenine pathway is useful, it doesn't necessarily show which way the pathway proceeds.

A metabolomics approach that investigates all cellular components and not just those of the kynurenine pathway in an untargeted manner will be able to provide clarification on what exactly is happening in the kynurenine pathway and wider cellular metabolism in ASCs. Furthermore, this same approach can be applied to the whole cell lysate, rather than just the media. This will allow for understanding of intracellular changes rather than just secretions. Additionally, while IFN-γ and quinolinic acid did not impact ASC confluency, cell cycle parameters were not investigated, so it would be favourable to investigate cell cycle parameters in future studies.

As the kynurenine pathway has been identified in many diseases, it would also be interesting to compare healthy to diseased patient samples. For example, in multiple sclerosis the kynurenine pathway is overactivated [360]. Tryptophan catabolites and their derivates are utilised as a strategy to treat T helper-1 mediated autoimmune diseases like multiple sclerosis [362]. It would be worthwhile isolating ASCs from multiple sclerosis patients and comparing these to healthy ASCs while investigating the kynurenine pathway.

5. Conclusion

To date this is the most comprehensive characterisation of the kynurenine pathway in ASCs. This data confidently demonstrates that the kynurenine pathway of tryptophan metabolism is activated by IFN-γ in human ASCs. Treating ASCs with IFN-γ resulted in a reduction in tryptophan and an increase in kynurenine. However, it is difficult to determine exactly what changes in the downstream parts of the kynurenine pathway, but it appears that there is a slight increase in the production of kynurenic acid and anthranilic acid. Additionally, high levels of quinolinic acid were detected in ASCs both with and without IFN-γ treatment. Quinolinic acid is capable of negatively impacting other cell types in the body. ASCs appear to be resistant to the deleterious effects of IFN-γ and quinolinic, which may be unique among other stem cell types in the body.

Alterations of the kynurenine pathway occurs in many diseases, Alzheimer's [349,350], amyotrophic lateral sclerosis [351,352], dementia [353], Huntington's [354,355], multiple sclerosis [356], AIDS [353], schizophrenia [357] HIV and neoplasia. ASCs have potential to serve as a minimally invasive means of understanding the role of the kynurenine pathway in these diseases, and ultimately acts as a way to study the disease itself. Furthermore, modulation of the kynurenine pathway has therapeutic potential [360], and ASCs are already being utilised as a treatment for many of these diseases.

This study has showcased the importance of investigating the kynurenine pathway in ASCs, moving forward this project needs to be expanded, namely, to evaluate the proteome, to investigate more metabolites, and to study diseased populations such as multiple sclerosis patient ASCs.

Chapter Five

Multiple sclerosis: made simple

1. Introduction

1.1 Background

As discussed at length in Chapter 1, unproven stem cell treatments are fuelled by online communities of hope which continually depict stem cell treatments in a positive light, often despite a lack of scientific evidence [92]. This is because sharing health experiences online gives individuals a sense they are participating in the creation of health information, and reading these first-person accounts affect other individuals health predominantly through the memorability of a story and the building an online community [95]. It should also be recognised that the internet has resulted in information being more readily accessible, as too are online communities. However, it is well acknowledged that social media itself is a vehicle for excessive hype [100]. When coupled with the minimal level scientific knowledge that the general public has, it is not surprising that patients often use digital media to access unproven treatments [92]. This lack of scientific knowledge is demonstrated by the results of the Australian literacy survey conducted in 2013, with 1515 respondents segmented and weighted to represent the nation's population by gender, age and residential location [451]. The survey involved asking a series of basic scientific questions, the results of which reinforce the need to increase scientific literacy. When asked the time it takes for the Earth to orbit the sun, 30% of those surveyed responded that it takes a single day, while 59% responded that it took a year. This lack of understanding of a fundamental concept affecting an individual's daily life clearly highlights the lack of basic scientific knowledge held by the general public [451]. This was not unique to Australia, with a similar survey conducted in America that covered the same core questions. When asked if the earliest humans lived at the same time as dinosaurs, 41% of Americans answered this as being true compared to the 27% of Australians who answered the same. In order to minimise the exploitation of patient hope due to this apparent lack of scientific knowledge and understanding, there is a desperate need for an increase in

the quantity and quality of resources for patients, and for organisations and industry with leverage to encourage this outreach [97].

Most diseases impact a broad demographic, many of who have little to no scientific knowledge. This can become problematic when they are diagnosed with a disease and aren't provided with the resources, they need to understand it. For example, multiple sclerosis is typically defined as a chronic demyelinating inflammatory disease[1] and it is often assumed naively that patients will understand this complex terminology because simple and direct explanations are not available. However, the scientific literacy of patients, in this case the multiple sclerosis community, cannot simply be assumed and the underlying science needs to be communicated for the empowerment of patients and their families.

Multiple sclerosis predominantly occurs in young adults with a mean onset of symptoms at 30 years of age [452]. There are estimated to be more than 2 million people affected worldwide [453,454] and the debilitating nature of this disease is exemplified through the long-term survival of patients [455]. Quality of life is significantly decreased for MS patients, primarily due to the extensive psychological stress that results from impairment of daily function and the unpredictable nature of the disease [456]. Exacerbating this is the overwhelming amount of scientific information patients are expected to understand. The empowerment of multiple sclerosis patients, their friends and families, through the provision of essential knowledge of the underlying science behind multiple sclerosis would be expected to increase quality of life.

1.2 Chapter proposal

This project aims to improve scientific literacy for the subject of multiple sclerosis using a video series and quantifying whether an increase in scientific literacy has taken place. The first three episodes of the series will effectively communicate exactly what multiple sclerosis is, with the first episode clarifying what a chronic illness is, the second describing demyelination, and the third explaining inflammation, all in the context of multiple sclerosis. This approach also brings the participant on a journey, starting with things they can relate to and are familiar with, such as disease course and relapses, and slowly integrates scientific

concepts that may be less familiar to them, such as describing internal organs such as the brain, and then moving on to a cellular level such as neurons. This approach will assist in providing participants with a sound understanding of the underlying science behind multiple sclerosis. These basic definitions are often overlooked in explanations of multiple sclerosis, resulting in a lack of knowledge which this project is going to address. Once armed with this scientific knowledge, the participants will be exposed to more complex science that is carefully presented to increase their understanding and empower them with knowledge. Episodes four through six will be focused around disease modifying drugs, the suspected causes of multiple sclerosis and finish with the current state of research. The final episode is an opportunity to highlight the realistic potential of current scientific findings, and to provide participants with an understanding of the foundations of scientific research. This is fundamental knowledge for the multiple sclerosis community, and it will help participants to navigate the plethora of science available online and allow them to differentiate between realistic hope and excessive hype.

The production of the videos will follow a learning design approach in which pedagogies, adult learning principles and instructional design models will inform the creation of the video content (storyboards) [457]. The first step will be to formulate achievable learning outcomes for the viewers. For this purpose, the SOLO taxonomy will be used to ensure active verbs that promote higher order thinking are present [458]. Then, constructive alignment will ensure the video content leads to development of these learning outcomes and assessment tasks (quizzes). Additionally, multimedia learning principles based on cognitive load theory will be applied to the development of the storyboard and the on-screen presentation of content [459]. Producing the storyboards will ensure the videos will be succinct, coherent and engaging for the users to watch and learn. The evaluative approach of the videos will include pre and post quizzes to measure knowledge acquisition and self-regulation questions to gauge how viewers adapt, for example, their environment to maximise learning with the videos [460]. It will also gather user feedback on how to improve the resources. There is a body of literature that showcases that educational videos properly designed can improve learning and retention and that the cognitive architecture of the brain mediates that learning and retention [461]. When users are presented with information that follows a stepwise progression using meaningful examples in a visual manner, this facilitates the coding of the information into the schema,

also called long-term memory [462]. The knowledge that sits into this domain stays for life. For the intervention to succeed, it is necessary to ensure the pedagogical integrity of the video content [463].

