# Characterisation of pathogenic pathways within the bone microenvironment of Gaucher Disease which contribute to bone pathology and haematological malignancy.

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**Signature** **Date**

## Abstract

**Introduction:** Gaucher disease is a recessive disorder, mutation of the GBA1 gene leads to a reduction in β-glucocerebrosidase (GBA) activity, accumulation of glucosylceramide and abnormal levels of other sphingolipids. Features include hepatosplenomegaly, cytopenia and bone disease including osteopenia and osteonecrosis. Enzyme replacement is the most common therapy however bone manifestations can be slow to respond, some patients show no improvement or continue to suffer bone events. Gaucher patients are reported to have a higher incidence of multiple myeloma with an estimated risk ratio up to 51.1.

**Aim:** To investigate the bone marrow microenvironment in GD by partial recreation of this environment using co-culture systems.

**Methods:** Co-culture of up to 3 cell types, histochemical staining, immunofluorescence imaging, functional assay, enzyme activity, therapy and lipid assays, cytotoxicity assay, flow cytometry.

**Results:** Gaucher patient (GD) peripheral blood mononuclear cell derived osteoclast cultures (OC) generated more osteoclasts and at earlier time points than control cultures. GD osteoclasts were larger, had more nuclei and resorbed more bone. Addition of GBA inhibitor CBE to control OC increased osteoclast generation, size and nuclei number. Addition of glucosylceramide to OC increased osteoclast numbers in both control and GD cultures. In vitro GD osteoclast numbers correlated with active bone disease, bone pain and anaemia. Addition of GD-specific therapies to GD OC decreased osteoclast generation, also observed in a selection of patients receiving GD-specific therapies. Uncoupling between GD osteoblast precursors and osteoclasts was observed. Culture of control OC with plasma cell line NCI-H929 increased osteoclast generation. CBE inhibition of human osteoblast cell line SaOS-2 reduced calcium deposition. Glucosylceramide increased NCI-H929 cell number after 7 days. NCI-H929 viability increased when in contact with osteoclasts in SaOS-2 co-cultures.

**Conclusion:** Osteoblast and osteoclast dysregulation may contribute to bone disease in GD. The GD bone microenvironment may provide a pro-survival environment for plasma cells.

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## List of abbreviations

| | |
|---|---|
| **2D** | Two dimensional |
| **3D** | Three dimensional |
| **4-MUG** | 4-methylumbelliferylglucopyranoside |
| **ACE** | Angiotensin converting enzyme |
| **AE** | Adverse event |
| **ALP** | Alkaline phosphatase |
| **APC** | Allophycocyanin |
| **APRIL** | A proliferation-inducing ligand |
| **AR** | Alizarin red |
| **AVN** | Avascular necrosis |
| **BAFF** | B-cell activating factor |
| **B-ALP** | Bone alkaline phosphatase |
| **Bcl-2** | B-cell lymphoma 2 |
| **Bcl-XL** | B-cell lymphoma-extra large |
| **b-FGF** | Basic fibroblast growth factor |
| **BMD** | Bone mineral density |
| **BMP** | Bone morphogenic protein |
| **BMT** | Bone marrow transplant |
| **BMU** | Basic multicellular unit |
| **BRC** | Bone remodelling compartment |
| **BSP** | Bone sialoprotein |
| **CBE** | Conduritol-β-epoxide |
| **CBFA-1** | core-binding factor subunit alpha-1 |
| **CCL** | Chemokine (C-C motif) ligand |
| **CD** | Cluster of differential |
| **Cer** | Ceramide |
| **c-fms** | Colony-stimulating factor-1 receptor |
| **CGRP-1** | Calcitonin gene-related peptide-1 |

| | |
|---|---|
| **CHIT1** | Chitotriosidase gene |
| **CLN** | Ceroid lipofuscinosis neuronal 8 |
| **CNS** | Central nervous system |
| **COX-2** | Cyclooxygenase-2 |
| **CT** | Calcitonin |
| **CTHRC-1** | Collagen triple helix repeat-containing protein 1 |
| **CTX** | Collagen type 1 cross-linked C-telopeptide |
| **CXCL** | Chemokine (C-X-C motif) ligand |
| **Cy** | Cyanine |
| **DC-STAMP** | Dendritic cell-specific transmembrane protein |
| **DEXA** | Dual energy X-ray absorptiometry |
| **DiGalCer** | Digalactosylceramide |
| **DKK1** | Dickkopf-related protein 1 |
| **Dlx-5** | Distall-less homeobox 5 |
| **DMEM** | Dulbecco's modified Eagle medium |
| **DMSO** | Dimethylsulphoxide |
| **DNA** | Deoxyribonucleic acid |
| **Dpyr** | Deoxypyridinoline |
| **DS3** | Disease severity scoring system for Gaucher disease |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ER** | Endoplasmic reticulum |
| **ERAD** | Endoplasmic-reticulum-associated protein degradation |
| **ERK** | Extracellular signal-regulated kinase |
| **ERT** | Enzyme replacement therapy |
| **EU** | European Union |
| **FACS** | Fluorescence activated cell sorting |
| **FBS** | Foetal bovine serum |
| **FDA** | Food and drug administration |
| **FITC** | Fluorescein isothiocyanate |

| | |
|---|---|
| **FOXO1** | Forkhead box protein O1 |
| **Fro** | Fragilitas ossium |
| **GalCer** | Galactosylceramide |
| **GAUSSI-I** | Gaucher Disease Severity Score Index – Type I |
| **GBA** | β-Glucocerebrosidase |
| **GBA1** | β-Glucocerebrosidase gene |
| **GBAP** | Glucocerebrosidase pseudogene |
| **GC** | Lysosomal β-glucocerebrosidase |
| **GD** | Gauchers Disease |
| **GlcCer** | Glucosylceramide |
| **GM1** | Ganglioside monosialotetrahexosyl |
| **GM2** | Ganglioside monosialotrihexosyl |
| **GM3** | Ganglioside monosialodihexosyl |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **HSCT** | Haematopoietic stem cell transplantation |
| **ICGG** | International Collaborative Gaucher Group |
| **Ig** | Immunoglobulin |
| **IGF** | Insulin-like growth factor |
| **IL** | Interleukin |
| **iNKT** | Invariant natural killer T cell |
| **iPSC** | Induced pluripotent stem cell |
| **ISCT** | International society for cellular therapy |
| **JNK** | Janus kinase |
| **LacCer** | Lactosylceramide |
| **LGR4** | Leucine-rich repeat-containing G protein-coupled receptor 4 |
| **LIGHT** | homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with HSV **g**lycoprotein D for binding to **h**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes |

| | |
|---|---|
| **LIMP2** | Lysosome membrane protein 2 |
| **LSD** | Lysosomal storage disorder |
| **M1** | Classically activated macrophages |
| **M2** | Alternatively activated macrophages |
| **MAPK** | Mitogen-activated protein kinases |
| **MCSF** | Macrophage colony stimulating factor |
| **M-CSFR** | Macrophage colony stimulating factor receptor |
| **MEMα** | Alpha minimal essential medium |
| **MGUS** | Monoclonal gammopathy of undetermined significance |
| **MIP1** | Macrophage inflammatory protein |
| **miRNA** | Micro ribonucleic acid |
| **MM** | Multiple myeloma |
| **MMP-9** | Matrix metalloproteinase-9 |
| **MMR** | Macrophage mannose receptor |
| **MPR** | Mannose-6-phosphate receptor |
| **MREC** | Multi-centre Research Ethics Committee |
| **mRNA** | Messenger ribonucleic acid |
| **MRI** | Magnetic resonance imaging |
| **MSC** | Mesenchymal stem cell |
| **mTOR** | Mechanistic target of rapamycin |
| **MTS** | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium |
| **NK** | Natural killer cell |
| **NKT** | Natural killer T-cell |
| **Notch** | Neurogenic locus notch homolog protein 1 |
| **NPC** | Niemann Pick type C |
| **NS** | No significant difference |
| **nSMase2** | Neutral sphingomyelinase 2 |
| **NTX** | Collagen type 1 cross-linked N-telopeptide |

| | |
|---|---|
| **OC** | Osteoclast culture |
| **OC-STAMP** | Osteoclast-stimulatory transmembrane protein |
| **OPG** | Osteoprotegerin |
| **OsteoMacs** | Osteal macrophages |
| **Osx** | Osterix |
| **p62** | Nucleoporin 62 |
| **PARC** | Pulmonary and activation-regulated chemokine |
| **PBMC** | Peripheral blood mononuclear cells |
| **PBS** | Phosphate buffered saline |
| **PCA-1** | Plasma cell antigen 1 |
| **PCT** | Pharmacological chaperone therapy |
| **PDGF** | Platelet derived growth factor |
| **PE** | Phycoerythrin |
| **PERCP** | Peridinin chlorphyll protein |
| **PGP** | P-glycoprotein |
| **PGS3** | Pediatric Gaucher Severity Scoring System |
| **PI** | Propidium iodide |
| **PICP** | Procollagen I carboxyterminal propeptide |
| **PINP** | N-terminal propeptide of type I collagen |
| **PSAP** | Prosaposin |
| **PTH** | Parathyroid hormone |
| **PTH1-R** | Parathyroid hormone 1 receptor |
| **PTHrP** | Parathyroid hormone related protein |
| **RANK** | Receptor activator of nuclear factor κB |
| **RANKL** | Receptor activator of nuclear factor κB ligand |
| **RPMI** | Roswell Park Memorial Institute medium |
| **RunX2** | Runt-related transcription factor 2 |
| **S1P** | Sphingosine-1-phosphate |
| **S1PR** | Sphingosine-1-phosphate receptor |

| | |
|---|---|
| **S-ACE** | Serum-angiotensin converting enzyme |
| **SCARB2** | Scavenger receptor class B member 2 |
| **SDF-1α** | Stromal cell-derived factor 1α |
| **SEM** | Standard error of the mean |
| **sFRP** | Secreted frizzled-related protein |
| **Shc** | Src Homology 2 Domain Containing |
| **SMURF-1** | SMAD Ubiquitination Regulatory Factor 1 |
| **Src** | Sarcoma- Proto-oncogene tyrosine-protein kinase |
| **SRT** | Substrate reduction therapy |
| **STAT** | Signal Transducers and Activators of Transcription protein |
| **TCR** | T-cell receptor |
| **TGF-β** | Transforming growth factor beta |
| **TGN** | Trans-golgi network |
| **Th** | T-helper cell |
| **THF** | Follicular helper phenotype |
| **TIM barrel** | Triose phosphate isomerase barrel or $(\beta\alpha)_8$-barrel |
| **TLR9** | Toll-like receptor 9 |
| **TNF-α** | Tumour Necrosis Factor alpha |
| **TRACP-5b** | Tartrate-resistant acid phosphatase isoform 5b |
| **TRAP** | Tartrate resistant acid phosphatase |
| **US/USA** | United states of America |
| **VCAM-1** | Vascular cell adhesion molecule-1 |
| **VDR** | Vitamin D receptor |
| **VEGF** | Vascular endothelial growth factor |
| **VLA-4** | Very late antigen-4 |
| **VNR** | Vitronectin receptor |
| **Wnt** | Wingless-related integration site |

# 1 Introduction

## 1.1 Lysosomes

Lysosomes were first described by Christian de Duve in 1955 when he discovered membrane bound organelles containing enzymes with lytic actions and named these organelles lysosomes, for which he won the Nobel Prize in Medicine or Physiology in 1974 (1). Subsequent electron microscopic studies showed lysosomes constitute up to 5% of the intracellular volume of mammalian cells, vary in size from 100-500nm in diameter (2) and are distinguished from their precursor, the late endosome, by their lack of mannose-6-phosphate receptors (MPR's) (3).

Lysosomes contain more than 60 different types of proteases, lipases and hydrolases (4) and are primarily responsible for the degradation of proteins, lipids and polysaccharides into their respective basic units i.e. amino acids, free fatty acids and monosaccharides (5,6). Lysosomal hydrolases are produced in the endoplasmic reticulum and transported through the trans-golgi network (TGN), many of which acquire mannose-6-phosphate residues to enable sorting through pre-lysosomal compartments via MPR's (3,7).

While MPR mediated transport is the most common (3), other mechanisms have recently been discovered. Patients with mucolipidosis type II (I-cell disease) are deficient in N-acetylglucosaminyl phosphotransferase which means proteins cannot be labelled with mannose-6-phosphate resulting in many of the hydrolases being secreted instead of transported to the lysosome (8). However, some proteins, including β-glucocerebrosidase (GC), are still transported to the lysosome. In the case of GC this transport is mediated through direct binding with LIMP2 in the endoplasmic reticulum, remaining bound until dissociation in the lysosome due to the low luminal pH of ~4.6 (2,9).

Extracellular components for degradation are transported by endocytosis and intracellular components by autophagy. Endocytosis begins with fission of the plasma membrane to form endocytic vesicles which subsequently undergo a maturation process to become early then late endosomes. Mature late endosomes

fuse with lysosomes to become endolysosomes. This fusion not only provides the material to be degraded but also TGN hydrolases essential for lysosomal function (6,8). Intracellular organelles no longer fit for purpose and protein aggregates are transported to the lysosome by autophagosomes which can fuse indirectly via late endosomes or directly to lysosomes to form autolysosomes (11). Once degraded, the products are exported for energy homeostasis or for reutilisation in biosynthetic pathways (10). Lysosomal trafficking can be mediated through several pathways, these include lysosomal exocytosis, present in all cell types (12) in which lysosomes fuse with the plasma membrane, retrograde trafficking in which MPR's are required for hydrolase transport to the lysosome and insoluble lipids are recycled back to the TGN (10) and lipid exporters including NPC-1 which is theorised to be the exporter for cholesterol, mutations in which lead to cholesterol and sphingolipid accumulation in lysosomes resulting in the lysosomal storage disorder Niemann-Pick type C (13).

## 1.2 Lysosomal storage disorders

Lysosomal storage disorders (LSD's) are rare metabolic diseases caused by the abnormal accumulation of material in lysosomes (14). Although individually rare their collective incidence is estimated at 1 in 5000 live births due to there being over 50 such diseases currently described (15). The majority of the first clinical reports, later identified as LSD's, were published over a century ago - Tay, 1881; Gaucher, 1882; Fabry, 1898; Niemann, 1914; Hunter, 1917. However, as previously mentioned the lysosome was not discovered until 1955 by Christian de Duve and LSD's were not defined until 1965 when H.G. Hers discovered that Pompe disease, a glycogen storage disorder, exhibited a deficiency in acid alpha glucosidase and defined the term "lysosomal enzyme deficiency states" (16). The majority of LSD's are autosomal recessive however several, including Fabry's disease (alpha galactosidase A deficiency) are X-linked (17). LSD's are generally classified by the accumulation of the substrate. These classifications include: Glycogen storage disease type II (Pompe disease), Mucopolysaccharidoses (I-VII), Oligosaccharidoses,

Lipidoses and Sphingolipidoses (18). There are other classifications which include LSD's caused by non-enzymatic defects but the majority of disorders are caused by mutations in lysosomal hydrolases (19) and so included in the classifications stated above. However, these classifications are limited by the fact that many lysosomal hydrolases are not specific for one substrate. For example, β-galactosidase is involved in the degradation of several substrates including keratan sulphate, sphingolipids and oligosaccharides, a deficiency in which results in the accumulation of all of these substrates but is classified as GM1 gangliosidosis (20).