Online video content has become ubiquitous and can be found across social platforms like Facebook, Instagram and Vine, and are utilised across higher education institutions as a means of blended learning to reinforce concepts. Thousands of videos providing instructional content can be found on YouTube including open course platforms like Khan academy [464]. It is important that a video series encapsulates what is currently known in the literature to improve learning. A large-scale study that utilised 6.9 million video watching sessions established that shorter videos are much more engaging than their longer counterparts [464] and for this reason all videos in this series will be between 3-10 minutes of length, ensuring they are short enough to maintain participant interest. When navigating educational scientific videos on YouTube, professional and amateur science communicators compete for views and engagement. While there are a greater number of professionally generated videos, user generated content is significantly more popular, and this is likely due to having a consistent science communicator [465]. This series will therefore utilise a single consistent science communicator, particularly because informal talking-head videos are reported to be more engaging [464].

The style of videos also significantly contributes to their ability to successfully communicate. An example of this significance is a study comparing narrative videos to expert interview videos, where the content was illustrating gender bias [466]. The participants reported that the narrative videos caused them to be more immersed in the story and identify with the characters, while the expert interviews resulted in increased logical thinking and perceptions of the expert being knowledgeable of the content delivered. When compared to control videos both variables increased awareness of gender bias, influenced knowledge of gender inequity, self-efficacy beliefs, and the recognition of bias in everyday situations, however it was only the expert interview videos that had an impact on the participants intentions to confront unfair treatment [466]. This clearly highlights the benefits of having an expert while having an entertaining narrative video with a relatable presenter. The series of videos created through this project will aim to address both of these variables, by creating narrative video

content with a relatable presenter who is also already perceived by the public as a scientific expert. Design and aesthetics have a profound impact on how users perceive information and learn, judge credibility and usability, and ultimately assign value to an online experience [467].

Investigation of how these videos improve participant understanding will involve pre and post questionnaires for each video. These questionnaires will involve quantitative questions to assess improved learning and qualitative questions to investigate self-regulated learning. Self-regulated learning strategy effects are not as well understood in the online context when compared to the traditional classroom, warranting further investigation of online self-regulated learning as unexplored factors may be more important within this context [468]. Self-regulated learning and motivation have been shown to mediate the effects of emotions on academic achievement. In particular, positive emotions foster academic achievement only when they are mediated by self-regulated learning and motivation [469]. There is a need to understand how students can best apply self-regulated learning strategies to achieve academic success within the online environment reinforcing the necessity to investigate self-regulated learning within this project [468]. Dr Jorge Reyna has over 10 years' experience in designing, implementing and evaluating blended learning in higher education, and this project will utilise his expertise in learning theories and theoretical frameworks to develop these questionnaires and analyse the resulting data from this study. The hypotheses are that educational videos on multiple sclerosis will improve self-regulation skills for online learning in the multiple sclerosis community, and that educational videos on multiple sclerosis will improve knowledge of multiple sclerosis topic amongst participants.

2. Method

A literature review was conducted in order to gather information on multiple sclerosis that would be of interest to the broader multiple sclerosis community. This content was placed into a story board and a script was written by Naomi Koh Belic. Amalia Sablada filmed and edited the videos. Questions were written by Naomi Koh Belic under the guidance of Jorge Reyna. 2

2.1 Videos

Six episodes were filmed and released to participants. Multiple sclerosis is often described as a chronic demyelinating inflammatory disease, so the first three episodes focus on exactly that: Chronic(les) of MS, Lessons in lesions and Inflammation information. The subsequent three episodes cover the cause, treatment and current research into multiple sclerosis: Cause for thought, Tricky treatments and (Re)searching high and low.

Figure 1: Chronic(les) of MS.

Episode 1 Chronic(les) of MS is available at https://youtu.be/H74QFEVY1kI

Figure 2: Lessons in lesions.

Episode 2 Lessons in lesions is available at https://youtu.be/92VBg_nbbkY

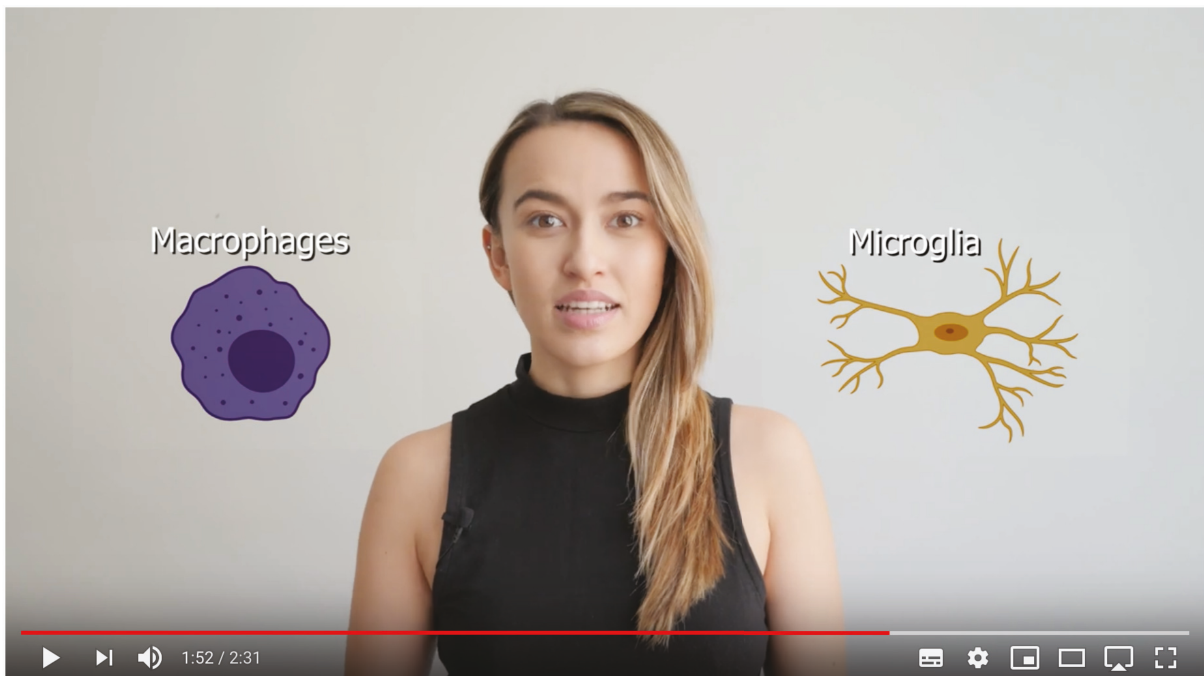

Figure 3: Inflammation information.

Episode 3 Inflammation information is available at https://youtu.be/jquOBqtEq9A

Figure 4: Cause for thought.

Episode 4 Cause for thought is available at https://youtu.be/3qWTxYyGmi8

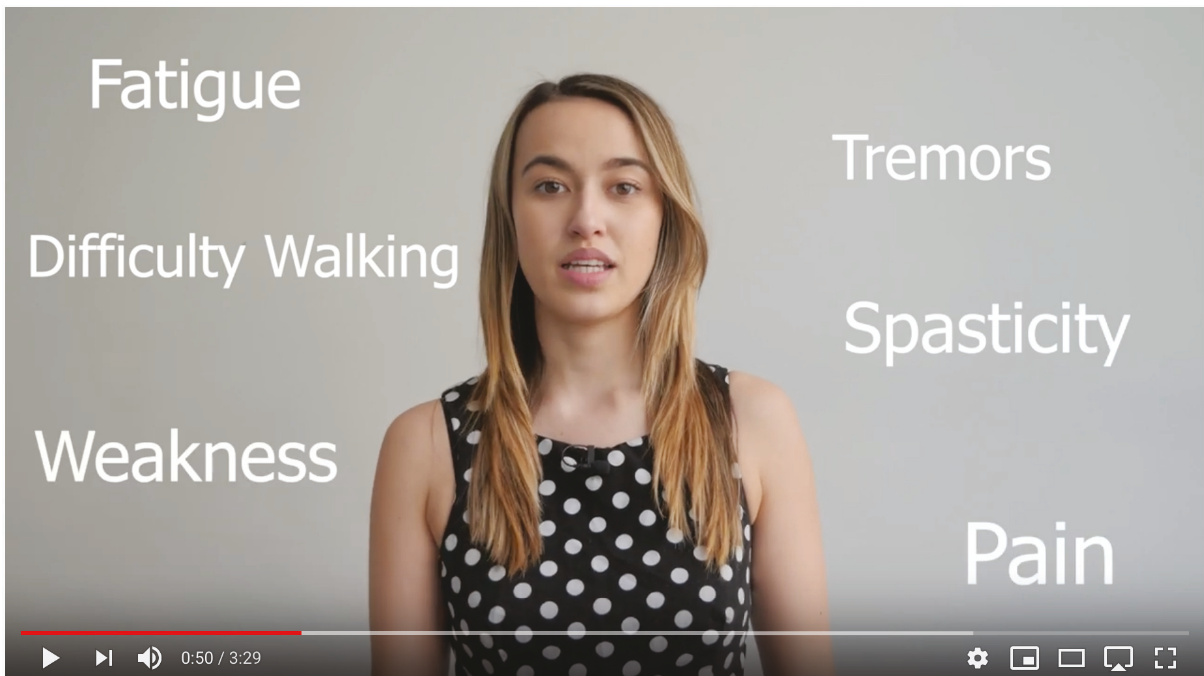

Figure 5: Tricky treatments.

Episode 5 Tricky treatments is available at https://youtu.be/QzxerD3nHB8

Figure 6: (Re)searching high and low.

Episode 6 is available at https://youtu.be/OsnTL-kaZ2g

2.2 Self-regulation questions

These self-regulation questions collectively provide information on attitude to multiple sclerosis digital videos, ability to form task strategies, environmental structuring, time management and help seeking.

Questions 1-16 will be scaled: strongly disagree, disagree, agree and strongly agree

1. I learn about multiple sclerosis while watching online videos
2. Learning about multiple sclerosis using online videos is good
3. Online videos helped me to learn about multiple sclerosis
4. I enjoy learning about multiple sclerosis using online videos
5. I take notes from the online videos video to understand multiple sclerosis
6. I watch additional online videos about multiple sclerosis
7. I visit websites about multiple sclerosis
8. I choose the location where I watch the online videos on multiple sclerosis to avoid distractions
9. I find a comfortable place to watch the online videos on multiple sclerosis

10. I choose a time with few distractions for watching the online videos on multiple sclerosis
11. I allocate extra time to watch the online videos on multiple sclerosis
12. I schedule regular times a week to watch the online videos on multiple sclerosis
13. I follow my planned schedule for watching the online videos on multiple sclerosis
14. I find people who are knowledgeable in multiple sclerosis so that I can ask them for help
15. I share the difficulties I am having understanding multiple sclerosis with my peers
16. I seek help on the Internet about multiple sclerosis

Questions 17-23 are open ended questions

17. Did you feel you have the knowledge on multiple sclerosis at the time you started watching the online videos?
18. How did you find the online videos on multiple sclerosis usefulness for understanding the condition?
19. Did you feel there were uncontrollable factors beyond your knowledge that could affect your engagement watching the online videos on multiple sclerosis?
20. Did you feel anxious watching the online videos on multiple sclerosis?
21. What do you like most about the online videos on multiple sclerosis?
22. What do you like least about the online videos on multiple sclerosis?
23. Do you have any suggestions on how to improve these videos?

2.3 Scientific questions

These questions provide insight on the scientific literacy of participants in regard to their understanding of multiple sclerosis.

Questions 1-5 are for episode 1

1. What is the most common cause of neurological disability in young adults?
    a. Epilepsy
    b. Multiple sclerosis

c. Alzheimer's
d. Parkinson's

2. How do we determine what type of multiple sclerosis a person has?
   a. Number of lesions
   b. Type of symptoms
   c. How long they have had multiple sclerosis
   d. Progression of disease course
3. Is a relapse when neurological symptoms increase?
   a. True
   b. False
4. What percentage of people with multiple sclerosis have primary progressive multiple sclerosis?
   a. 10-15%
   b. 30-35%
   c. 50-55%
   d. 70-75%
5. Relapsing remitting multiple sclerosis can develop into secondary progressive multiple sclerosis
   a. True
   b. False

Questions 6-10 are for episode 2

6. What is the most complex organ in your body?
   a. Heart
   b. Liver
   c. Lung
   d. Brain
7. What are lesions?
   a. Scarring of brain and spinal cord
   b. Bleeding in the brain
   c. An open wound
   d. Excessive cellular growth in the brain and spinal cord

8. Myelin surrounds
    a. Dendrites
    b. Axon
    c. Cellular body
    d. Nucleus
9. Demyelination
    a. Leads to a reduction in a neurons ability to transmit electrical signals
    b. Causes lesions
    c. Is the damaging of myelin
    d. All of the above
10. You brain can send messages to the rest of the body without electrical signals
    a. True
    b. False

Questions 11-15 are for episode 3

11. What is the most common cause of demyelination?
    a. Lesions
    b. Inflammation
    c. Neurons
    d. Incorrect signalling
12. Inflammation is mediated by
    a. T cells and B cells
    b. Macrophages and microglia
    c. B cells and microglia
    d. T cells, B cells, macrophages and microglia
13. Which of the following statements are true?
    a. Antigens induce an immune response
    b. Antigens lead to the production of antibodies
    c. Antigens and antibodies are proteins
    d. All of the above
14. Which of the following statements about T cells are true?
    a. They contribute towards demyelination

b. They harm axons
c. They encourage cells to sites of inflammation
d. All of the above

15. Which of the following cells detect, engulf and destroy harmful organisms?
    a. T cells
    a. B cells
    b. Macrophages
    c. All of the above

Questions 16-20 are for episode 4

16. The exact cause of multiple sclerosis is known
    a. True
    b. False
17. Which of the following statements are true?
    a. A single virus has been identified as the cause of multiple sclerosis
    b. A combination of viruses have been identified as the cause of multiple sclerosis
    c. Both a single virus and a combination of viruses have been identified as the cause of multiple sclerosis
    d. Neither a single virus nor combination of viruses have been identified as the cause of multiple sclerosis
18. Multiple sclerosis is a hereditary disease
    a. True
    b. False
19. The further away from the equator, the greater the incidence of multiple sclerosis
    a. True
    b. False
20. Increased multiple sclerosis is associated with
    a. Lowered vitamin D and lowered ultraviolet radiation
    b. Increased vitamin D and lowered ultraviolet radiation
    c. Lowered vitamin D and increased ultraviolet radiation
    d. Increased vitamin D and increased ultraviolet radiation