Multiple organs and tissues are typically affected. Clinical manifestations may include enlarged liver and spleen (hepatosplenomegaly), cardiac disease, facial dysmorphia, immune defects and skeletal abnormalities including bone loss and remodelling (21). Severity of each LSD is difficult to define as each disease has a spectrum of severity ranging from patients with such a mild phenotype that they can go undiagnosed to a severity which is incompatible with life (22,23). However, many of the most severe forms involve the central nervous system and account for up to 75% of LSD's (24).

Many variants of mutations in LSD's result in misfolding or aberrant proteins which, although their catalytic activity is usually unaffected, are typically targeted to the endoplasmic reticulum associated degradation pathway instead of to the lysosome effectively resulting in decreased overall enzyme activity and are theorised to cause ER stress and contribute to disease severity (25,26).

Functional loss of certain lysosomal membrane proteins has been found to result in a number of LSD's. Mutations in lysosomal ion channels and transporters alter ion homeostasis within the lysosome impairing the trafficking and degradation of substrates leading to LSD's. Similarly, accumulation of lipids within the lysosome may alter the functionality of lysosomal membrane proteins such as ion channels or catabolite exporters further exacerbating the effect of the defect (27,28).

Recently, it has been shown that autophagic capacity is reduced in LSD's resulting in an increased number of autophagosomes that are never cleared by fusion with lysosomes leading to a blockage of autophagic movement to and from the lysosome

and plasma membrane, termed autophagic flux with the overall effect of an accumulation of substrate in the autophagosomes, however the potential impact of this remains unclear (20,29).

A number of therapeutic approaches have been developed to treat LSD's including enzyme replacement therapy (ERT), substrate reduction therapy (SRT), bone marrow transplantation and gene therapy (30). The most common therapies being ERT and SRT in which the defective enzyme is replaced or the creation of substrate is blocked, respectively. Pharmacological chaperones have also been recently developed which bind to misfolded proteins and enable these proteins to be transported to the lysosome at which point they dissociate due to the low luminal pH of the lysosome (31,32).

## 1.3 Sphingolipidoses

Sphingolipids are a major category of lipids and are present in all mammalian cells and lipoproteins (33). The simplest sphingolipid is ceramide which consists of a fatty acid residue attached by an amide link to a sphingosine backbone and is thus regarded as a central metabolite in sphingolipid processing (34). Further additions to the head group result in more complex sphingolipids. Addition of phosphocholine or phosphoethanolamine leads to a group termed sphingomyelins while addition of sugar residues produces the glycosphingolipids group which is subdivided into cerebrosides (a single glucose or galactose residue), sulphatides (sulphated cerebrosides), globosides (more than one sugar residue) and gangliosides (three or more sugar residues, one of which must be a sialic acid (35), shown in figure 1.1. Cerebrosides are present in all cell types, globosides in visceral organs, galactosylceramides and their sulphatides are generally restricted to myelin and kidney while gangliosides are typically found in neuronal membranes (35).

Sphingolipidoses are a group of diseases in which the main storage substrate is a sphingolipid caused in the majority by a mutation in a lysosomal hydrolase resulting in a reduction in catabolic activity (19), these diseases include Gaucher, Fabry, Tay-Sachs, Niemann-Pick and metachromatic leukodystrophy among others (36), see

figure 1.2. Mutations in transporters can also lead to sphingolipidoses. For example mutations in NPC-1 or NPC-2 genes cause the cholesterol trafficking disease Niemann–Pick type C disease which due to blocked endosome-lysosome fusion results in storage of glycosphingolipids in the late endosome/lysosomes (37).

Recent research in unrelated diseases have also shown that sphingolipids are highly bioactive and have been theorised to play a role in a number of conditions including cancer (38), diabetes (39), dermatological diseases (40), lung inflammation (41), intestinal immunity and allergy (42) which may help to shed some light on the range of pathologies present in sphingolipidoses and LSD's in general.

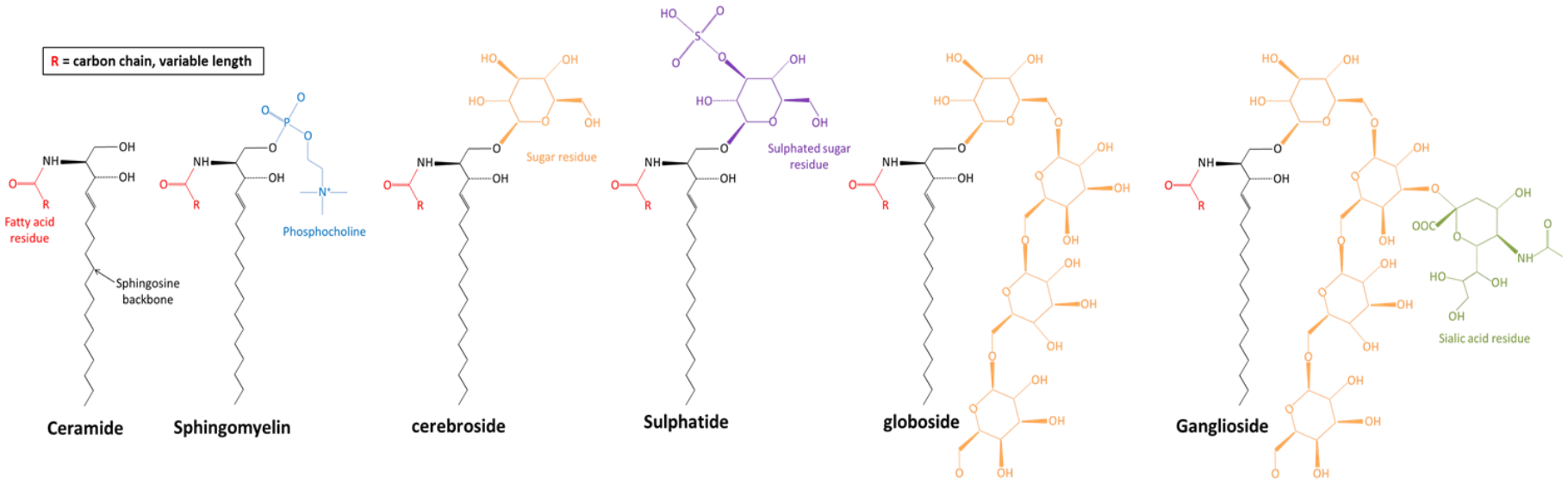



**Figure 1.1** Example diagrams of the basic chemical structures of sphingolipid categories. Own figure.

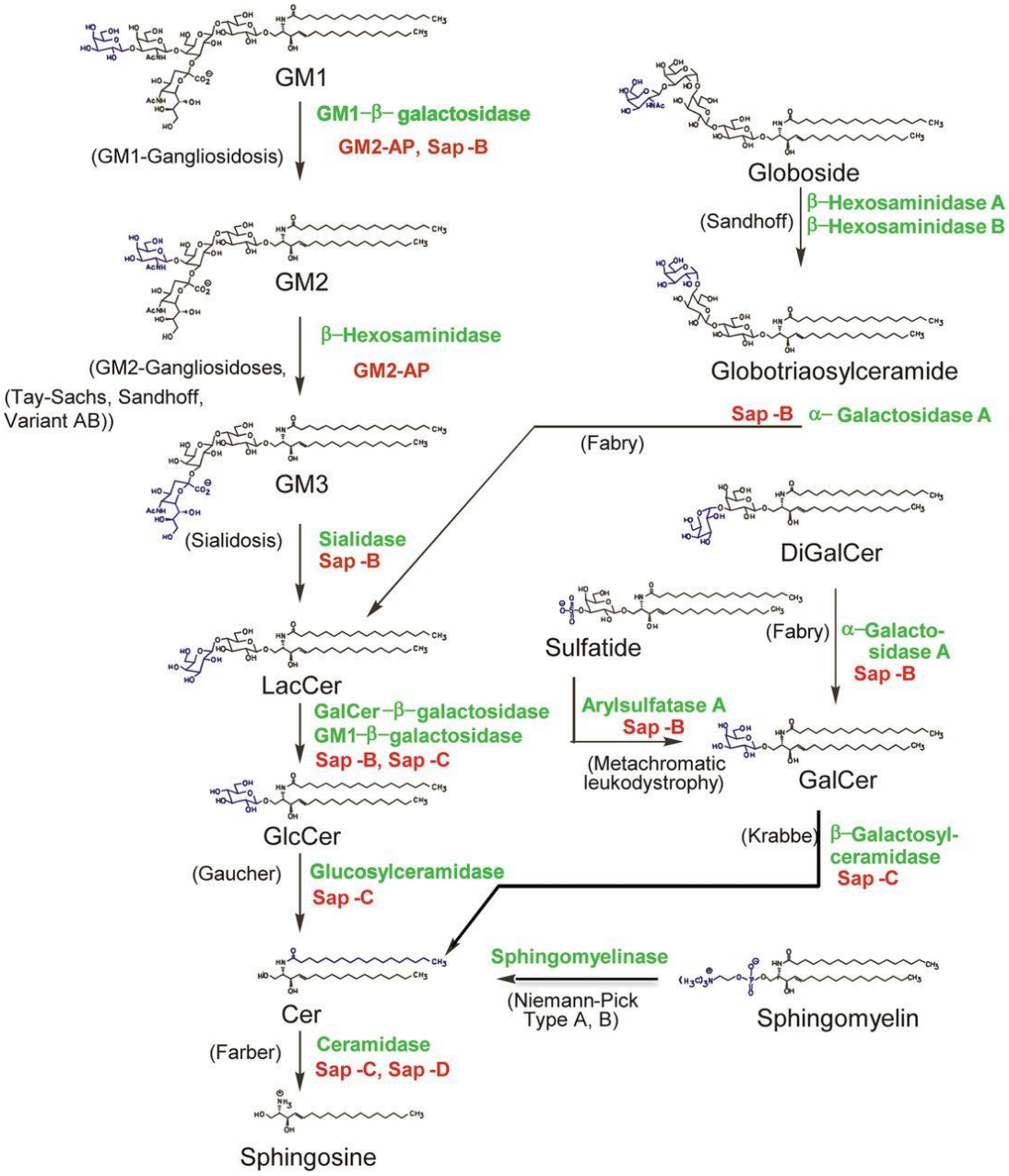



**Figure 1.2** Diagram of sphingolipid metabolism. Source: Metabolic and cellular bases of sphingolipidoses. Konrad Sandhoff. Biochemical Society Transactions. Dec 01, 2013.

## 1.4 Gaucher disease

### 1.4.1 Introduction

Gaucher disease was first described in 1882 by Phillipe Gaucher. As a medical student he examined a 32 year old female and provided descriptions of an enlarged spleen and subsequent microscopic study revealed engorged cells, initially thought to be malignant (43). Subsequent to this report more cases of patients with enlarged cells, identified as macrophages, were reported and given the name 'Gaucher cells' (44), the term still used today. The cause of the disease, deficient activity of β-glucocerebrosidase, was discovered in 1965 by Brady et al. Further investigation identified β-glucocerebrosidase as a lysosomal enzyme in 1972 and patients with Gaucher disease were described as having a lysosomal storage disorder thereafter (45). Storage of the enzymes substrate, glucosylceramide, in the macrophage results in cells with a small eccentrically placed nuclei surrounded by a bright, crinkled cytoplasm (46). The macrophage being the primary storage cell due to their function of degrading senescent blood cells, cell debris and microbes providing an additional peripheral source of substrate (47).

The gene encoding β-glucocerebrosidase (GBA) was found to localise to chromosome 1q21 in the mid 1980's (48) and was sequenced in 1989 (49). It is now known that GD is an autosomal recessive disorder for which more than 300 mutations and polymorphisms have been reported (50). However, the exact incidence of GD is not clear. Estimated incidence based on screening data from an Australian population, published in 1999, was 1:57,000 (51), prevalence in Caucasians was estimated at 1:40,000 to 1:60,000 in a report in 2011 (52) while a new born screening study in Hungary gave a higher prevalence of 1:13,341 in 2012 (53). Incidence is significantly higher in the Ashkenazi Jewish population with incidence estimated at 1:850 (54) and carrier frequency reported between 1:10 to 1:17.5 (55,56).

### 1.4.2 GBA1 gene, protein and pseudogene

Lysosomal β-glucocerebrosidase (GC) is encoded by the GBA1 gene located on chromosome 1q21 and consists of 11 exons, 10 introns and is 7.8 kilo bases in size. (49). In addition, a pseudogene, GBAP (glucocerebrosidase A pseudogene) 5.7kb in size, is located downstream of GBA1 which appears to have arisen due to a tandem duplication despite having 96% sequence homology it is missing large portions of exons two, four, six, seven and nine (49).

GBA1 cDNA is about 2kb in length and is translated into a protein of 497 amino acid residues (49). Synthesis is initiated from one of two ATG codons within the sequence. Transcription occurs predominantly from the upstream initiator, but deletion of either ATG codon does not affect overall expression levels in vitro (57). GBA1 also has two promoters, one of which has an associated CpG island, a common feature of housekeeping genes and possibly explains the expression of GC in the majority of cell types (58).

GC is a 60kDa protein comprising of three domains identified by X-ray crystallography in 2003. The third domain contains the catalytic site and consists of a TIM barrel (8 α-helices, 8 β-strands) formed from amino acid residues 76-381 and 416-430 (59).

### 1.4.3 GBA1 mutations

Over 250 pathogenic mutations have been reported for the GBA1 gene including 203 missense, 18 nonsense, 36 insertion/deletions, 14 splice junction variants and 13 complex alleles (50) carrying two or more mutations resulting from gene recombination or conversion with the pseudogene (60). Pathogenic mutations have not been described in exon 1, with the majority of mutations occurring in two exon blocks (exons 5-7 and 8-11) (50).

The first mutations to be described were the missense mutations L444P (exon 10) in 1987 (61) and N370S in 1988 (exon 9) (62) and are the most common (50,63). However, allele frequency varies with ethnicity and geographical location. In the

Ashkenazi Jewish population, N370S, L444P, R496H, V394L (all missense), 84GG (insertion), and ISV2+ 1G>A (splice junction) account for roughly 96% of mutant alleles (50,64). The frequency of N370S is higher among Iberians (Portuguese: 53.7%, Spanish: 46.2%) than other non-Jewish European groups (65,66). In contrast, the N370S and 84GG mutations have not been identified among Chinese and Japanese GD patients with the most prevalent alleles being L444P (54%) and RecNciI (25%) among the Chinese and L444P (41%) and F213I (14%) in the Japanese populations (67).

A number of the mutations described, including N370S which is located at the interface between the second and third domains but not in the active site, cause destabilising changes in the protein structure which although only partially reduce catalytic activity result in retention in the endoplasmic reticulum and subsequent degradation (59,68,69). The L444P mutation causes a structural change in the hydrophobic core of the Ig-like second domain which is theorised to affect its interaction with Saposin C (59), an essential co-factor for lysosomal GC catalysis of glucosylceramide (70). The insertion mutation 84GG, which denotes a second guanine base insertion at residue 84, results in premature termination of transcription which is predicted to severely compromise GC as homozygosity for this mutation has not been reported to date (63).

In addition to pathogenic mutations in the GBA1 gene mutations in the PSAP gene, which encodes for prosaposin, a polyprotein which is cleaved into four different saposins referred to as Sap A-D (71), also result in a variant form of Gaucher disease. As mentioned, saposin C is an essential cofactor for GC, functioning as an activator for GC's activity. While extremely rare, with only 6 cases reported to date (72), mutations including p.C315S, p.342_348FDKMCSKdel, p.L349P, p.C382G and p.C382F have been identified to cause saposin C deficiency (70).