Questions 21-25 are for episode 5

21. There is a particular treatment that is optimal for treating multiple sclerosis
    a. True
    b. False
22. Multiple sclerosis symptoms can be treated with
    a. Physical treatments
    b. Medical treatments
    c. Physical and medical treatments
    d. Neither physical or medical treatments
23. How many disease modifying drugs are available for relapsing remitting multiple sclerosis in Australia?
    a. 1-5
    b. 6-10
    c. 11-15
    d. 16-20
24. Disease modifying therapies do not target the immune system
    a. True
    b. False
25. Which factors should a neurologist consider when choosing the most appropriate multiple sclerosis treatment?
    a. History of multiple sclerosis
    b. Effectiveness of previous treatments
    c. Cost
    d. All of the above

Questions 26-30 are for episode 6

26. One century ago multiple sclerosis was an untreatable disease
    a. True
    b. False
27. Lesions were first viewed in
    a. 1940s
    b. 1960s

c. 1980s
d. 2000s

28. The first disease modifying therapy for primary progressive multiple sclerosis was approved in
    a. 2011
    b. 2013
    c. 2015
    d. 2017
29. Identifying biomarkers for multiple sclerosis will aid in earlier diagnosis, therefore earlier treatment, and improved patient outcomes
    a. True
    b. False
30. Symptoms and rehabilitation can not be managed with physiotherapy and psychology
    a. True
    b. False
    c.

2.4 Demographic questions

These questions provide insight on the participants that can be used to stratify the data,

1. What is your gender
    a. Male
    b. Female
    c. Transgender female
    d. Transgender male
    e. Gender variant/non-conforming
    f. Prefer not to answer
2. What is your age?
3. What is your postcode?
4. Is English your first language?
    a. Yes

    b. No (please state your first language)
5. What is the highest level of education you have completed?
    a. High school graduate
    b. Trade/technical/vocational training
    c. University degree (please state degree)
    d. Postgraduate (please state degree)
6. What is your employment status?
    a. Full-time employed
    b. Part-time employed
    c. Casual employed
    d. Not employed
7. Do you have multiple sclerosis?
    a. No
    b. Yes (please state when were you diagnosed and what type of multiple sclerosis do you have)
8. Do you have a relative or friend who has multiple sclerosis?

2.5 Instructions to participants

Participants registered their interest (https://forms.gle/4gTe7NLFfr1E6Qoy9) and were sent an Information sheet and consent form (UTS Ethics Approval ID: ETH18-2127) and were asked to complete these tasks in order:

1. Answer Introduction self-regulation questionnaire
2. Answer Introduction scientific questionnaire
3. Watch Chronic(les) of MS video
4. Answer Chronic(les) of MS questionnaire
5. Watch Lessons in Lesions video
6. Answer Lessons in Lesions questionnaire
7. Watch Inflammation Information video
8. Answer Inflammation Information questionnaire

9. Watch Cause for Thought video
10. Answer Cause for Thought questionnaire
11. Watch Tricky Treatments video
12. Answer Tricky Treatments questionnaire
13. Watch (Re)searching High and Low video
14. Answer (Re)searching High and Low questionnaire
15. Answer Conclusion self-regulation questionnaire
16. Answer Conclusion demographic questionnaire

3. Results

At the time of thesis submission, there were 69 individuals registered to participate, but only 19 of which have completed the study. This number is very low, and no significant conclusions can be drawn from this data. However, this was largely due to the University of Technology Sydney Information Technology Division accidentally deleting the account and associated google drive that stored all the live forms and data for this study. These new 69 participants signed up between the 25th of February and the 3rd of April. If participants continue signing up and completing the study at this rate, it is safe to assume that by the end of 2020, there will be enough participants for the analysis to be repeated on the data and generate results that have statistical significance. It is not possible to delay the submission of this thesis for a year simply to recollect data that was deleted and for this reason the subsequent analysis will be conducted on n=19 and will simply be an indication of trends to expect once future analysis is repeated.

Participants filled in multiple choice questionnaires based on the content from the videos before and after viewing each episode. Their grades were measured as a percentage and illustrated in Figure 7.

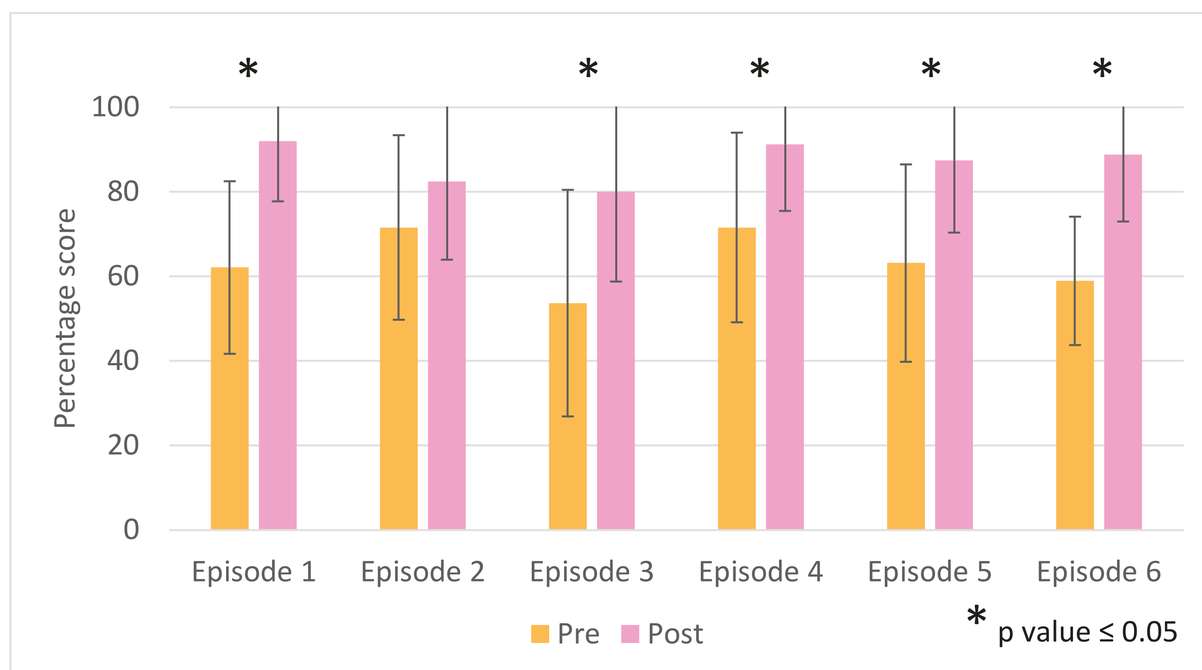

Figure 7: Percentage scores of scientific questions pre and post episode.

It can be seen that after watching the videos the percentage score for the questionnaire increased. This increase was statistically significant for episode 1, 3, 4, 5 and 6, but not for episode 2.

The self-regulation questionnaire answered were given numerical values where strongly disagree = 1, disagree = 2, agree = 3 and strongly agree = 4. These questions were then split up based on their category, questions 1-4 formed the category attitude to videos, 5-7 formed task strategies, 8-10 formed environment structuring, 11-13 formed time management and 14-16 formed help seeking. This was done for both pre (Table 1) and post (Table 2) video.

Table 1: Self-regulation questionnaire results pre videos.

	Attitude to videos	Task strategies	Environment structuring	Time management	Help seeking
Minimum	1.00	1.00	1.00	1.00	1.00
Maximum	4.00	4.00	4.00	4.00	4.00
Mean	2.33	2.06	2.30	1.81	2.26
Std. Dev.	1.18	1.11	1.18	0.96	1.04
Variation	1.39	1.24	1.39	0.93	1.08
Mode	1.00	1.00	1.00	1.00	3.00

Table 2: Self-regulation questionnaire results post videos.

	Attitude to videos	Task strategies	Environment structuring	Time management	Help seeking
Minimum	1.00	1.00	1.00	1.00	1.00
Maximum	4.00	4.00	4.00	4.00	4.00
Mean	3.52	2.48	3.17	2.02	2.67
Std. Dev.	0.59	0.89	0.82	0.78	0.90
Variation	0.34	0.79	0.68	0.60	0.81
Mode	4.00	2.00	3.00	2.00	3.00

It is clear from Table 1 and 2 that the answers from participants increased in numerical value from pre to post watching the videos. This can be seen clearly with the mean and mode values. In addition, the standard deviation pre video is greater than the standard deviation observed post video.

These values were combined with the numerical output from the scientific tests (out of 5) to form two groups, pre video and post video. These two groups were compared using an ANOVA and the output is displayed in Table 3 and Table 4. As there are 5 categories from the self-regulation questionnaire and 6 videos, this leads to n=11 per group (pre and post).

Table 3: Pre and post video ANOVA data summary.

	n	Mean	Std. Dev.	Std. Error
Pre video	11.00	2.71	0.61	0.18
Post video	11.00	3.63	0.92	0.23

Table 4: Pre and post video summary of ANOVA findings.

	Degrees of freedom	Sum of squares	Mean square	F ratio	p value
Between groups	1.00	4.68	4.68	7.706	0.012
Within groups	20.00	12.15	0.608		
Total	21.00	16.84			

The F ratio is the ratio of the two mean square values. An F ratio with a value closer to 1 indicates there is no variation among groups, and a higher number indicates variations between the group. This F ratio indicates that there is variation between pre and post groups. The p value is less than the significance value of 0.05 therefore indicating that there is a difference between the pre and post groups.

It is very difficult to categorise the responses from the open-ended questions (questions 17-23 from the self-regulation questions) due to the small number of participants. In the meantime, a few responses have been shared instead as they provide insight on the participants experience.

Table 5: Open ended answer examples question 18.

	How did you find the online videos on multiple sclerosis usefulness for understanding the condition?
Participant 1	Very helpful, easy to understand and very informative
Participant 10	good, very clear and precise
Participant 11	I found they were very helpful, and short duration made the challenge easier to absorb.

These examples of answers are encouraging and suggest that the videos were useful and that the short length of the videos was beneficial.

Table 6: Open ended answer examples question 21.

	What do you like most about the online videos on multiple sclerosis?
Participant 4	I liked that complex research and information was made simple so that laypeople such as myself can digest it quickly and easily.
Participant 13	Short videos help with understanding a complicated topic by dividing it up into achievable learning modules.
Participant 14	They are informative without too much animated information. The animations were useful to know how to spell some of the words or to see what axons etc look like.

The example responses here also suggest the short length of the videos was beneficial, that the content was simple and that the amount of animations was appropriate.

Table 7: Open ended answer examples question 22.

	What do you like least about the online videos on multiple sclerosis?
Participant 2	Pace was too fast
Participant 6	Speed of speech and hand movements were distracting for me. That's just my issues though.
Participant 13	The lack of text means that sometimes a video needs to be viewed again to fully comprehend the information being presented.

It is suggested from these examples of answers that the videos are too quick, and that in future more reinforcing text would be useful.

Table 8: Open ended answer examples question 23.

	Do you have any suggestions on how to improve these videos?
Participant 2	more animation guides to help navigate the dense scientific bits
Participant 3	yes - more animation , pictures help me learn
Participant 16	No- these would have been so helpful when I was first diagnosed!

These examples responses indicate that in future the videos should contain more animations and that the animations served their purpose of educating the multiple sclerosis community.

While demographic data was collected, no substantial analysis could be made due to the low number of participants. One question is visualised as it indicates a high level of education among current participants.

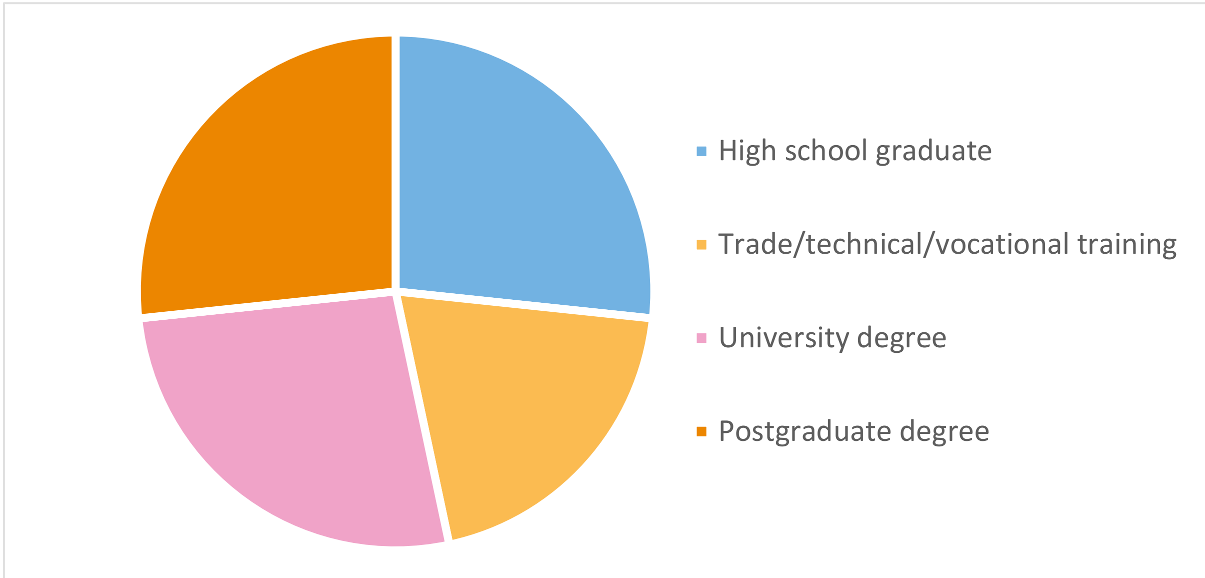

Figure 8: Education level of participants.

It is seen in Figure 8 that all participants have graduated high school, and more than half of the current participants are university educated.

4. Discussion

4.1 Videos for multiple sclerosis education

In this work, participants completed a questionnaire pre and post watching individual episodes of an educational video series about multiple sclerosis to determine whether such media could improve the scientific literacy of the participants. It must be reiterated that only 19 participants completed this study. This value is exceptionally low, and no significant conclusions can be drawn from this data. While statistical analysis was still conducted on the data, it is simply to indicate trends in the existing data that may still be present once more participants have completed the study. In saying that, promising findings were still observed. In Figure 8, the percentage score for the questionnaire increased after watching the videos and this increase was statistically significant for episode 1, 3, 4, 5 and 6, but not for episode 2. This confirms that educational videos improve multiple sclerosis knowledge amongst participants.