Despite elucidation of the mutations, protein structure, processing and activation of GC there is still poor correlation of genotype with phenotype. Age of onset and disease progression can vary between patients homozygous for the same mutation (73), even between twins (74) and mutations can be present across a spectrum of severity (75). While the N370S mutation has been found to correlate with mild,

non-neuronopathic disease homozygotes can display a wide range of symptoms from being asymptomatic to having severe organomegaly and bone involvement (73). Similarly, the L444P allele, commonly associated with a severe neuronopathic form, has been shown to be present in patients ranging from moderate systemic and neurological symptoms to seizures and severe developmental delay (76).

Therefore, to address this variability, potential candidates for disease modifiers have been identified. These candidates include the GBA2, SCARB2 gene (encoding the LIMP2 protein), CLN8 and microRNA MiR-127-5p. GBA2 is a non-lysosomal GC which has been suggested to have a direct involvement in neurological disorders (77). LIMP2 is involved in the mannose-6-phosphate independent trafficking of GC (78) while increased CLN8 expression has been suggested to reduce disease severity (79). MicroRNA's, small non-coding RNA's, have been found to be involved in a number of biological processes and diseases. Recently, MiR-127-5p was found to reduce GC activity and protein level by down regulation of LIMP-2. In addition, other MiRNA's including miR-16-5p and miR-195-5p were found to upregulate GC activity (80).

### 1.4.4 Clinical presentation

Gaucher disease is currently considered a continuum of phenotypes from asymptomatic to severe (81,82). However, to aid diagnosis GD is generally classified into three major clinical types namely types 1, 2 and 3 of which type 1 is the most common. Analysis of 1,698 individuals in the International Gaucher registry reported 94% as type 1, <1% as type 2 and 5% as type 3 (63).

Type 1 is characterised by the presence of hepatosplenomegaly (enlargement of liver and spleen, with a spleen up to 20 times normal size (4)), clinical or radiological evidence of bone disease (including osteopenia, osteonecrosis, osteosclerosis, osteolytic lesions, pathological fracture and bone remodelling (84,85)), anaemia, thrombocytopenia, lung disease (86), gammopathy, bleeding diathesis, liver fibrosis, growth retardation, fatigue, weight loss, pulmonary hypertension

(17,60,87–90) and the absence of neurological involvement of the central nervous system (91), the presence of which characterises types 2 and 3.

Although historically classified as a non-neuronopathic phenotype investigation into neuronopathic features of type 1 GD patients have revealed occurrence of peripheral neuropathy and neurological changes secondary to bone complications in a multi-centre, multinational cohort in 2008 (92). In addition a link has been established between type 1 GD and the development of early onset Parkinsonism (93). These observations led to the finding that there is a high prevalence of GBA1 mutations in non-GD Parkinson's disease patients with pathogenic variants identified in 5-10% of individuals with Parkinson's disease (94), making heterozygosity for GBA1 mutations a risk factor for developing early onset Parkinsonism (95). The mechanism behind this association is under investigation but several have been suggested including accumulation of mutant GC within Lewy bodies (96) and impaired mitochondrial function (97). In addition, a 2016 study has shown increased dimerization of α-synuclein (the protein associated with classical Parkinson's disease) in the red blood cells of GD patients correlating both with glucosylceramide levels and the increased oxidative stress observed in GD (98).

GD patients with central nervous system involvement are classified into type 2 (acute/infantile) and type 3 (subacute/chronic/juvenile) according to age of onset of neurological symptoms and rate of disease progression (99). Type 2 is the most severe form with symptoms observed as early as 6 months of age followed by rapid progression and limited psychomotor development leading to death by 2 to 4 years of age. Neurological involvement can include bulbar signs (stridor, squint, swallowing difficulty), pyramidal signs (opisthotonus, head retroflection, spasticity and trismus) and cognitive impairment (100). Type 3 is generally less severe; onset can occur before 2 years of age but often with slower progression leading to death by the third to fourth decade. Symptoms can include ocular apraxia, seizures and myoclonic epilepsy (101,102). It has been suggested that type 3 should be divided into three subclasses (72,103), types 3a to c, in which type 3a would have early onset neurological disease with seizures and horizontal supranuclear gaze palsy but mild visceral symptoms. Type 3b would have later onset of seizures but significant

hepatosplenomegaly as well as kyphoscoliosis and a barrelled chest. Although type 3b is regarded as the 'Norrbottnian type' (found in high frequency in the Norbotten region of Sweden) the clinical distinction between types 3a and 3b is difficult therefore these sub-classifications are rarely used (104). Type 3c, more commonly referred to as the cardiovascular form, is a distinct subtype predominated by cardiovascular disease with calcification of the mitral and aortic valves with mild splenomegaly, supranuclear ophthalmoplegia and corneal opacities. This form is specifically associated with patients homozygous for the D409H mutation (105,106). In contrast, a subtype of type 2 often viewed as a distinct class, the perinatal-lethal form is a rare subset with considerable genotypic variability (107). Symptoms can include hepatosplenomegaly, pancytopenia, microscopic skin changes (108). Distinctive facial features and arthrogryposis have been reported in 35-43% of patients (109).

In addition, pathogenic mutations in GBA2, a related gene to GBA1 encoding for a non-lysosomal glucosylceramidase, have been shown to cause an autosomal recessive form of cerebellar ataxia with spasticity (110) and autosomal recessive hereditary spastic paraplegia (111).

### 1.4.5 Pathophysiology of type 1 GD

Deficiency in GC activity leads to accumulation of its substrate glucosylceramide in macrophages, the primary storage cell in GD, resulting in the formation of foamy macrophages classically referred to as 'Gaucher cells' (44,46). This storage affects all of the cells of the mononuclear phagocyte system including tissue macrophages of the spleen, liver (Kupffer cells), lung (alveolar macrophages), central nervous system (microglial cells), bone (osteoclasts), bone marrow, lymph nodes, skin, urinary tract and gastro-intestinal tract (112). Interestingly, a post mortem investigation of the lipid composition of a type 2 GD patient found the fatty acid composition of glucosylceramide in cells of the central nervous system (CNS) was different to those in the liver and spleen. Glucosylceramide in the CNS was predominantly composed of steric acid whereas visceral organs were

predominantly composed of palmitic acid suggesting a different origin of glucosylceramide in these cells (113).

Gaucher cells have been shown to accumulate lipid in membrane-bound tubular structures by X-ray crystallography (114), express monocytic markers, are tartrate resistant acid phosphatase (TRAP) positive and have the phenotype of alternatively activated macrophages (115). They are 20-100μM in diameter, accumulate in sheets within tissue and are associated with deposition of extracellular sphingolipid, inflammatory infiltrate and fibrotic reaction (116,117).

Hepatosplenomegaly is a clinical hallmark of GD. In the liver, sheets of lipid laden Kupffer cells are present but hepatocytes do not appear to store glucosylceramide possibly due to excretion into the bile (118). Hepatic pathology includes fibrosis, extramedullary haematopoiesis with a suggested increase in gallstones. Other rare pathology includes cirrhosis, liver failure, portal hypertension, oesophageal varices and hepato-pulmonary syndrome (89,119). Four cases of GD patients which had undergone liver transplant, reported by Ayto et al in 2010, were found to have favourable outcomes after up to 10 years post-transplant and was therefore regarded as a valid option for GD patients with liver failure (119). Splenic pathology includes Gaucher cell infiltration, fibrosis, extra-medullary haematopoiesis, infarction and nodules which can be mistaken for cancerous infiltration (120). However, despite the elevated levels of glucosylceramide in these tissues they cannot account for the increase in volume observed. The spleen for example can have up to a 25 fold increase in size but the glucosylceramide accounts for <2% of that mass (116).

Bone disease is a common feature of GD with radiological evidence described in up to 93% of patients (121). However, the severity of bone involvement can range from complete absence of symptoms to severe osteonecrosis and pathological fractures (122). The bone disease can be partially explained by bone marrow infiltration of Gaucher cells suggested to cause displacement of marrow cells to the periphery (123) which may lead to marrow infarcts including osteonecrosis of joints (122) and is likely associated with bone marrow fibrosis and impaired haematopoiesis (120). In addition, Gaucher cells are hypothesised to elicit an

inflammatory response which may affect bone metabolism (122). However, the range of severity and the variety of types of bone disease indicate the involvement of several mechanisms which cannot be explained by Gaucher cell infiltration alone.

Pulmonary disease in the form of interstitial lung disease is common for types 2 and 3 but rare in type 1 GD (120). However, alveolar macrophages have been shown to be affected when macrophages were examined by light and electron microscopy from a broncho-pulmonary lavage of a young GD patient with respiratory symptoms (124). In addition, infiltrates of Gaucher cells have been reported in the lung interstitium and alveolar air spaces (125). Evidence for the prevalence of pulmonary disease is unclear with one study of 95 type 1 GD patients showing 68% having pulmonary function abnormalities (126) while a later study of 150 patients found only 5 with clinical evidence of pulmonary involvement (127). Pulmonary hypertension is suggested to occur more often in asplenic patients (90) but splenectomy is now rarely performed (120,128).

Renal pathology is rare in GD but it has been described in a small number of patients and is associated with storage of glucosylceramide in glomerular mesangial and endothelial cells and interstitial cells of the kidney (129).

### 1.4.6 Molecular and cellular pathology of type 1 GD

Several cell types have been reported to be affected in GD. Some of these have been linked to the presence of Gaucher cells. An immunohistochemical study of splenic Gaucher cells by Boven et al in 2004 identified the presence of large M2-like anti-inflammatory cells surrounded by smaller M1 cells positive for IL-6, weakly expressing IL-1β and secreting MIP-1β. These cells have been theorised to trigger the induction of $CD4^+$ T-cells with Th1 or Th2 phenotype (115,122). GD patient macrophages have been found to have impaired chemotaxis in response to CCL3, CCL5, and CXCL12 and reduced reactive oxygen species production when cultured in vitro (130). Macrophages derived from the peripheral blood monocytes of an N370S homozygous GD patient by Aflaki et al in 2015 showed an increased secretion of IL-1β and IL-6 in comparison to control cells. Interestingly they also

found elevated levels of autophagic adaptor p62 which was preventing the delivery of inflammasomes to autophagosomes suggesting that dysfunction of the inflammasome activation may contribute to visceral pathology and the increased prevalence of Parkinson's disease in GD (131). In addition, a cellular model in which macrophages were differentiated from induced pluripotent stem cells (iPSC) of a GD patients also expressed higher levels of IL-6, IL-1β and TNF-α than control cells (132). Alterations in monocytes, precursors to macrophages, have also been found. Monocytes isolated from the peripheral blood of untreated GD patients have a decreased capability of SDF-1α dependent migration (133) and have also been found to have an altered ratio of anti-inflammatory $CD14^+$ $CD16^-$ to pro-inflammatory $CD14^+$ $CD16^+$ in favour of the pro-inflammatory monocytes when compared to control cells (134).

Immune cell dysregulation has also been reported with a marked decrease in NK cells, CD4+ and CD8+ T cells and altered responsiveness of plasmacytoid dendritic cells to TLR9 which is theorised to contribute to a decreased response to pathogens and favour the development of malignancies (135,136). In addition, a new population of type 2 natural killer T cells (NKT) with follicular helper phenotype (THF), identified by Nair et al in 2015, have been found in higher percentages in GD patients peripheral blood mononuclear cells (PBMC's) and are thought to be capable of regulating B cell activity (137).

Mesenchymal stem cells (MSC), which can differentiate into several cell types including osteoblasts, adipocytes and chondrocytes, have also been found to be affected. A study of 10 GD patient bone marrow samples found morphological and cell cycle abnormalities and impaired growth potential with a decreased capacity to differentiate into osteoblasts (138). However, assessment of bone marrow MSC from a GD patient with N370S/L444P mutations were found to have normal growth and differentiation capacities but a marked increase in prostaglandin E2, COX-2, CCL2 and IL-8 compared to normal controls (139). Models of progenitor cells have also found altered phenotypes. GD patient iPSC differentiated to hematopoietic progenitor cells demonstrate an increased commitment to myeloid lineage differentiation and decreased erythroid differentiation and maturation (140). While

a model using the irreversible inhibitor of GC, conduritol β-epoxide (CBE), to mimic GD in mesenchymal progenitors found inhibition led to progressive impairment of proliferation (141). Assessment of erythrocyte morphology by electron microscopy of 9 untreated GD patients by Bratosin et al in 2011 found they have abnormal morphology with reduced viability and CD47 expression which are theorised to facilitate erythrophagocytosis in untreated patients (142).

Analysis of GD patient serum and plasma has found an elevation in a number of cytokines and chemokines including IL-1β, IL-1 receptor agonist, TNF-α, IL-6, soluble IL-2 receptor (143), osteopontin (144), CD163 (145) amongst others which are probably due to secretion by activated macrophages. A summary is provided in table 1.2. Increased levels of IL-1β, TNFα and IL-6 may contribute to activation of coagulation and hypermetabolism (87,112). Other cytokines which promote monocyte differentiation into osteoclasts found to be elevated in GD patient plasma and serum, in addition to IL-1β, TNFα, IL-6 include IL-10 (146,147), macrophage inflammatory protein (MIP) 1α, MIP1β, macrophage colony stimulating factor (MCSF) and pulmonary activation regulatory cytokine (PARC) (147–149).

In addition, recent analysis of the lipid composition of GD patient plasma and urine found elevated levels of 20 plasma and 10 urinary lipids including species of phosphatidylcholine, sphingomyelin and ceramides (150). With several publications showing substantially higher levels of glucosylsphingosine (151,152), the de-acylated form of glucosylceramide, in GD plasma and serum demonstrates that the deficiency of GC affects the synthesis and degradation pathways of many sphingolipids several of which are known to be bioactive and involved in other diseases, mentioned in section 1.3. Some of the altered sphingolipid concentrations measured in GD patient plasma are shown in table 1.1 below.

| **Sphingolipid** | **Change (ref)** | **Sphingolipid** | **Change (ref)** | **Sphingolipid** | **Change (ref)** |
|---|---|---|---|---|---|
| Ceramide | ↑(150) | Sphingosine | ↑(153) | Sphingomyelin | ↑(150) |
| Lactosylceramide | ↓(154) | Sphingosine-1-phosphate | ↑(153) | Phosphatidyl-choline | ↑(150) |
| Glucosylceramide | ↑(155) | Glucosylsphingosine | ↑(152) | | |

**Table 1.1** Sphingolipid concentrations altered in GD plasma. Up and down arrows indicate higher or lower concentrations relative to control cohorts.

| Cytokine | Origin | Action | References |
|---|---|---|---|
| IL-1α<br>IL-1β<br>IL-1RA | Monocytes<br>Macrophages<br>Dendritic cells<br>Lymphocytes<br>Granulocytes<br>Fibroblasts | Pro-inflammatory<br>Leukocyte migration | (143,146,156,157) |
| sIL-2R | T-lymphocytes | Lymphocyte stimulation<br>Pyrogen<br>Pro-inflammatory | (143) |
| IL-6<br>IL-6 mRNA | Macrophages<br>T-cells<br>Plasma cells<br>osteoclasts | B-cell stimulation<br>Pro-osteoclastogenic<br>Pro or anti-inflammatory response | (143,146,156) |
| IL-8 | Macrophages<br>Epithelial cells<br>Lymphocytes | Neutrophil chemotaxis<br>Granulocyte chemotaxis<br>B-cell stimulation<br>Pro-inflammatory | (143,147,156) |
| IL-10 | Monocytes<br>Macrophages<br>Lymphocytes | B-cell survival and proliferation<br>Anti-inflammatory | (146,157) |
| sCD163 | Monocytes<br>Macrophages | Anti-inflammatory | (145) |
| MCSF | Osteoblasts<br>Bone marrow stromal cells | HSC differentiation to macrophage | (147,156) |
| MIP1-α | Macrophages | Pro-inflammatory<br>Leukocyte stimulator | (148) |
| MIP1-β | Macrophages | Monocyte chemotaxis<br>NKT chemotaxis | (148) |
| Osteopontin | Macrophages<br>Osteoblasts<br>Osteocytes<br>Dendritic cells | Osteoclast maturation<br>Bone remodelling | (144) |
| PARC | Monocytes<br>Macrophages<br>Eosinophils | T-cell chemotaxis<br>Lymphocyte chemotaxis | (149,157) |
| TNF-α | Monocytes<br>Macrophages<br>Fibroblasts | Inflammation<br>Neutrophil chemotaxis<br>Macrophage phagocytosis | (143,146,156,158) |