The answers to the self-regulation questionnaire across the categories of attitude to videos, task strategies, environment structuring, time management and help seeking is shown in Table 1 for pre video and Table 2 post video. It is clear that that the answers from participants increased in numerical value from pre to post watching the videos and is particularly clear when looking at the mean and mode values. Furthermore, the standard deviation pre video is greater than the standard deviation observed post video.

In order to determine if the multiple sclerosis educational videos improve self-regulation skills for online learning, the combined scientific and self-regulation data were compared for pre and post video in an ANOVA (Table 3 and Table 4 respectively). The F ratio confirms that there is variation between pre and post groups and the p value indicates that there is a statistically significant difference between the pre and post groups. This consequently demonstrated that multiple sclerosis educational videos improve self-regulation skills for online learning in the multiple sclerosis community. Interestingly all participants have graduated high school, and more than half of the current participants are university educated as shown in Figure 8. This

further supports the importance of creating educational video content, as even the highly educated participants improved their multiple sclerosis knowledge.

The open-ended questions are yet to be sufficiently analysed (Tables 5-8), but they suggest that the videos were useful, that the content was simple, and they served their purpose of educating the multiple sclerosis community. However, there was also opposing statements such as the short length of the videos was beneficial, but also that they were too quick. Additionally, the minimal amount of animations was praised and criticised. While considerably more participants are needed for any conclusions to be drawn, these initial findings are promising, but also demonstrates the wide range of learning styles that are inherent across the population and the difficulty of addressing them all for maximum effect.

Participants not only have an improved understanding of the scientific concepts behind multiple sclerosis but will also hopefully have a knowledge of how scientific research is conducted which can shape their ability to explore scientific resources, accurately identifying reliable sources and separating scientific research with excessive hype from research that provides realistic hope. This project has led to a sound understanding of how this video series improved learning, and how participants self-regulate learning. This knowledge can be adapted to produce better scientific educational video content beyond the multiple sclerosis community. This project has not only provided a means to educate the multiple sclerosis community through increased scientific understanding but will facilitate future research into improving digital media learning for the multiple sclerosis and broader community in a diverse range of medical topics.

4.2 Future directions

This study is not complete, and the primary future aim is to continue collecting participant responses. After the google drive on which the live forms and data was stored for this project was accidentally removed by the University of Technology Sydney, 69 new participants signed up between the 25th of February and the 3rd of April. Should participants continue signing up and completing the study at this rate it is safe to assume that by the end of 2020, there will

be enough participants for the analysis to be repeated on data that has sufficient statistical power. Two reputable multiple sclerosis foundations, MStranslate and Multiple Sclerosis Research Australia, are promoting this study. When taking into account the current rate of participant interest and promotion from these leading organisations, it is in the best interest to reanalyse this data for future publication once enough participants have signed up.

While this project has led to improved quality of video content for the purposes of self-directed learning, there are still significantly more areas to expand on. For example, more complex scientific literature can be explained as there is no lack of novel multiple sclerosis research being produced that needs disseminating. Future projects should investigate different styles of digital media, such as verbal and written styles, as this would have meaningful contributions to communicating with the community.

5. Conclusion

This project aimed to improve scientific literacy for the subject of multiple sclerosis and quantify whether an increase in scientific literacy has taken place. A series of six videos were produced that explain the science behind multiple sclerosis. Investigation of how these videos improve participant understanding involved pre and post questionnaires for each video. These questionnaires involved quantitative questions to assess improved learning and qualitative questions to investigate self-regulated learning. This project aimed to provide a sound understanding of how this, and by implication other video series, improve learning and how participants self-regulate learning. The participants in this project will not only have an improved understanding of the scientific concepts behind multiple sclerosis, but will also have a knowledge base of how scientific research is conducted which will shape their ability to explore scientific resources, accurately identify reliable sources and separate scientific research with excessive hype from research that provides realistic hope. This knowledge can be adapted to produce better scientific educational video content beyond the multiple sclerosis community. For example, this information can shape the way educational online resources are created to combat unproven stem cell treatments.

Chapter Six

Stemming the tide of misinformation

As described throughout this thesis, stem cells are defined by their ability to self-renew and differentiate into multiple cell types [1]. Because of this, stem cells have the potential to repair or replace damaged tissue and are therefore of great interest for regenerative medicine which is advancing at an astonishing rate and has genuine potential [34]. To date there has been success with stem cell treatments for cancers such as leukaemia and multiple myeloma. However, with the exception of hematopoietic stem cells (HSCs), all other stem cell therapies are still in the research, pre-clinical or clinical phases [37], a fact that has not stopped them being utilised in unproven treatments by predatory clinics. The development of standard medical therapies follows a linear model: basic research, applied research and development, and production and diffusion [44]. While there appears to be considerable investment into both preclinical and clinical trials of stem cells, this has only resulted in minimal success for their implementation of routine treatments [36]. This raises concern that clinical trials are being poorly designed and is ultimately slowing the progression of successful clinical translation [36]. Adult stem cells are the most common stem cell being used in clinical trials, however both embryonic (ESCs) and induced pluripotent stem cells (iPSCs) are also being used [36]. Pluripotent stem cells are used in situations where adult stem cells are difficult to isolate or expand [36].

A recurring theme throughout this thesis, and one of the original drivers of this work, is that there are stem cell treatments available that have not been through clinical trials, which are referred to as unproven stem cell treatments. While the term 'unproven stem cell treatment' carries negative connotations, it is important to note that the term also encompasses some legitimate research that is still in-progress and is yet to be sufficiently proven [67]. It is incredibly difficult to determine the number of patients who have received unproven stem cell treatments, but it is suggested to be in the tens of thousands or even higher [68]. There is little to no evidence that these treatments are safe, let alone that they work and there is also insufficient evidence that stem cell therapies will provide more benefits than they do harm [103,104]. Stem cell treatments are invasive and their potential is often misrepresented without

sufficient supporting scientific evidence [69]. And while there is considerable interest from patients, patient demand should not mean that stem cell treatments are provided in a commercial context outside of clinical trials that prove safety and efficacy and until there is sufficient evidence, governments should restrict their availability [83,102]. Regulation of stem cell therapies in Australia is reactive rather than proactive as it requires complaints to be made [85]. In Australia, autologous adipose stem cells (ASCs) are being offered outside of clinical trials for osteoarthritis, cartilage repair, stroke, multiple sclerosis, retinal neuropathy, spinal cord injury, amyotrophic lateral sclerosis and autism [83]. The lack of evidence behind these therapies can be seen with examples like the treatment of stroke, where there is no substantial indication that stem cells treatments can replace damaged cells, reconstruct neural circuits or improve loss of function [77-79]. Despite this, the number of private clinics offering autologous stem cell therapies is continuing to grow in Australia [84]. These predatory clinics are offering stem cell treatments that have little to no scientific evidence of safety, let alone efficacy [86]. This practice is extremely unethical, but the unfortunate reality is that predatory clinics are continuing to exploit vulnerable patient populations around the world [85]. Despite regulatory efforts to reduce the commercial promotion of unproven stem cell treatments, they are still a growing global problem [87]. Australia has the 5th highest total number of stem cell clinics and the 5th highest number of clinics per capita in the world [88].

These unproven stem cell treatments that lack rigorous clinical evidence is detrimental to scientific progress, hinders the implementation of successful of stem cell treatments and damages the public perception of medical practitioners and scientists. Interestingly, the growth of unproven autologous stem cell treatments can be attributed to the realisation that liposuction can be used to extract stem cells [83]. The process of extracting ASCs is minimally invasive in comparison to sourcing other adult stem cells. Despite the rampant used of ASCs in unproven stem cell treatments, there is surprisingly little research characterising them. This project sought to clarify this in Chapter 2 by creating the most comprehensive proteomic catalogue of ASC proteins to date. This was achieved through the identification and quantification of proteins from the ASC whole cell lysate, membrane bound fraction, extracellular vesicles and select secreted cytokines. This project was necessary to increase the overall understanding of ASCs and the data from this project can shape the use of ASCs,

particularly in the clinic. Furthermore, this will serve as a comprehensive library of proteins that can be used to support the use of ASCs as research models.

Research such as that presented in Chapter 2 create a well-established baseline understanding of ASCs, and this is important as only then can complex scientific questions be answered. A proteomic approach was employed as characterisation of proteins is crucial for developing a sound understanding of the complexity of biological samples as they are a dominant feature of cellular phenotype [183]. Furthermore, analysis of the proteome provides information on the functional complexity as proteomes differ both spatially and temporally within cells and tissues [183]. Chapter 2 successfully detected a total of 2290 proteins that were conserved across 8 patient ASCs. A PANTHER analysis was conducted on all these proteins to further elucidate their function by investigation of the following categories: molecular function, cellular compartment, biological process, protein class, and protein pathway. Proteins that were unique to a single cellular fraction were further investigated with STRING networks that highlighted functional enrichments of interest. Proteins detected across multiple fractions were quantified and clustered in a heatmap, with each individual cluster of proteins being further investigated in its own STRING network with particular functional enrichments shown. Select secreted cytokines were also quantified to provide a more complete understanding of ASCs. Collectively the research in Chapter 2 revealed a comprehensive ASC proteome and provided clarity on proteins expressed by ASCs isolated from fresh patient lipoaspirates. This immense data showcases the complexity of ASC proteins and their interactions.

While useful, this only provides some insight on how ASCs are being used in the clinic. There is a lack of standardisation of procedures for isolation, expansion and maintaining clinical standards of ASCs. For example, there are a variety of media available for culturing ASCs that contain fetal bovine serum (FBS) [10], but there has been a shift towards utilising systems that are xeno free and defined [302]. There has been minimal research into the impact that isolation and expansion with xeno free mediums has on ASCs, in particular there is a lack of proteomic characterisation. Chapter 3 investigated the proteome differences of cells grown in different media, and directly compared traditional media against a xeno free alternative. A xeno free media was investigated as FBS is known to be highly variable and consequently poorly defined

[294], and the use of bovine derivatives can also result in bacterial, viral and prion infections [23,295,296]. However, it should be noted with some irony that although xeno free media is 'defined', the manufacturers do not state what those defined components are. ASCs were isolated from 6 patient lipoaspirates and were cultured in either traditional or xeno free media. This research helps to close the gap in the lack of standardisation of procedures regarding ASC isolation and expansion.

The data in Chapter 3 highlighted that the fundamental understanding of changes that occur in cellular phenotype from things as routine as using a different media is lacking, and not just in stem cells but in the wider field of cell culture. It reinforces that clinical application of stem cell treatments should not be applied until comprehensive understanding is gained. When comparing traditional and xeno free expanded ASCs there is a clear shift in the proteome, though it is difficult to determine which isolation and expansion medium is preferable. While the bulk of scientific research has been done on traditional media, it is not safe to assume that this media is preferable. There is not enough data to support traditional media being the superior. The proteome across ASCs isolated from different patients and maintained in traditional media was much more homogenous than their counterparts isolated and expanded in xeno free media. When investigating the shift in the proteome of ASCs, it was clear that there was a shift in the abundance of proteins involved in metabolism. There were measurable differences in proteins involved in transcription, translation, and the synthesis and degradation of proteins. Furthermore, the cytokine profile was wildly different between ASCs isolated and maintained in traditional compared to xeno free media, and the absence of vital stem cell cytokines such as FGF basic suggests that traditional media may be preferential, however substantially more research is required to validate this.

It is not just xeno free and traditional media ASCs that are regularly administered to patients in both proven and unproven stem cell treatments. When looking at clinical uses of ASCs, both fresh and cryopreserved are utilised [311]. However, in the context of research, cryopreserved ASCs are more commonplace as this allows for storage for future use. Cryopreservation of stem cells has been extensively researched because it allows for easier use of ASCs for commercial and clinical applications [312]. There is a substantial amount of literature investigating the effect of cryopreservation on ASCs, and Chapter 3 contributes to

this through the characterisation of the proteome. Traditional media ASCs were expanded as fresh cells or were cryopreserved and subsequently revived and expanded. The proteomes of fresh and cryopreserved ASCs were compared, and a shift in the proteome was observed after cryopreservation. While this shift is not as pronounced as the one observed between ASCs isolated with traditional versus xeno free media, it is still substantial. This research suggests this change also manifests in the metabolome with evidence of particular pathways being altered in ASCs after cryopreservation, or alternatively that ASCs are using alternative sources of energy from the same media. Ultimately cryopreservation of ASCs appears to be reducing metabolic fitness, but again this requires further validation. The research in Chapter 3 clearly highlighted that isolation and expansion ASCs in different media, and using them fresh or after cryopreservation, causes substantial changes in the proteome and it appears these changes are likely metabolic. These differences require significantly more research as these ASCs are already being used in both registered clinical trials and in unproven stem cell treatments.

This also highlighted fundamental flaws in the way we currently culture ASCs and reinforces the need to characterise ASCs outside of the clinic. While pluripotent stem cells are used less commonly in a clinical setting, they are used most often in research models. Using human stem cells as a research model not only offer an alternate to animal models, but they can be used to model particular diseases and provide insight on the phenotypes of the disease and to generate potential therapies [106]. Disease models can be derived from autologous patient cells, for example iPSCs have been used to understand disease pathophysiology, to identify novel drug targets, and to increase clinical success of new drugs [107]. While the bulk of stem cell models use pluripotent cells, multipotent stem cells can still be used as they are capable of differentiating into cells of interest, they can be exposed to different conditions and variables, they can be isolated from patients to recapitulate aspects of their disease, or they can be modified to exhibit characteristics of a disease. Monolayer models are often favoured because they are comparatively cheap and reproducible. Not only are they simple to use but they allow for the investigation a homogenous cell culture [109]. Monolayer stem cell models were used in Chapter 2, 3 and 4 for these reasons.

Chapter 4 examined particular metabolites involved in the kynurenine pathway in a single monolayer of ASCs. Investigation of ASC metabolites has been highlighted as an area that

warrants further research in Chapter 2 and 3. The kynurenine pathway was chosen as it accounts for the metabolism of more than 95% of tryptophan [346]. Tryptophan is one of 9 essential amino acids that cannot be synthesised by cells and must therefore be acquired elsewhere. Synthesis of tryptophan is not energetically favourable and even organisms that can synthesize tryptophan will favour acquiring it over synthesizing it [340,341]. Changes in the kynurenine pathway have been implicated in many diseases such as Alzheimer's [349,350], amyotrophic lateral sclerosis [351,352], dementia [353], Huntington's [354,355], multiple sclerosis [356], HIV/AIDS [353], schizophrenia [357] and neoplasia. Chapter 4 successfully produced the most comprehensive characterisation of the kynurenine pathway in ASCs. This data confidently demonstrated that the kynurenine pathway of tryptophan metabolism is activated by IFN-γ in human ASCs. Treatment of ASCs with IFN-γ resulted in a reduction in tryptophan and an increase in kynurenine. ASCs also appear to be resistant to the deleterious effects of IFN-γ and quinolinic acid, a product of the kynurenine pathway that has shown toxicity in other cells. This research contributes to the notion that ASCs have potential to serve as a minimally invasive means of understanding diseases, in this case the regulation of the kynurenine pathway. Furthermore, modulation of the kynurenine pathway has therapeutic potential [360], and ASCs are already being utilised as a treatment for many of these diseases. Chapter 4 reinforced the importance of investigating the kynurenine pathway in ASCs, moving forward this project needs to be expanded, namely, to evaluate the proteome and to investigate more metabolites. This can also be improved by studying diseased populations, for example analysis of the kynurenine pathway in multiple sclerosis patient ASCs. This research encourages the use of ASCs as a stem cell research model.