**Table 1.2** Plasma and serum cytokines, chemokines and growth factors elevated in GD.

### 1.4.7 Clinical biomarkers

The plasma of GD patients is commonly used to monitor disease by analysis of biomarkers. Common biomarkers include chitotriosidase activity (159,160), PARC (CCL18) (149), TRAP (160), serum antigen converting enzyme (S-ACE) (160) and ferritin (159). Chitotriosidase is primarily secreted by macrophages, increased expression of chitotriosidase by Gaucher cells leads to increased plasma chitotriosidase levels (161). Chitotriosidase activity has been found to correlate with disease burden and decreases when GD patients are treated with GD specific therapies (162). However, approximately 6% of the population are homozygous for a null mutation in the gene encoding chitotriosidase, CHIT1, resulting in undetectable enzyme activity and up to 34% of individuals heterozygous for the null mutation which is expected to have half the measurable activity of wild type protein (163,164). PARC/CCL18 is a chemokine which is also overexpressed by Gaucher cells (115) and has been shown to parallel chitotriosidase activity, correlate with visceral bulk, decrease when GD patients are treated with GD specific therapies (149) and can be used to monitor disease severity in GD patients with the null mutation for chitotriosdase (165). TRAP, an acid phosphatase expressed in osteoclasts and dendritic cells has been shown to be elevated in GD however serum levels have not been found to reflect bone disease severity in GD patients (116,166). S-ACE is elevated in the serum of GD patients and can be used to monitor disease severity and response to GD specific therapies (167). However, ACE is expressed in several tissues types (168) and has a common polymorphism resulting in variable expression (169). Serum ferritin levels are raised in GD patients (170) and are thought to be caused by macrophage activation due to substrate accumulation (159) however this can also be the case for several diseases and conditions and therefore may not reflect the severity of GD (171,172). Recently, a number of potential biomarkers have been suggested for assessing severity and response to therapy. Glucosylsphingosine is highly elevated in GD plasma and has been found by Fuller et al in 2015 to correlate with changes in chitotriosidase activity for patients receiving GD specific therapies (173). Similarly osteopontin, produced by immune cells and macrophages, has also been found to be elevated in

GD patient plasma, to decrease when patients receive GD specific therapies and to correlate with chitotriosidase activity (144). Soluble CD163, again elevated in GD patient plasma, is actively shed from the plasma membrane of monocytes/macrophages and has been found to correlate with disease severity and chitotriosidase activity. In addition it was found to be significantly elevated in patients with abnormal BMD and patients with pulmonary hypertension (145). Neopterin is a catabolic product of guanosine triphosphate, synthesised by macrophages and indicative of a pro-inflammatory immune response. This has also been found to be elevated in GD patient plasma and to decrease when GD patients receive GD specific therapies (174). However, the most applicable biomarker is probably glucosylsphingosine as its elevation in plasma is a direct consequence of a reduction in GC activity. Although the other markers also correlate with disease severity and the majority decrease when the patient receives GD specific therapy they are primarily increased due to sphingolipid storage in the macrophage inducing cellular dysfunction and are therefore surrogate biomarkers

### 1.4.8 Disease severity scoring systems

The Zimran scoring system has historically been used to document disease burden. However, this scoring system was developed before therapies were available for GD and before the discovery of biomarkers. This system incorporates weighted scoring domains for age of presentation, spleen status, bony disease, liver function, hepatomegaly, splenomegaly, cytopenias and clinical signs of liver disease (175). However, substantial weighting is given to irreversible factors such as splenectomy, osteonecrosis and imaging abnormalities making the scoring system insensitive to changes in response to GD specific therapies and therefore not ideal for following disease progression (176). The Gaucher disease severity scoring index for type 1 (GAUSSI-I) is a more recent scoring index, published in 2008, which consists of six domains: skeletal, haematological, lung, neurological, visceral and biomarker, evaluated in 51 type 1 GD patients and suggested to be more sensitive for monitoring response to treatment than the Zimran scoring system (177). However,

it has been suggested that the use of the GAUSSI-I scoring system may be limited due to its complexity and technological requirements (178). Subsequently, other scoring systems have been developed for adult and paediatric GD type 1 patients namely the DS3 and PGS3 systems published in 2015 by Weinreb et al and 2013 by Kallish et al respectively. The DS3 scoring system was validated in 133 type 1 GD patients and consists of 3 domains: bone, haematologic and visceral and was found to be effective for assessing disease burden and response to GD specific therapies (179). The PGS3 system, designed specifically for paediatric GD type 1 patients, was evaluated in 26 patients and found to correlate with disease severity at diagnosis and over time. Unlike the other scoring systems, the PGS3 is designed to be informative for clinical management rather than to follow response to therapy as many of the patients will not have started therapy (178). Comparison of the two most recently developed adult scoring systems, GAUSSI-I and DS3, show a marked difference in the contribution of the bone domain to the total score with a maximum of 26% in GAUSSI-I but a maximum of 42% in DS3 suggesting the need to follow bone disease in response to treatment in GD may be of increasing relevance clinically for GD patients.

### 1.4.9 Diagnosis

Gaucher disease may be suspected in individuals with characteristic bone disease, hepatosplenomegaly, haematological changes and CNS involvement however clinical findings alone are not regarded as sufficient for diagnosis (54,60). The gold standard is the demonstration of deficient GC activity, estimated between 5-25% of normal activity, in leukocytes or other nucleated cells (60,121). However, carrier testing by enzyme assay is unreliable due to overlap between carrier and non-carrier enzyme activities. The finding of reduced enzyme activity can be further supported by genetic sequencing of the GBA1 gene to confirm the presence of two pathogenic alleles. Discovery of a previously undescribed mutation would require confirmation of pathogenicity by confirming the deficiency in activity of GC (60).

### 1.4.10 Prognosis for type 1 GD patients

Analysis of data recorded in the International Collaborative Gaucher Group (ICGG) Gaucher Registry of type 1 GD patients found an average life expectancy of 68 years compared to 77 years for the reference population from the USA. Splenectomised patients were found to have a shorter life expectancy (64 years for splenectomised versus 72 years with intact spleen). However, as the earliest current therapy was only introduced in 1995, life expectancy is anticipated to increase (180,181).

### 1.4.11 Treatment for type 1 GD patients

Prior to the development of GD specific therapies the most common treatments included bone marrow transplantation (BMT)/haematopoietic stem cell transplantation (HSCT) (182–184) for severe type 1 and 3 GD patients and splenectomy for treatment of painful splenic infarcts, cytopenias and diagnostic purposes (185,186). However, while HSCT has been found to improve cytopenias and hepatomegaly in addition to correcting the metabolic defect (187) it is also associated with significant morbidity and mortality (10-25%) (187,188). Splenectomy increases the risk of bacterial infection, worsening bone disease and development of pulmonary hypertension (112,176,189). Worsening bone disease is likely to be due to one of the spleens functions of phagocytosing effete erythrocytes which is achieved through having a high number of resident macrophages. In GD this may mean the spleen stores a large proportion of the lipid laden macrophages therefore splenectomy may force circulating macrophages to reside in other tissues such as the bone marrow, increasing Gaucher cell burden and thus worsening bone disease. Due to these risks both options are rarely used since the development of effective therapies for type 1 GD. Recently, some groups have suggested a re-evaluation of HSCT as advances in technologies such as high-resolution human leukocyte antigen typing enables more accurate donor recipient matching (190) and improved techniques for mobilisation and collection of stem cells (191) may make this treatment safer and more effective (192).

#### 1.4.11.1 Enzyme replacement therapy (ERT)

The rationale for ERT is based on the observation that most cells secrete small quantities of lysosomal enzymes which could be internalised by other cells. It was therefore suggested that infusion of recombinant GC would result in its internalisation and trafficking to the lysosomes where it metabolises the accumulated substrate glucosylceramide (193). In 1976-77 methods for large scale purification of GC from human placenta were developed leading to the first infusion of GC into patients (194,195). However, initial studies were not as effective as expected. This was explained in subsequent research by Furbish et al in 1978 in which infusions given to rats demonstrated that unaltered placental GC was localising to hepatocytes, cells which store very little glucosylceramide (196). Further investigations of the placental GC found it contained a high percentage of galactose-terminated oligosaccharide side chains which targeted the enzyme to the hepatocytes (197). To re-target the GC the monosaccharide residues were sequentially removed from the side chains using the enzymes neuraminidase, β-galactosidase and β-N-acetylglucosaminidase to expose mannose residues which could bind to the macrophage mannose receptor (MMR), be internalised by the macrophages and trafficked to the lysosomes (193,198).

The first trial of the mannose-terminated GC, published in 1991 by Barton et al, resulted in a reduction in hepatosplenomegaly and improvement of haematological parameters with no serious adverse events (193). Subsequently, this therapy was approved by the Food and Drug Administration (FDA) (199) and was marketed by Genzyme as alglucerase (Ceredase). However, the preparation of this therapy required a large supply of placentas and was therefore both very limited in terms of supply and very expensive. In 1994 Genzyme obtained FDA approval for a recombinant form of GC produced in Chinese hamster ovary cells named imiglucerase (Cerezyme) (200,201). An outcome survey of data recorded in the ICGG registry of type 1 GD patients receiving imiglucerase for 10 years showed significant improvements in haemoglobin levels, platelet count, bone crises, liver and spleen volumes (202).

Two other ERT's have since been developed and given FDA approval: velaglucerase alfa (VPRIV, Shire Pharmaceuticals) a gene activated form produced in human fibroblast-like cell line (203) and taliglucerase alfa (Elelyso, Protalix/Pfizer) produced in carrot cells, the first drug made in plant cells to be given FDA approval (204).

A 9 month, randomised control study comparing the efficacy of imiglucerase and velaglucerase alfa found no difference in their effectiveness although a slightly higher risk of drug related adverse events (AE) was found with velaglucerase alfa treatment with 8/17 patients with an AE when receiving velaglucerase versus 6/17 AE for patients receiving imiglucerase (205). In addition, these therapies are well tolerated with approximately 10-15% of individuals developing antibodies to imiglucerase and 1% of individuals developing antibodies to velaglucerase alfa (206,207). Taliglucerase alfa is a much more recent therapy, obtaining approval for use in paediatric patients in 2014 (208) therefore there is less clinical data available but initial reports show reduction of hepatosplenomegaly, improvement in haematological parameters (209), increased bone marrow fat fraction (210) and stability in disease parameters when switching from imiglucerase (211). Taliglucerase alfa is also the only ERT which is deemed kosher by the US Orthodox Union. A basic comparison of the ERT's are shown in table 1.3 below.

| Enzyme | Product name | Produced in | Biochemical differences |
|---|---|---|---|
| Imiglucerase | Cerezyme | Chinese hamster ovary cell line (CHO) | Amino acid substitution at residue 495 (R495H) |
| Velaglucerase alfa | VPRIV | Human fibroblast cell line | Native human enzyme |
| Taliglucerase alfa | Elelyso | Carrot root cell | Native human enzyme with plant glycosylation |

**Table 1.3** Comparison of commercially available ERT's.

#### 1.4.11.2 Substrate reduction therapy (SRT)

SRT is an alternative treatment strategy to ERT by inhibiting glucosylceramide synthase and thus reducing the amount of substrate, glucosylceramide, for the defective GC to process (212). This approach was developed to meet the need of

patients for whom ERT is not a therapeutic option due to allergy, hypersensitivity or poor venous access (213).

The first compound approved for SRT in mild to moderate GD patients was N-butyldeoxynojirimycin (miglustat, marketed as Zavesca by Actelion) (214). The main advantage of which is its oral administration (215). It has been approved in a number of countries including the USA, Canada, EU, Switzerland and Israel (60). Miglustat treatment has been shown to result in significant decrease in hepatosplenomegaly after 6 to 18 months with bone and haematological parameters remaining stable (214,216). Increase in bone density at the lumbar spine and femoral neck was reported as early as 6 months (218). However, side effects including gastrointestinal dysfunction, peripheral neuropathy and tremors most likely caused by its non-specific inhibition of other enzymes including intestinal disaccharides with the most common adverse outcomes in clinical trials being weight loss, bloating, flatulence and diarrhoea (60,218). Due to these side effects miglustat is only recommended for patients with a mild to moderate phenotype who are not suitable for ERT (213).

Recently, a second SRT has been approved by the FDA called eliglustat tartrate (Genzyme) a synthetic analogue of 1-phenyl-2-decanoylamino-3- morpholino-1-propanol formulated as a tartrate salt (219). Eliglustat is designed to be a more specific inhibitor of glucosylceramide synthase, experiments with which have shown that even at relatively high micromolar concentrations it did not inhibit intestinal disaccharides or lysosomal GC (220). In a placebo-controlled phase 3 study of therapy naïve patients those given eliglustat for 9 months had a 28% decrease in spleen volume and a 7% decrease in liver volume with improvement in haematological parameters (221). A positive effect on bone was also suggested by a significant increase in lumbar spine T-scores in 19 patients enrolled in a phase 2 study for up to 4 years (222). Reported side effects include diarrhoea, arthralgia, fatigue and headache (223).

#### 1.4.11.3 Pharmacological chaperone therapy (PCT)

PCT is a rapidly developing field with numerous new compounds under development for several diseases in which abnormal protein folding or defective trafficking can lead to enzyme deficiency (224). PCT's are low molecular weight compounds designed to stabilise or aid correct folding of the native conformation of the mutant enzyme in the endoplasmic reticulum (ER) enabling the enzyme to avoid aggregation and degradation by the ER associated degradation pathway (ERAD) (225). The stabilised protein can then be transported through the trans-golgi network for maturation to the lysosome (226). The PCT is designed to bind to the active site at neutral pH and dissociate at the acidic pH present in the lysosome (227). Several pathogenic mutations in GD are thought to result in premature ERAD mediated degradation including the most common N370S (228). One of the first candidates for PCT for GD was the unmodified iminosugar isofagomine which binds to the active site of GC and can therefore behave as a competitive inhibitor (229). Initial experiments demonstrated isofagomine to be an enhancer of GC activity increasing the enzyme activity and protein levels of several mutant GC's (230), including N370S (230), however phase II and III trials found suboptimal improvement in key GD clinical parameters (231). It was theorised by Sánchez-Fernández et al in 2016 that the reason for this may be that the transport of isofagomine to the ER and lysosome is inefficient. In addition, it is thought that the balance between its action as a competitive inhibitor and chaperone may require very accurate dosage both in terms of amount given to the patient and the period between doses (225).

A second PCT, ambroxol hydrochloride found by screening 1040 FDA approved drugs (227), has also been shown to increase GC activity and protein levels (232). Ambroxol is a mucolytic approved for treatment of respiratory diseases (233) but is not currently approved as a therapy for GD. A pilot study in which 12 type 1 GD patients received a relatively low dose of ambroxol for 6 months showed no adverse effects. The study produced limited clinical effects most likely due to the low dosages used but trials with higher doses are planned (234).