Furthermore, more complex models can also be created such as organoids. Organoids have been made from both pluripotent and multipotent stem cells [129]. Organoids have attracted substantial interest as they recapitulate spatial organization of cells, cellular interactions and particular physiological functions, consequently providing insight on the stem cell niche [130]. In future it would warrant further investigation into characterising the proteome of stem cell organoid models. Organoids are also useful for drug testing, as they will likely be more accurate representations of the human body when compared to animal models and can consequently reduce the loss of animal life [129]. Organoid models are considerably more complex than other stem cell models, and this also means that standardization is going to be

significantly harder to achieve. This will be a continual challenge for the field as this lack of standardisation will make it difficult to compare different studies [130]. There will also be challenges when trying to characterise organoids, as there will be variation in cellular phenotype dependant on where the cell is in the organoid. An approach that could be considered is single cell proteomics, but this raises the issue of minimising proteome changes while separating the organoid into single cells. Ultimately this research emphasises the need of further research into stem cell models, particularly more complex disease models. This will also be pivotal in further developing the understanding of ASCs, which is vital as they are still being broadly used for treatments.

While stem cell treatments have immense potential, unproven stem cell treatments are widespread and threatening the entire field. This is in part due to the media painting an overly optimistic picture of stem cell therapies which will ultimately cause patients to be disappointed and disillusioned with the treatments they do receive [90]. On top of this, online digital media has allowed for patients to generate their own perception of significant and successful treatments [92]. Communities of hope are created and maintained online where they continue to depict stem cell treatments in a positive light, often despite the lack of scientific evidence [92]. Sharing health experiences online gives individuals, a sense they are participating in the creation of health information, and reading these first-person accounts affect other individuals health predominantly through the memorability of a story and building an online community [95]. It is important to recognise that it is not only stem cell clinics, but patients and media outlets that amplify stem cell treatments. Many researchers contribute to this excessive hype by overstating their findings in order to secure funding for their own research. The relationship between patients and experts in the field has also been altered because of this direct-to-consumer marketing of stem cell therapies [73]. There is a lack of comprehensive information and reliable resources available to combat the existing predatory online stem cell market [68]. What is required is the production of reliable resources for patients, and for organisations and industry to push for this outreach [97]. Chapter 5 produced reliable scientific information in the form of educational videos and delivered it through the online social media platform YouTube. While the focus of Chapter 5 was not specifically on stem cells, the findings can also be applied to better education in the stem cell field.

Chapter 5 aimed to improve scientific literacy for the subject of multiple sclerosis and quantify whether an increase in scientific literacy has taken place. A series of six videos were produced that explain the science behind multiple sclerosis and an investigation of how these videos improve participant understanding was conducted using of pre and post questionnaires for each video. These questionnaires involved quantitative questions to assess improved learning and qualitative questions to investigate self-regulated learning. This chapter successfully provided a sound understanding of how this online video series, and by implication other online video series, improve learning and how participants self-regulate learning. The participants in this project not only had an improved understanding of the scientific concepts behind multiple sclerosis, but also have some insight on how scientific research is conducted which will hopefully shape their ability to accurately identify reliable sources and separate scientific research with excessive hype from research that provides realistic hope. This knowledge from this project can be adapted to produce better scientific educational video content beyond the multiple sclerosis community, and in particular should be employed to educate the broader community about stem cell therapies, as it is known that patients use digital media to access unproven treatments [92].

In order to directly contribute to improving scientific literacy, the author of this thesis Naomi Koh Belic set out to engage with the community, provide reliable resources on stem cell therapies and to make science more accessible. The clear success of the educational videos for digital media learning from Chapter 5, showcase the potential that science communication has to combat the online false scientific claims that have been highlighted throughout the thesis regarding unproven stem cell therapies. It is vital for researchers to recognise the desperate need for their science to be communicated to the public. After all, what is the point of conducting research if this knowledge isn't applied within the community. For this reason, Naomi has engaged with the scientific community through her research presentations at 15 conferences, 3 of which were oral presentations. She regularly attends the annual Lorne Proteomics Symposium and was invited to co-chair the Interactomics Session in 2020. Naomi has also attended the International Society for Stem Cell Research Annual Meeting every year since 2017. While attending she noticed that the Stem Cell Ethics session had the lowest attendance, despite how essential it is for stem cell researchers to understand the ethics of their field, and she discussed this in the article she was invited to write for the International

Society for Stem Cell Research in 2019. She has also taken her research to the general public through a variety of scientific competitions starting in 2016 with the University of Technology Three Minute Thesis where she won the Science Faculty final and competed in the University finals. In 2017, she competed in the NSW state finals and National finals of FameLab, and in 2018 when she competed in the National finals of Amplify Ignite. Engagement with the public was also shown at Sydney Science Festival where in 2018 she organised and hosted an event at Paramatta Library, presented her own research at Manly Library, was a guest on the live podcast recording for In Situ Science and in 2019 presented her research at the live podcast recording of ABC Ockham's Razor. The excessive hype of stem cell therapies was discussed at length in her presentation at Pint of Science in 2019. And there has been substantial discourse on the same topic on a variety of podcasts including Global Caveat, Ockham's Razor, In situ Science, Eastside FM and Diffusion Radio. In order to ensure the next generation of scientists understand the importance of science communication she has developed and delivered a science communication workshop for the subject Medical Biotechnology at the University of Technology Sydney. She was also awarded a Learning and Teaching Grant from the University of Technology Sydney to create an educational video series on science communication for the subject Career Management for Scientists, where she was also awarded a Learning and Teaching Team Award. Engagement with the wider community is maintained through her active use of social media platforms for science communication such as Instagram, TikTok, Twitter and Facebook. Naomi featured in an education video for U by Kotex to improve scientific literacy around vaginal health for teenagers which was released on social media, the Facebook video alone having over 250,000 views. She has assisted in making science accessible to the general public through featuring on educational yet approachable shows like the 10 episodes of Dr Karl's Outrageous Acts of Science that was released on Discovery Channel that she was a Biology Expert for, or through the online science series Sciencey with ABC that she hosted.

Ultimately this PhD provided much needed proteomic characterisation of ASCs, with a focus on isolation, expansion and storage methods used in clinical applications, and investigation of their use as a research model with a focus on the kynurenine pathway. In order to ensure the findings of this project influenced changes in the community, Naomi actively sought out to communicate her science and utilised her PhD to further investigate how to improve digital

media education. Scientists have a perceived level of trust in the public eye, and the current global pandemic reinforces the importance of scientific education. Knowledge is power, and it is the responsibility of scientists to ensure that science is accessible for everyone.

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