Due to the fact that PCT's are chemical compounds optimization strategies can be employed to improve pharmacokinetics, toxicity and bio-distribution (235). One such example is a modification to the structure of ambroxol resulting in doubling the maximum GC activity enhancement in N370S homozygous GD fibroblasts (225).

#### 1.4.11.4 Gene therapy

GD is considered a potential candidate for gene therapy for several reasons. 1. It is a monogenic disorder with studies showing demonstrable cross correction (193) 2. only 11-15% of expression was sufficient for correction in vitro (236). In addition, the GBA1 gene and the main affected cells have been well characterised. Recent studies involving adenovirus (237) and lentivirus (238) vectors demonstrated increased expression of GC in mouse models with the study using lentivirus stating this expression cured the type 1 GD model used (238). However, several hurdles still need to be overcome before this can be considered a viable therapy (239).

### 1.4.12 Gaucher disease and cancer risk

Patients with GD have been reported to have an elevated risk of developing both haematological and non-haematological cancers when compared to the general population (240,241). Reported haematological cancers include: multiple myeloma (157,241–243), acute lymphoblastic leukaemia (244), acute myeloid leukaemia (245), chronic lymphocytic leukaemia (246), chronic myeloid leukaemia (247,248), large B-cell lymphoma (249), T-cell lymphoma (250) and Hodgkin lymphoma (251). Non-haematological malignancies include: liver (157,252), lung (253), bone (254,255), colon (243), kidney (157,256), testis (256), thyroid (243), prostate cancers (243), glioblastoma multiforme (257), dysgerminoma (258) and melanoma (242).

Several studies have been carried out to investigate the incidence of cancer in GD patients. In 1982, Lee et al compiled a registry of 239 type 1 GD patients. Of the 35 patients who died in this cohort 54% (19/35) were attributed to cancer (256). A

study in 1993 by Shiran et al reported an increased cancer risk of 14.7 fold for haematological and 3.4 fold for non-haematological cancers among 48 type 1 GD patients. However, it has been suggested that this study may have overestimated the risk as these patients were selected due to referral to a cancer centre and thus introduced referral bias (245,259). Zimran et al in 2005 carried out a study of 505 type 1 GD patients of Ashkenazi Jewish ancestry but found no overall increased risk of haematological malignancy with the exception of multiple myeloma (260). However, this was a relatively young cohort with a mean age of 37 years which probably resulted in underestimation of cancer incidence. A similar issue was present in a study by Rosenbloom et al in 2005 which included 2742 type 1 GD patients from the ICGG registry. Again this study found no increase in overall cancer risk but did record a 5.9-fold increased risk for multiple myeloma. However, the population studied was also a relatively young age group and so may have led to an underestimation of cancer incidence.

In 2006 de Fost et al assessed a population of 131 type 1 GD patients from Western Europe. This study found an increased risk of 2.5 fold for cancer and 12.7 for haematological malignancies. Interestingly, this study also found a higher incidence of hepatocellular carcinoma with a standardised rate ratio of 141.3 (157). Taddei et al in 2009 assessed a cohort of 403 type 1 GD patients which were either homogenous or heterogeneous for the N370S mutation. As the majority of the patients were of Ashkenazi Jewish ancestry the relative risk was calculated in relation to the Israeli population. In this study 55 cancers were reported in 46 patients with an overall risk of cancer of 1.8 and 3.45 for haematological cancers excluding myeloma (242). A study in 2010 by Lo et al assessed a cohort of 403 type 1 GD patients 9 of which developed up to 3 sequential cancers. The authors of this study suggesting that type 1 GD patients may be at risk of developing multiple malignancies and that splenectomised patients appeared to have an elevated risk of developing cancer (243). Based on the number of case reports AL amyloidosis is suggested to occur more frequently in GD compared to the general population (261). Similarly, non-Hodgkin lymphoma has also been found to be more common in a cohort of GD patients when compared to the general population (240).

### 1.4.13 Gaucher disease and multiple myeloma

The incidence of polyclonal gammopathy and paraproteinaemia has been reported to be increased in several type 1 GD patient cohorts (262). The incidence of polyclonal gammopathy in adult cohorts has been reported in up to 60% of patients, with IgG the most common immunoglobulin to be elevated (87,146,262). In addition, the incidence of polyclonal gammopathy has been suggested to be higher in paediatric GD populations (263). Analysis of the incidence of polyclonal and monoclonal gammopathy of unknown significance (MGUS) in type 1 GD patients has been shown in large cohorts to follow a temporal sequence with 32% having polyclonal gammopathy at a mean age of 62 years, 3.2% MGUS at a mean age of 78 years, and 2.2% multiple myeloma at a mean age of 84 years (242,262). Population studies have demonstrated that approximately 1% per year of patients with MGUS progress to develop multiple myeloma (264). However, it is unknown if this rate of progression applies to GD patients (265).

A number of studies, including those mentioned in section 1.4.12. have shown an increased risk of multiple myeloma for type 1 GD patients (146,157,241,242,266). In addition, based on the number of case reports multiple myeloma is the most common cancer reported in GD (157,241,259) with myeloma and B cell lymphomas regarded as the leading cause of death in GD (157,241,242). In the 2006 de Fost study, multiple myeloma risk is estimated to be 51.1 fold which was accompanied by an increased prevalence of MGUS: 16% in the Netherlands and 7% in Germany compared to an estimated 1-2% in a population of a similar age (157). Similarly, Taddei in 2009 found the highest risk in their cohort was for multiple myeloma with a relative risk of 25 fold. However, this rate was determined only for the first incidence of cancer in an individual. When all incidences of multiple myeloma were included the relative risk rose to 37.5 fold (242). Landgren et al in 2007 carried out a study of 1,525 adult male U.S. veterans with type 1 GD and found no increased risk of multiple myeloma. This study has been criticised for its young population and the use of ICD codes 272.2 and 272.7 to identify patients with GD are not specific for GD and include other lipid storage diseases as well as dyslipidemias (245,267). In regards to age, the 2005 study by Rosenbloom showed that 9/10 patients with

multiple myeloma were older than 60 years of age (241) suggesting focusing on a relatively young cohort will cause a significant underestimation of prevalence in the GD population.

### 1.4.14 Gaucher Disease and myeloma – potential mechanisms

Several mechanisms have been proposed to contribute to an increased incidence of cancer in general and multiple myeloma in particular in GD. As previously mentioned several reports have shown that type 1 GD patients may have a dysfunctional immune system (268) with marked decreases in NK cells, CD4+ and CD8+ T cells and peripheral blood dendritic cells (135,136,269). A study of 5 type 1 GD patients found defective T-cell function through a reduction of E-rosetting capacity proposed to result from hyperferritinaemia which is commonly found in GD (269). In addition, ferritin release from Gaucher cells has been suggested to reduce T-cell function and IgM release from B cells (270,271).

One suggested mechanism causing the immune dysregulation are the Gaucher cells and alternatively activated macrophages surrounded by highly inflammatory macrophages in tissues (115). Several anti and pro-inflammatory cytokines have been reported to be elevated in the serum of GD patients including IL-1, MCSF, IL-6 and TNF-α which may result in chronic stimulation of the immune system (132,147). These cytokines have also been proposed to directly underlie the occurrence of gammopathies in GD with IL-6 known to stimulate B-cells consistently elevated in GD serum (272,273).

Recently, the role of sphingolipids as bioactive molecules in cancer has been suggested to contribute to the increased incidence of cancer in GD. Invariant natural killer cells (iNKT) are known to participate in anticancer immunosurveillance. They are a subset of αβ T-cells with an invariant TCR alpha chain that recognize lipids, one of which has been shown to be endogenous glucosylceramide, also shown to be a major signal for both murine and human iNKT cells (274,275). Glucosylceramide has also been linked with cell proliferation (276). MDCK (Madin-Darby Canine Kidney Epithelial) cells treated with an inhibitor of GC,

conduritol β-epoxide, showed a time dependent accumulation of glucosylceramide which correlated with increased cellular proliferation (277). In addition, glucosylsphingosine has also been suggested to mediate cellular dysfunction (272) and to lead to increased levels of sphingosine-1-phosphate which has been shown to have proliferative pro-mitogenic, anti-apoptotic and pro-angiogenic effects (278).

Studies have also demonstrated that several multidrug resistant cell lines have increased cellular levels of glucosylceramide including the breast cancer cell line MCF-AdrR and epidermoid carcinoma cell line KB-V-1 (279). A comparable increase has also been documented in human tumor specimens (280) with drugs such as tamoxifen and verapamil shown to markedly decrease glucosylceramide levels (274). In this regard, glucosylceramide has been suggested to be involved in drug resistance rather than proliferation (281,282) as it has been shown to upregulate the expression of the multidrug efflux pump P-glycoprotein (PGP) and to block chemotherapeutic-induced oxidative cell death (281–283).

The role of ceramides, also found to be elevated in GD plasma, in relation to inflammation and cancer have also expanded (284). Not only has ceramide been shown to be pro-apoptotic and a mediator of pro-apoptotic pathways but also an anti-inflammatory and that a metabolite of ceramide, ceramide-1-phosphate, is a potent pro-inflammatory mediator (278,285,286). Recent research has also suggested unique anti and pro-cancer effects depending on the length of the acyl chain of the ceramide species (285,287). In addition, several anti and pro-cancer effects are linked to the sphingosine-1-phosphate/ceramide biostat theory which purports that sphingosine-1-phosphate is pro-oncogenic and pro-metastatic while ceramide regulates anti-cancer fate with ceramide-based therapeutics emerging as anti-cancer agents (38,278,285).

In regards to potential genetic modifiers, several genes in inflammatory pathways have been studied but no consistent correlations have been observed (245).

## 1.5 Bone remodelling

The skeleton is a very specialised and dynamic organ that undergoes continuous regeneration (288). Bone modelling occurs during bone formation and takes place from the skeletal development in the foetus to the second decade of life (289). Modelling is distinct from remodelling as it involves bone formation at sites that have not undergone prior resorption and therefore leads to modification of bone mass and form. Remodelling occurs throughout life. Very small regions of bone are resorbed by osteoclasts followed by recruitment of osteoblast precursors that differentiate and deposit bone matrix to replace the amount of bone removed (289). Remodelling is crucial for adaption to physical stress, removal of old bone and repair of bone damaged by daily physical load to maintain mechanical integrity (290,291). Remodelling processes occur asynchronously at different stages of progress at anatomically distinct sites throughout the skeleton (292). 5-10% of the skeleton is replaced every year and the entire skeleton is completely replaced every 10 years (293). Osteoclasts and osteoblasts are not present on the bone surface simultaneously as the remodelling process can take up to 6 months (294). Early studies by Eriksen et al published in 1984 showed bone resorption in the trabeculae of the iliac crest takes approximately 3 weeks (295) while bone formation takes a further 3-4 months (296). Between these two activities there is a currently poorly understood reversal phase (297) which lasts for approximately 5 weeks (295).

Precise co-ordination of bone removal and replacement is essential to ensure an equivalent amount of bone replaces the bone removed (289). To achieve this a basic multicellular unit (BMU) is formed. The concept for the BMU originally included only osteoclasts and osteoblasts but over recent years the number of cell types involved has expanded as other contributors to bone remodelling have been discovered. These cells include T-cells, osteocytes, macrophages and precursors of osteoclasts and osteoblasts (291,298,299). The BMU differs according to whether remodelling is occurring on the bone surface i.e. trabecular bone or within the bone i.e. cortical bone. In trabecular remodelling the BMU forms on the surface with a canopy of cells predominantly of mesenchymal stem cell origin over either osteoclasts or osteoblasts. In this form of BMU the osteoclasts remove an amount

of bone and the surface is cleaned up by lining cells and probably macrophages (289,300). Osteoblast precursors then fill the resorbed space, differentiate and deposit new bone (289). Within the bone the BMU consists of a cutting zone led by osteoclasts which are followed by differentiating osteoblasts with remaining space filled by blood vessels, nerves and connective tissue, both BMU's shown in figure 1.3 obtained from Sims and Martin et al 2014 (289). However, it should be noted that 90% of bone remodelling is found on previously resorbed bone on the trabecular surface and so the trabecular BMU is the most commonly referred to (301,302).

The tight regulation between osteoclast and osteoblast activity is referred to as coupling (289). To ensure this a second structure forms around the BMU, this is referred to as the bone remodelling compartment (BRC). This consists of a canopy of bone lining/osteoblast cells and a nearby capillary covering the BMU (303). Hauge et al have provided direct evidence that when the need for a remodelling event has been detected bone lining cells separate from the underlying bone and form the raised canopy before bone resorption begins (304). This initiation event is thought to be orchestrated by osteocytes as evidence indicates that they can act as mechanosensors (305,306). The main purpose of the BRC is hypothesised to provide a compartment for intercellular communication between bone cells, epithelial, vascular and immune cells which may be accessible via the blood supply ensuring maintenance of local concentrations of coupling signals and the correct positioning of cells for contact based communication (289).

The potential influence of immune cells has been of growing interest as it has been shown that T-cells can mediate the anabolic effect of parathyroid hormone (PTH) known to induce bone formation at low concentrations but bone resorption at high concentrations (307,308). In addition, several cytokines produced by T-cells are known to either be promoters or inhibitors of osteoclast formation including IL-17, IL-4, RANKL and Wnt10b (309–311). A recent finding that B-cells produce Wnt-1, known to promote bone formation, further implicates the immune system in modulation of bone remodelling (312). It has also been suggested that the canopy of bone lining cells may play an important role in the reversal phase as biopsies

from postmenopausal and glucocorticoid induced osteoporotic patients exhibited incomplete canopies at sites where there was reversal phase arrest i.e. bone was resorbed but osteoblast precursors were not recruited and so the bone was not replaced (313,314).

Locally generated cytokines are regarded as the key influences on bone remodelling which in turn are often regulated by hormones and the nervous system (289). Recent research has shown that signals go in both directions between osteoclasts and osteoblasts. One proposed mechanism in addition to cellular and hormonal signalling is the release of cytokines from the bone matrix following resorption by the osteoclasts (294). Bone is composed of inorganic salts and organic matrix. The inorganic material consists of phosphate and calcium ions which form hydroxyapatite crystals and make up approximately 65% of the bone composition (315,316). 90% of the organic bone matrix consists of collagenous proteins which are predominantly type 1 collagen (315). The non-collagenous proteins include a range of cytokines and growth factors including transforming growth factor beta (TGF-β), bone morphogenic protein-2 (BMP-2), platelet derived growth factor (PDGF), insulin-like growth factors (IGF's), osteopontin, osteonectin, fibronectin and bone sialoprotein II (317–321). All are deposited by the osteoblasts during matrix production and released by resorption (319). Some of which are then activated by plasminogen activators and matrix metalloproteinases (322,323). Their main actions are to stimulate osteoblast progenitors including their recruitment (324,325), migration (324,326,327) and differentiation (326,328).

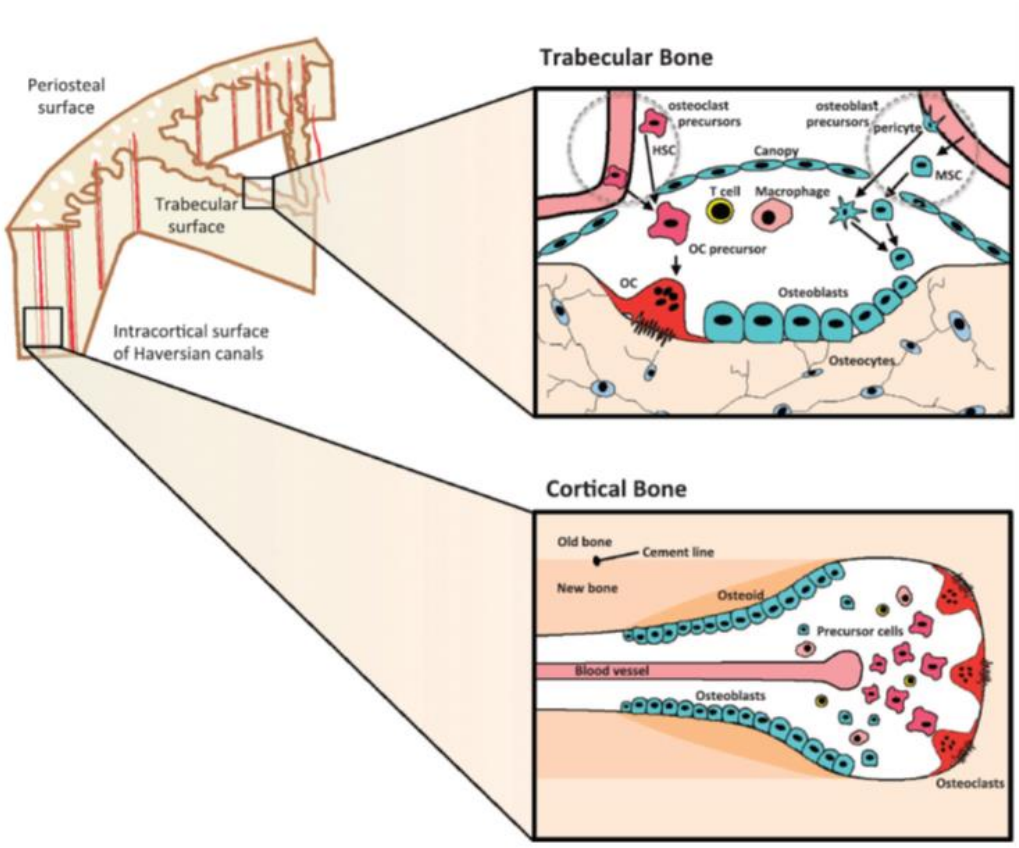



**Figure 1.3** Bone remodelling. Basic multicellular units (BMU) in trabecular bone and cortical bone. Sims and Martin. BoneKey reports, 2014 (289).

## 1.6 Osteoclasts

Osteoclasts are terminally differentiated giant multinuclear cells (329) formed by the fusion of cells of the monocyte/macrophage lineage and are the only known cell type to form ruffled membranes and degrade bone (330). Cell-cell fusion occurs primarily via the dendritic cell-specific transmembrane protein (DC-STAMP), regarded as the master regulator of osteoclastogenesis (331). However, recent research by Witwicka et al published in 2015 has shown that a second fusion protein, osteoclast-stimulatory transmembrane protein (OC-STAMP), is also essential for osteoclastogenesis and is not interchangeable with DC-STAMP (332). In addition, DC-STAMP is thought to play a role in bone homeostasis as evidence suggests it regulates the differentiation of both osteoblasts and osteoclasts (331). Osteoclastogenesis is primarily driven by macrophage colony stimulating factor (MCSF), secreted by a number of cells including mesenchymal stem cells (MSC's)

and osteoblasts (333,334), and receptor activator of nuclear factor κB ligand (RANKL) secreted in the main by osteoblasts and osteocytes (335,336). Binding of MCSF to its receptor (M-CSFR; c-fms) on osteoclast precursors stimulates their proliferation, inhibits apoptosis and promotes expression of the RANKL receptor (RANK) increasing their sensitivity to RANKL (337,338). The binding of RANKL to its receptor induces osteoclast formation (339). In the presence of MCSF, RANKL stimulates DNA synthesis and cell proliferation during the early phase of osteoclastogenesis but in the later phase exerts an anti-proliferative action (340). It is therefore theorised that RANKL optimises osteoclast formation by ensuring a sufficient number of pre-osteoclasts for cell-cell contact and fusion (341). To mediate osteoclastogenesis RANKL is bound by osteoprotogerin (OPG), a soluble decoy receptor secreted by a number of cell types including MSC's and osteoblasts, preventing RANKL from binding to RANK thus inhibiting osteoclastogenesis (342). OPG has also been shown to induce apoptosis of both osteoclast precursors and mature osteoclasts via the classic Fas/FasL pathway (343). Recent research by Luo et al published in 2016 has identified a second decoy receptor, leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4) whose expression is induced by RANKL signalling and therefore acts as a negative regulator of osteoclastogenesis in addition to OPG (344).

Once formed, osteoclasts are short lived and undergo apoptosis within a few days (341). They are defined by their ability to resorb bone, dissolving both inorganic and organic components of the bone matrix, by secreting protons and collagenolytic enzymes (345). During bone resorption polarization of the osteoclast occurs. This results in the formation of four osteoclast membrane domains: the sealing zone and ruffled border, which are in contact with the bone surface and the basolateral and functional secretory domains which are not in contact with the bone surface (346,347). The formation of the ruffled border involves the reorganisation of the actin cytoskeleton to form an F-actin ring which creates a podosome, isolating an area of the membrane which forms the ruffled border. Maintenance of the ruffled border is essential for osteoclast activity and is formed by intense trafficking of lysosomal and endosomal components (315). The sealing zone is formed by the

binding of αVβ3-integrin (vitronectin receptor) to bone matrix proteins such as bone sialoprotein (BSP), osteopontin and vitronectin creating a sealed central region in which the ruffled border is located (348). In the ruffled border protons and enzymes such as tartrate resistant acid phosphatase (TRAP), matrix metalloproteinase-9 (MMP-9) and cathespin K are transported into a compartment called the Howship Lacuna to dissolve the bone matrix (346,349–351). The degradation products are endocytosed across the ruffled border and transcytosed to the plasma membrane (347).

Osteoclast formation and activity is controlled by both systemic and local factors. Systemic factors include parathyroid hormone (PTH), calcitonin (CT), estrogen and vitamin D3 (1,25(OH)2 vitamin D3) (288). PTH is secreted by the parathyroid glands in response to changes in serum calcium levels (352). Chronic elevation of PTH levels leads to trabecular bone loss however its primary action is on MSC's and osteoblasts, increasing RANKL and decreasing OPG production (353) leading to increased osteoclast formation. CT is secreted by the thyroid C cells and tissue specific alternative splicing of the calcitonin gene results in the production of calcitonin gene-related peptide-1 (CGRP-1). Their effects are primarily mediated through the calcitonin receptor and calcitonin receptor like receptor respectively (354,355), both expressed on osteoclasts (356) resulting in morphological changes in osteoclasts inhibiting their motility and activity (357). Estrogen is a major hormonal regulator of bone metabolism in both men and women (288). Estrogen blocks RANKL/MCSF mediated osteoclast differentiation by reducing c-Jun activity (358), suppressing osteoclast differentiation. In addition, estrogen has also been shown to modulate a number of cytokines including TNF-α, MCSF, IL-1, IL-6 and prostaglandins (359,360). Vitamin D3 is recognised as being essential for the normal development and maintenance of bone (288) primarily through endocrine actions to stimulate intestinal calcium and phosphate absorption (361). However, research by Takeda et al published in 1999 has shown that vitamin D3 has localised effects in the bone microenvironment, stimulating osteoclastogenesis by binding to vitamin D3 receptor (VDR) expressed by osteoblasts and osteocytes inducing the secretion

of RANKL (362). In addition, evidence suggests vitamin D3 can also directly regulate osteoclast precursor proliferation and fusion (363,364).

As research into the regulation of bone remodelling progresses an ever increasing number of local factors influencing osteoclast formation and function are being discovered, shown in table 1.4. Many of these factors ultimately affect the main system of osteoclast regulation, namely the RANKL/RANK/OPG axis (342). Factors such as bone morphogenic proteins (including BMP 2 and 7) (365), interleukins (including IL-6 and IL-10) (366,367), oncostatin M (368), Wnt proteins (including Wnt3a, 5a, 16) (369,370) indirectly affect osteoclast formation and maturation by altering the expression and secretion of RANKL and OPG by osteoblasts. While other factors such as caveolin 1 (371) and forkhead box protein O1 (FOXO1) (372) affect the expression of RANK by osteoclast precursors, altering their sensitivity to RANKL.

RANK/RANKL expression in osteoclasts is also regulated by microRNA's which are non-coding RNA sequences of ~22 nucleotides which can prevent the transcription of mRNA. In osteoclasts, these miRNA sequences can be either pro-osteoclastogenic e.g. miR-148a or anti-osteoclastogenic e.g. miR-503 (373). With the continual discovery of different miRNA sequences, many more are likely to be found to regulate osteoclastogenesis.

Several lipids have also been found to modulate osteoclast formation and function. These include cholesterol which has been suggested to affect osteoclast viability (374), ceramide which has been shown to reduce osteoclast activity by inhibiting actin ring formation (375) and lactosylceramide which has been shown to increase RANK expression in osteoclasts (376). Furthermore sphingosine-1-phosphate (S1P) has been found to be a chemotactic factor, regulating precursor osteoclast migration between the bone marrow and the blood (377). S1P is produced by cells in the vasculature (378) and at high levels by osteoclasts (379). Regulation of osteoclast recruitment is mediated through two receptors, S1PR1 (chemoattractant) and S1PR2 (chemorepulsant). High concentrations of S1P in the blood cause internalisation of S1PR1 and surface expression of S1PR2 on precursor osteoclasts resulting in chemorepulsion of the precursors from the blood stream to

the bone marrow. Equally, a higher concentration in the bone marrow can cause migration to the blood thus regulation of the S1P gradient can affect recruitment and formation of osteoclasts in the bone marrow niche (377,380). In addition, S1P has also been found to increase expression of RANKL by osteoblasts, further enhancing osteoclast generation (380).

| Factor | Origin | Generation | Activity | Ref |
|---|---|---|---|---|
| Arginase 1 | Osteoclasts | ↓ | Unknown | (381) |
| BMP 2 and BMP-7 | Osteoblasts, osteoclasts | ↑ | ↑ | (365) |
| Caveolin 1 | Monocytes, macrophages | ↑ | Unknown | (371) |
| FOXO1 | Monocytes, macrophages | ↓ | ↓ | (372) |
| IL-1β | Osteoblasts, macrophages Osteoclasts | ↑ | ↑ | (382) |
| IL-6 | Osteoblasts, macrophages Plasma cells | ↑ | Unknown | (366) |
| IL-8 | Macrophages | ↑ | ↑ | (383) |
| IL-10 | Monocytes, macrophages T-cells | ↓ | ↓ | (367) |
| IL-11 | Osteoblasts | ↑ | Unknown | (384) |
| IL-17 | T-helper cells | ↑ | ↑ | (385) |
| IL-23 | NK cells, dendritic cells | ↑ | ↑ | (386) |
| LGR4 | Osteoblasts, monocytes, macrophages | ↓ | ↓ | (344) |
| MCSF | Osteoblasts, osteocytes, fibroblasts | ↑ | ↑ | (387) |
| Micro RNA-148a | Monocytes, pre-osteoclasts | ↑ | Unknown | (373) |
| Micro RNA-503 | Monocytes, pre-osteoclasts | ↓ | Unknown | (373) |
| MIP-1α, MIP-1β | Macrophages, lymphocytes | ↑ | ↑ | (388) |
| Notch | Macrophages, osteoblasts | ↓ | Unknown | (389) |
| Oncostatin M | Macrophages, osteoblasts | ↑ | Unknown | (368) |
| OPG | Osteoblasts, MSC's | ↓ | ↓ | (342) |
| RANKL | Osteoblasts, osteocytes, MSC's | ↑ | ↑ | (342) |
| Semaphorin 3b | Osteoblasts | ↑ | ↑ | (390) |
| TGF-β | Osteoblasts | ↑ | ↑ | (391) |
| TNF-α | Monocytes, macrophages | ↑ | ↑ | (392) |
| Wnt3a | MSC's, osteoblasts | ↓ | ↓ | (369) |
| Wnt5a | MSC's, osteoblasts | ↑ | ↑ | (370) |
| Wnt 16 | MSC's, osteoblasts | ↓ | ↓ | (370) |

**Table 1.4** Local extracellular and intracellular factors affecting osteoclast generation and activity**.** Ref = reference.

## 1.7 Osteoblasts

Osteoblasts are derived from MSC's with initial commitment requiring the expression of specific genes including the synthesis of BMP's and Wnt proteins (393) to become precursor osteoblasts which have been found to circulate in the blood as well as being resident in the bone marrow (394). Interestingly, it has been shown by Gray et al in 1996 that osteoblast precursors can sense the size and shape of pits formed by osteoclast resorption and preferentially attach, mature and form bone matrix in those locations (395). Osteoblast differentiation is largely mediated through the expression of Runt related transcription factor 2 (RunX2), regarded as the master gene as RunX-2-null mice lack osteoblasts, in conjunction with Distall-less homeobox 5 (Dlx-5) and osterix (Osx) (396,397). A recent study by Chen et al published in 2015 has also shown mTORC1 to have a distinct role in osteoblast maturation (398). Maturation is characterised by a morphological change becoming large cuboidal cells with a high expression of Osx and the secretion of bone matrix proteins (396).

Once mature, osteoblasts synthesise bone matrix which occurs in two phases, deposition of organic matrix followed by mineralisation. In the first phase, osteoblasts secrete collagen proteins, predominantly collagen type 1, non-collagenous proteins including osteonectin, bone sialoprotein II, osteopontin and proteoglycans such as decorin and biglycan (315,399,400). This is followed by the mineralisation phase which itself has two stages, the vesicular and fibrillar phases (401,402). Matrix vesicles, loaded with calcium ions bound to proteoglycans, bud from the apical membrane and are deposited onto the organic matrix. The proteoglycans are degraded by osteoblast secreted enzymes and the calcium ions are released into the vesicle via annexin calcium channels (401–403). Secretion of alkaline phosphatase (ALP) by osteoblasts degrades phosphate-containing compounds, releasing phosphate ions which react with the calcium ions to form hydroxyapatite crystals. The fibrillary phase occurs when the hydroxyapatite crystals rupture the vesicle due to supersaturation leading to the release of these crystals onto the organic matrix and its mineralisation (404,405). Additionally, osteoblasts produce cytoplasmic processes towards the bone matrix which can

come in contact with osteocyte processes leading to further communication and modulation of function (406). Finally, osteoblasts can undergo apoptosis or change function to become osteocytes or bone lining cells (407,408).

As with osteoclasts, osteoblast formation and function is regulated by both systemic and local factors, many of which affect both cell types. Systemic osteoblast factors include PTH, parathyroid related protein (PTHrP), CT, estrogen, vitamin D3, insulin-like growth factor 1 (IGF-1) and androgens (321,409–413). PTH, while being able to stimulate osteoclastogenesis has also been found to increase proliferation and differentiation of precursor osteoblasts (414) and decrease apoptosis of mature osteoblasts (415). Daily, low dose injections of PTH have been shown to increase osteoblast numbers and bone formation while continuous exposure leads to osteoclastogenesis and bone loss (416). PTHrP has very similar effects to PTH as both signal through the same receptor, namely PTH1-R (410). However, PTHrP is encoded by a different gene to PTH and while PTH is secreted by the parathyroid gland PTHrP is secreted by numerous tissues such as skin, blood vessels, smooth muscles, bone, kidney and neuronal glial tissues (410).

Calcitonin has been reported to enhance osteoblast differentiation by inducing BMP-2 expression (417) and to inhibit apoptosis of osteoblasts and osteocytes (418). Similarly, estrogens have been shown by Kousteni et al in 2001 to inhibit osteoblast apoptosis and increase their lifespan by downregulating JNK and activating the Src/Shc/ERK signalling pathway (419). Vitamin D3 (1,25(OH)2 vitamin D3) has been shown to stimulate differentiation of MSC to osteoblast (420), and to affect osteoblasts directly via the vitamin D receptor, VDR, stimulating bone formation and mineralization (412). IGF-1 circulates systemically, is secreted locally in the bone marrow and is one of the most abundant proteins in the bone matrix (321).

IGF-1 has been suggested to induce MSC migration to the bone marrow as in cell culture IGF has been shown to enhance the expression of genes that promote cell migration (421). In addition, bone matrix IGF-1 has been shown to induce MSC differentiation to osteoblasts by activating mTOR (325) and to stimulate osteoblast function and survival (288).

Androgens have been shown to increase collagen type 1, osteocalcin and alkaline phosphatase secretion leading to increased osteoblast differentiation and mineralisation (422,423). Deletion of androgen receptor in the osteoblasts and osteocytes of mice led to decreases in trabecular bone mass in male mice (424,425). Additionally, in an orchidectomised mouse model, activation of androgen receptor by dihydro testosterone prevented osteoblast apoptosis (426) suggesting androgens directly regulate both osteoblast development and apoptosis.

As for osteoclasts, research into local factors influencing osteoblast formation and function has also led to the discovery of numerous factors both in the extracellular matrix and within MSC's and osteoblasts, some of the most recent are shown in table 1.5. Many of these factors including BMP's 2 and 7, Wnt's 2, 3a, 5a, 6a,10b and numerous micro RNA's directly influence MSC differentiation into precursor osteoblasts and their subsequent maturation (369,373,427–430). In addition, a number of these factors, again including BMP's 2 and 7, Wnt 3a, 5a influence both osteoblasts and osteoclasts (365,369,370,427,428) suggesting dual roles depending on which cells are present in the BRC and may act as coupling factors connecting the activity of osteoclasts with the recruitment of osteoblasts.

Research is also revealing potential roles of sphingolipids in osteoblast recruitment and activity. S1P produced by osteoclasts has been shown to be a chemoattractant for MSC's expressing its receptors, S1PR1 and S1PR2, resulting in their migration to the bone marrow and thus also acting as a coupling factor between osteoclasts and osteoblasts (431). In addition, catabolism of sphingomyelin by neutral sphingomyelinase 2 (nSMase2) has been shown by Khavandgar published in 2011 to be essential for mineralisation in a *fro/fro* mouse model (432).

| Factor | Origin | Generation | Activity | References |
|---|---|---|---|---|
| BMP – 2 | T-cells | | ↑ | (427) |
| 4 | Osteoblasts | ↓ | | (433) |
| 7 | Osteocytes | | ↑ | (428) |
| 9 | | ↑ | | (434) |
| Claudin-1 | Osteoblast | ↑ | | (435) |
| Cthrc1 | Osteoclast | ↑ | ↑ | (436) |
| Irisin | Skeletal muscle | ↑ | ↑ | (437) |
| miR-15b | MSC's | ↑ | ↑ | (373) |
| 20a | Precursor- osteoblasts | ↑ | | (438) |
| 29a | Osteoblasts | ↑ | | (439) |
| 125b | | ↓ | | (373) |
| 129-5p | | ↑ | | (440) |
| 138 | | ↓ | | (373) |
| 138-5p | | ↓ | | (441) |
| 204 | | ↓ | ↓ | (442) |
| 338-3p | | ↓ | | (443) |
| 675-5p | | ↓ | | (441) |
| 705 | | ↓ | | (444) |
| 3077-5p | | ↓ | | (444) |
| MMP's | Osteoblasts | ↑ | ↑ | (445) |
| Sclerostin | Osteocytes | ↓ | ↓ | (446) |
| Semaphorin 4D | Osteoclasts<br>T-cells | ↓ | ↓ | (447) |
| Smurf1 | Osteoblast | ↓ | ↓ | (448) |
| Wnt 1 | B-lymphocytes | | ↑ | (312) |
| 2 | Osteoblasts | ↑ | | (449) |
| 3a | T-cells | ↑ | | (449) |
| 5a | | ↑ | | (429) |
| 6a | | ↑ | | (430) |
| 10a | | | ↑ | (430) |
| 10b | | ↑ | | (450) |

**Table 1.5** Local extracellular and intracellular factors affecting osteoblast generation and activity.

## 1.8 Macrophages

In GD the macrophage is the primary storage cell due to their function of degrading senescent blood cells, cell debris and microbes providing an additional peripheral source of substrate. Gaucher macrophages have been found to be altered as a consequence of this storage with impaired chemotaxis, reduced oxygen species production and increased secretion of several cytokines including IL-1β, IL-6 and MIP-1β all shown to stimulate osteoclastogenesis.

In the 1980's macrophages were found to be distributed near the bone surface in Balb/c mice (451) and early studies found enhanced differentiation of mouse marrow stromal cells or calvarial osteoblasts when co-cultured with cells of the monocyte/macrophage lineage leading to increased alkaline phosphatase activity and collagen type 1 synthesis from stromal cell cultures indicating their differentiation into functional osteoblasts (452,453). These reports also found macrophages in close proximity to bone cells in vivo and secretion of BMP-2 leading to increased osteoblast survival and differentiation (454). In 2008, Chiang et al identified a discrete population of macrophages dubbed osteal macrophages (OsteoMacs) intercalated throughout murine and human osteal tissues (455). These OsteoMacs were located adjacent to mature osteoblasts at sights of active bone remodelling in a murine model with over 75% of osteoblasts on the surface of the cortical bone covered with a canopy of OsteoMacs (456). The removal of OsteoMacs from mouse calvaria osteoblast preparations led to a decrease in bone nodule formation in vitro (455). The co-culture of primary mouse macrophages with bone marrow stromal cells led to an activation in IL-6 secretion by both bone marrow macrophages and stromal cells, enhancing MSC migration and differentiation when in vitro (457).

In vitro human systems have shown that monocytes and macrophages could promote osteoblast differentiation from MSC's and that this required direct cell-cell contact. This contact resulted in the production of oncostatin M by macrophages with the osteoblast differentiation dependent on oncostatin M, prostaglandin E2 and cyclooxygenase 2 (458,459). In addition, oncostatin M has been shown to increase mineralisation both in vitro and in vivo (460,461). Interestingly,

macrophages co-cultured with MSC's led to a polarisation of macrophages to an alternatively activated (M2) phenotype with increased expression of CD206 and production of IL-10, IL-12 with a decrease in production of TNF-α, IL-6 (462,463). A separate study by Schlundt et al published in 2015 also found that induction of M2 macrophages through IL-4 and IL-13 in a murine model significantly enhanced bone formation during their 3 week investigation (464). Other research by Asada et al published in 2013 has also suggested that macrophages communicate with osteocytes in a murine model to influence MSC migration (465). Conversely, classically activated macrophages (M1) are known to secrete several pro-osteoclastic cytokines including TNF-α, IL-1β and IL-6 (466). As inflammatory macrophages in addition to OsteoMacs are present in the bone marrow it suggests a fine balance needs to be maintained between M1/M2 macrophages to regulate bone remodelling (464).

## 1.9 Multiple myeloma and bone

Initially, multiple myeloma (MM) begins as monoclonal gammopathy of undetermined significance (MGUS), progressing to smouldering myeloma (asymptomatic) and finally becoming overt myeloma resulting in bone marrow infiltration (467). However, recent findings suggest that the onset of MGUS can coincide with skeletal fragility, suggesting the bone destruction may occur at a very early stage (468). While MGUS can develop into a number of malignancies including lymphoma, amyloidosis, chronic lymphocytic leukaemia and solitary plasmacytoma, the most common is MM with an estimated 1% of patients progressing from MGUS to MM per year (264). MM is a plasma cell disorder characterised by end organ damage including renal impairment, anemia and hypercalcemia (469). In addition, more than 80% off MM patients develop bony lesions leading to pain and fractures with lytic lesions most common in the spine, skull and long bones with widespread osteopenia also a common feature (470). MM bone disease is regarded as distinct from other metastatic diseases as in MM there is no bone formation unlike for example prostate and breast cancers in which there is increased activity of both osteoclasts and osteoblasts (471).

Patients with MM are associated with increased numbers of osteoclast progenitor cells in the peripheral blood (472), with osteoclast numbers increased in the bone marrow of patients with monoclonal gammopathy, increasing further in patients with malignant disease (473). Serum TRAP, C-terminal telopeptide (CTX) and hydroxyproline (markers of bone turnover) have been shown to be elevated in MM patients and to be higher in patients in stage B (472), confirming elevated osteoclast activity.

The serum and bone marrow of MM patients have been found to have high levels of several cytokines promoting osteoclast formation and activity including RANKL, IL-3, IL-6, IL-7, MIP-1α, stromal derived factor 1α (SDF1α), TNFα, VEGF, osteopontin and MMP-13 (474–479). Many of which are secreted by the myeloma cells, including RANKL, TNF-α, MIP-1α, VEGF, osteopontin and MMP-13 directly increasing osteoclastogenesis (474,477–480). Myeloma cells further enhance osteoclastogenesis by downregulating the expression of OPG produced by osteoblasts (481).

Physical interaction has also been found to play a role as histological studies have shown that osteolytic lesions occur adjacent to plasma cells in the bone marrow specimens of MM patients (482). Myeloma cells adhere to stromal cells and osteoclasts via the very late antigen 4 (VLA-4) – vascular cell adhesion molecule 1 (VCAM-1) interaction which has been shown by Abe et al in 2009 to induce the production and secretion of MIP-1α and MIP-1β, stimulating osteoclastogenesis (483). Levels of MIP-1α in the bone marrow of MM patients correlate with severity of bone disease and high levels have been associated with poor prognosis (483,484). It is also hypothesised that myeloma cells fuse with precursor osteoclasts to form osteoclast-like cells capable of resorbing bone with one study by Andersen et al 2007 finding 30% of osteoclast nuclei in MM patients having a myeloma cell origin (485).

In turn, osteoclasts have been shown to support myeloma cell survival and enhance proliferation by firstly upregulating signalling pathways including via signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein kinases (MAPK's) and secondly by secreting soluble factors such as IL-3, IL-6,

osteopontin, B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) (475,486,487). In addition, resorption of the bone matrix results in the release of growth factors such as TGF-β and IGF-1 which also promote myeloma cell survival and proliferation (488).

Reduced bone formation due to decreased osteoblast differentiation and activity also contributes to the bone disease in MM (489). Several factors secreted by myeloma cells including Dickkopf-1 (DKK1), secreted frizzled related protein-2 (sFRP-2) and sclerostin have been shown to inhibit osteoblast maturation (490–492). DKK-1, sFRP-2 and sclerostin block Wnt signalling, preventing osteoblast maturation and activity (490–492). Other factors elevated in the serum and bone marrow of MM patients such as IL-3 and IL-7 have dual affects, promoting osteoclastogenesis and inhibiting osteoblast differentiation and maturation (493,494).

A study by Giuliani et al published in 2005 showed co-culture of myeloma cells with osteoblast precursors led to the downregulation of Runx2/CBFA-1, a transcription factor essential for osteoblast differentiation (494). The same study showed a marked reduction in the number of Runx2/CBFA-1 positive cells in the bone marrow biopsies of MM patients (494). In addition, MSC's have been shown to support progression by expressing RANKL and IL-6 (495) while osteoblasts have been suggested to suppress myeloma by secreting decorin, a small leucine-rich proteoglycan (496). A flow cytometry based study by Noll et al published in 2014 of MM patient bone marrow noted an increased number of colony forming MSC which correlated with disease burden at time of diagnosis, suggesting the balance between MSC's and osteoblasts may play an important role in the proliferation and survival of myeloma cells (495).

Studies have shown that osteocytes, the most abundant bone cells, are also reduced in number in MM bone marrow (497). A second study by Delgado-Calle published in 2016, utilising a murine model of human MM, found myeloma cells physically interact with osteocytes resulting in caspase-3 dependent apoptosis of osteocytes. Induction of apoptosis led to an increase in RANKL expression. In addition, osteocyte – myeloma cell contact reciprocally activated Notch signalling

increasing Notch 3 and Notch 4 receptor expression stimulating MM cell growth (498).

Studies have also shown that macrophages infiltrate MM bone marrow and that myeloma cells specifically attract peripheral blood monocytes by interacting with bone marrow stromal cells (499,500). This interaction results in an increase in expression of a number of chemokines including CCL2, CCL3, CCL14 and CXCL12 leading to chemoattraction of the monocytes to tumor sites (499,500). Myeloma cells affect macrophage polarization (500), increasing the expression of factors that stimulate the survival of plasma cell proliferation and survival including IL-6, IL-10, LIGHT and VEGF while lowering the expression of IL-12 and TNF-α (501–504).

## 1.10 Bone disease in GD

As previously mentioned, bone disease is a very common and debilitating feature of GD with patients suffering from a wide variety of manifestations including osteopenia, osteonecrosis, osteosclerosis, osteolytic lesions, pathological fracture and bone remodelling such as Erlenmeyer flask formation (84,85). A 2015 analysis by Grabowski et al of data on 681 type 1 GD patients entered into the International Collaborative Gaucher Group (ICGG) Gaucher Registry found an average of 84% of patients had bone lesions increasing to 96.3% for the N370S/84GG genotype (27 patients). The most common bone feature being osteoporosis present in an average of 75% of patients (101). A second study of 100 type 1 GD patients by Deegan et al in 2011 combining clinical and questionnaire data found evidence of Erlenmeyer flask formation in 59%, osteonecrosis in 43%, fragility fracture in 28%, osteomyelitis in 6% and lytic lesions in 4% of patients with mobility impaired in 32% and 15% experienced significant pain (84). In addition, Baris et al in 2016 highlighted that bone crises are not restricted to long bones. In a cohort of 100 patients small bone crises represented 31.5% of all bone crises (505).

In paediatric cohorts, a study by Ciana et al in 2012 of 18 patients found 44.4% had Erlenmeyer flask deformity and 16.6% had pathological Z scores (≤ -2.0) (506). A

study by Mistry et al published in 2011 of type 1 GD patients in the ICGG Gaucher registry found 44% (n = 43) of children and 76% (n = 41) of adolescents had low bone mineral densities with Z scores of ≤ -1. Demonstrating that the regulation of bone metabolism and development is disrupted at a young age in some GD patients which may suggest additional causes of bone manifestations to Gaucher cell bone marrow infiltration (510).

Since the advent of ERT several long term studies have shown significant increases in bone mineral density and reduction of bone crises in type 1 GD patients with improvement in bone pain recorded as early as 3 months after receiving ERT (506–510). However, a number of studies have also shown some patients continue to suffer bone events while on ERT. A study by Stirnemann et al published in 2010 found bone events occurred in 4 of 21 patients on ERT with a bone event prior to beginning ERT and 6 of the remaining 41 patients developed bone events only during ERT (85). Deegan et al published in 2011 found 8 patients developed new osteonecrosis after the start of ERT while Baris et al published in 2016 found small bone crises increased after the initiation of ERT (84,505). In 2008 de Fost et al reported 12 out of 40 type 1 GD patients continued to experience bone disease despite increasing doses of ERT (511). These observations suggest further research is required to identify the mechanisms behind the bone manifestations in GD to determine the most effective potential therapies for these refractive patients.

Bone disease in GD patients is generally assessed by dual X-ray absorbiometry (DEXA) in conjunction with magnetic resonance imaging (MRI) (123) however these techniques only provide a snapshot of disease progression and cannot provide information on when bone dysregulation begins or whether a patient is undergoing a period of bone loss. Therefore, to address this issue several studies have been carried out to assess the efficacy of markers of bone turnover. These studies have included the measurement of bone formation markers including osteocalcin, bone alkaline phosphatase (B-ALP), Procollagen I carboxyterminal propeptide (PICP), N-terminal propeptide of type I collagen (PINP) and bone resorption markers including collagen type 1 cross-linked C-telopeptide (CTX), tartrate-resistant acid

phosphatase 5b (TRACP 5b), cathepsin K, urinary hydroxyproline, deoxypyridinoline (Dpyr) and collagen type 1 cross-linked N-telopeptide (NTX) (508,512–517).

The most common marker assessed are osteocalcin and CTX however of the five studies measuring osteocalcin only two found a significant decrease (512,515) while the remaining three found no significant difference (508,513,516). In regards to CTX, two studies found a significant increase (513,514), a third found no difference (515) while a fourth measured a significantly lower concentration compared to the control population (512). However, in the fourth study a greater proportional decrease in osteocalcin was also detected and it was concluded that bone resorption exceeded bone formation (512). A decrease in PICP was found in two studies (513,514), an increase in cathepsin K in two studies (518,519) and TRACP 5b in another study (517). No significant changes in BALP, PINP, urinary hydroxyproline, Dpyr or NTX were found (508,513,515,516).

These findings suggest there may be both a reduction in bone formation due to the decreases in osteocalcin and PICP and an increase in bone resorption as shown by increases in CTX, cathepsin K and TRAP5b. However, due to conflicting data between studies and the fact that few studies have measured the same analytes in their respective patient cohorts it is not possible to draw a definite conclusion from these findings.

As previously detailed in table 1.2 numerous proteins have been found to be elevated in the plasma and serum of type 1 GD patients. Interestingly, the majority of these proteins including IL-1β, IL-6, IL-8, MCSF, MIP-1α, MIP-1β and TNF-α are known to stimulate osteoclast generation and activity (143,147,148,382,387,388,392,520). In addition, osteopontin, a negatively charged protein known to bind calcium and thus preventing bone mineralisation, is also elevated in GD serum (144). Systemic regulation may also be affected as two studies have shown that GD patients have a deficiency in 1,25(OH)2 vitamin D3 which is known to stimulate osteoblast differentiation (521,522). Taken together, these findings suggest GD bone marrow may have a highly pro-osteoclastogenic environment which may in part explain the bone loss observed in GD patients.

To further investigate the cellular aspects of bone disease in GD several animal and cellular models have been created. Currently, the only murine model with notable bone involvement is the Mistry model in which exons 8 through 11 of the GBA1 gene were conditionally deleted in the cells of the hematopoietic and mesenchymal lineages. These mice showed histological evidence of medullary infarction and associated avascular osteonecrosis in addition to osteopenia. Cellular examination showed reduced proliferation of bone marrow stromal cells and reduced osteoblast differentiation, confirmed by a reduction in alkaline phosphatase (ALP), bone sialoprotein (BSP), Runx2, and osterix mRNA expression. However, no alteration in osteoclast number or activity were found (272). Supporting evidence has also been provided by research published in 2015 by Zancan et al using Zebra fish models in which Gba1 loss of function was attained either by knock down using morpholino oligonucleotides or via genetic knockdown by introducing a point mutation in the splicing donor site of GBA1 exon 4. In both Zebra fish models loss of Gba1 function was associated with impaired canonical Wnt signalling, osteoblast differentiation and reduced bone mineralisation (523).

For cellular studies evidence suggests both osteoblasts and osteoclasts may be dysregulated in GD. Several studies investigating MSC's with Gba1 deficiency either by CBE inhibition or isolation of MSC from GD patient bone marrow found impairment in MSC proliferation and differentiation into both adipocytes and osteoblasts (138,139,141,524).

Our group began investigating the characteristics of Gaucher patient osteoclasts in 2005, presenting initial findings as posters in 2005, 2006, 2007 and 2008. Based on these findings this project was initiated in April 2012 to investigate both osteoclast and osteoblast function and their interaction in relation to GD. Since this projects instigation research by Mucci et al published in August 2012 demonstrated that treatment of osteoclast precursors with conditioned medium from CBE treated peripheral blood mononuclear cells (PBMC's) resulted in increased osteoclast differentiation (525). Mucci et al published in 2015 also found higher osteoclast generation of PBMC's isolated from type 1 GD patients when compared to controls confirming our findings (134).

Furthermore, T-cells and macrophages, both known to play a role in regulating osteoclast formation, have been found to be altered in GD patients. Research by Lacerda et al published in 1999 has shown changes in total T-lymphocyte numbers and alteration in CD4+/CD8+ T-lymphocyte ratio (527). Mucci et al, published in 2015, found an increase in the CD14+CD16+ pro-inflammatory monocyte subset found to produce several pro-osteoclastic cytokines in addition to increased expression of RANKL on the surface of T-cells (134).

Taken together, evidence suggests there is dysregulation of the bone microenvironment in GD leading to uncoupling between osteoclasts and osteoblasts involving both systemic and local factors.

## 1.11 Aims and hypotheses

Bone disease is a common feature of GD with radiological evidence described in up to 93% of patients ranging from osteopenia and bone remodelling to avascular necrosis. At the initiation of this project no group had published in vitro data on Gaucher patient osteoclasts or their interactions with osteoblasts in the bone microenvironment. However, investigations into the bone microenvironment by some groups have suggested there may be alterations in MSC function and increased resorption of control osteoclasts when cultured in CBE treated MSC conditioned medium. In addition, our previous findings suggested GD osteoclasts may have altered characteristics when compared to control osteoclasts therefore the aims of this project are:

(1) To understand the roles of osteoclasts in Gaucher patient bone disease.
(2) To understand the relationship between in vitro findings and clinical features of bone disease in GD patients.
(3) To understand the role of osteoblasts in GD bone disease.
(4) To understand the relationship between plasma cells and in vitro GD osteoclastogenesis.
(5) To understand the effects of interaction between osteoblasts, osteoclasts and plasma cells in Gaucher disease.

These aims are explored with the hypotheses detailed below.

## Chapter 3

**Hypothesis 1:** Circulating GD monocytes have an increased potential to form osteoclasts compared to control monocytes.

**Rationale:** The majority of GD patients have low BMD with an average of 75% presenting with osteopenia. This bone loss could result from increased osteoclast number and/or activity, decreased osteoblast number and/or activity or a combination thereof. Several groups have found increased pro-osteoclastic cytokines in the plasma and serum of GD patients. Sphingolipids including ceramide and sphingosine-1-phosphate, both found to be elevated in GD serum, have been reported to affect osteoclast migration and maturation.

**Methods:** Differentiation of monocytes isolated from the peripheral blood of GD patients and control subjects into functional osteoclasts by culture with established osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

**Hypothesis 2a:** Increased osteoclastogenesis is directly related to β-glucocerebrosidase deficiency rather than protein misfolding.

**Rationale:** GD results from pathogenic mutations in the GBA gene causing either a decrease in β-glucocerebrosidase activity or misfolding of the protein resulting in degradation by the ER associated degradation pathway (ERAD). Data generated by our group prior to this project and research published during this project by Mucci et al with the irreversible inhibitor of β-glucocerebrosidase CBE in cell lines showed inhibition resulted in increased osteoclastogenesis suggesting it is directly related to a decrease in to β-glucocerebrosidase activity.

**Methods:** Culture of control subject monocytes with CBE in the presence of osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

**Hypothesis 2b:** Addition of GD-specific therapies to GD osteoclast cultures reduces osteoclastogenesis.

**Rationale:** If increased osteoclastogenesis is directly related to β-glucocerebrosidase deficiency, replacement of the deficient enzyme or reduction of the substrate should reverse this effect.

**Methods:** Culture of GD or control subject monocytes with ERT or SRT in the presence of osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

**Hypothesis 3:** The altered sphingolipid profile found in GD patients causes increased osteoclastogenesis.

**Rationale:** A number of sphingolipids concentrations have been found to be elevated in GD serum and plasma including ceramide, sphingosine, S1P, glucosylsphingosine and glucosylceramide. Research has shown roles for sphingolipids such as ceramide, sphingosine-1-phosphate and lactosylceramide in the formation and function of osteoclasts. The elevated concentrations of these sphingolipids, possibly including glucosylsphingosine and glucosylceramide - the roles of which in relation to osteoclasts have yet to be elucidated, may cause increased osteoclastogenesis.

**Methods:** Culture of GD or control subject monocytes with a number of different sphingolipids added exogenously in the presence of osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically

positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

## Chapter 4.

**Hypothesis 1:** GD patients with active bone disease generate more osteoclasts in vitro.

**Rationale:** A number of bone diseases such as osteoporosis can be caused by increased osteoclast generation leading to increased bone resorption. The number of nuclei per osteoclast has also been shown to correlate to osteoclast activity.

**Methods:** Correlation of MRI based evidence of active bone disease with contemporaneous in vitro osteoclast generation and size.

**Hypothesis 2:** GD patients with anaemia generate more osteoclasts in vitro.

**Rationale:** A recent publication by Khan et al in 2012 has shown anaemia to be a risk factor for avascular necrosis in GD patients with anaemic patients 60% more likely to develop AVN with an adjusted odds ratio of 1.56.

**Methods:** Correlation of patients with levels of haemoglobin indicative of anaemia with in vitro osteoclast generation.

**Hypothesis 3:** Treatment of GD patients with GD-specific therapies will reduce in vitro osteoclast generation.

**Rationale:** Previous data in this thesis has shown increased osteoclast generation is directly related to a decrease in β-glucocerebrosidase activity and that addition of GD-specific therapy to GD in vitro cultures reduced osteoclast generation. Therefore, GD-specific treatments such as ERT and SRT designed to replace the enzyme or reduce the impact of its decreased activity will probably ameliorate the effect on osteoclastogenesis in vivo.

**Methods:** Compare osteoclast generation of naïve patients with patients receiving therapy. Longitudinal study of individual patients by taking blood samples for osteoclast culture at each patient visit. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

## Chapter 5.

**Hypothesis 1:** There is dysregulation between osteoblasts and osteoclasts, commonly referred to as uncoupling, in GD patients.

**Rationale:** Osteoblasts and osteoclasts are normally very tightly regulated primarily by each other. In GD increased osteoclast generation and activity may result in an imbalance between osteoclast and osteoblast number and function leading to bone loss.

**Methods:** Flow cytometry using markers identifying osteoblast precursor percentage in peripheral blood samples is correlated with the number of osteoclasts generated by contemporaneous in vitro osteoclast cultures.

**Hypothesis 2:** Osteoblast mineralisation is reduced when β-glucocerebrosidase activity is inhibited.

**Rationale:** Studies of a GD murine model by Mistry et al (272) showed reduced proliferation of bone marrow stromal cells and reduced osteoblast differentiation and two Zebra fish GD models developed by Zancan et al (524) demonstrated loss of Gba1 function was associated with impaired canonical Wnt signalling, osteoblast differentiation and reduced bone mineralisation.

**Methods:** Primary and cell line osteoblasts cultured with CBE under mineralising condition. Mineralisation quantified by alizarin red staining and solubilisation.

**Hypothesis 3:** Osteoblast mineralisation is reduced in the presence of exogenous glucosylceramide.

**Rationale:** Previous data in this thesis has shown exogenous glucosylceramide directly affects osteoclast generation by significantly increasing osteoclast numbers in both control and Gaucher cultures indicating that this is a bioactive sphingolipid and therefore may also affect osteoblast function.

**Methods:** Primary and cell line osteoblasts cultured with exogenous glucosylceramide under mineralising condition. Mineralisation quantified by alizarin red staining and solubilisation.

**Hypothesis 4:** Osteoblast function is not directly impacted by GD-specific therapies.

**Rationale:** Previous data in this thesis has shown GD-specific therapies reduce osteoclast generation in vitro both directly and when given to patients. However, a subset of patients have been reported to continue to suffer bone events while receiving GD-specific therapy suggesting a negative impact on osteoblast function is negating the reduction in osteoclast numbers. This may be via the indirect impact of reduced osteoclast activity having the overall effect of reducing bone matrix resorption which in turn means a reduction in osteoblast signalling factors released from the bone matrix.

**Methods:** Primary and cell line osteoblasts cultured with GD-specific therapies in the absence or presence of CBE under mineralising conditions. Mineralisation quantified by alizarin red staining and solubilisation.

## Chapter 6.

**Hypothesis 1:** GD osteoclastogenesis is further enhanced when cultured with myeloma plasma cells e.g. the NCI-H929 myelolma plasma cell line.

**Rationale:** Myeloma patient plasma cells and myeloma plasma cell lines have been reported to express cytokines such as IL-6, RANKL, TNF-α, MIP-1α known to stimulate osteoclastogenesis, contributing to the osteopenia and bone lesions found in over 80% of myeloma patients.

**Methods:** Co-culture of control or GD patient osteoclast cultures with a myeloma plasma cell line. Osteoclasts will be identified by being histochemically positive for TRAP.

**Hypothesis 2:** The altered sphingolipid profile in GD patients creates a pro-survival environment for plasma cells which in turn stimulate osteoclast generation, creating a positive effector loop between plasma cells and osteoclasts in GD.

**Rationale:** Several sphingolipids including glucosylsphingosine and glucosylceramide have been suggested to have roles as bioactive molecules in cancer. Both sphingolipids have been linked with increasing cellular proliferation and reducing apoptosis and glucosylceramide has been suggested to be involved in drug resistance.

**Methods:** Culture of a myeloma plasma cell line with a range of exogenous sphingolipids. Cell number determined by haemocytometer.

## Chapter 7.

**Hypothesis 1:** GD osteoblast-osteoclast interaction with plasma cells enhances overall bone loss.

**Rationale:** Plasma cells have been shown to increase osteoclastogenesis and to secrete cytokines which induce osteoclast generation and function and to decrease

differentiation of MSC's into osteoblasts. In addition, MGUS patients have been reported to have lower BMD.

**Methods:** Co-culture of primary and cell line osteoblasts with primary osteoclasts and myeloma plasma cell line. Mineralisation quantified by alizarin red staining and solubilisation.

**Hypothesis 2:** GD osteoclasts can protect plasma cells from apoptosis.

**Rationale:** Osteoclasts have been shown to secrete cytokines including IL-6 which stimulate plasma cell proliferation and survival.

**Methods:** Co-culture of primary and cell line osteoblasts with primary osteoclasts and myeloma plasma cell line. Viability assessed by flow cytometry.

**Definition:**

**Bone event –** clinical events such as avascular necrosis, bone infarct or fracture which occurred within 3 months of the initiation of an osteoclast assay for that patient. Excluding ongoing bone disease such as osteopenia and osteoporosis.

This definition applies to the term 'bone event' when used in results and discussion sections of this thesis.

# 2 Materials and methods:

## 2.1 Patient and healthy control recruitment.

This study was approved by the Royal Free Hospital Multi-centre Research Ethics Committee (MREC) to recruit healthy controls or individuals with either Gaucher disease or myeloma.

All patients received a patient information sheet 14 days prior to providing informed consent. Each recruited individual was assigned a unique identification code. Patient data was stored in a secure location on a password protected system and access was limited to the study investigators. Tissue specimens including bone marrow were acquired and stored in accordance with the Human Tissue Act.

Unless otherwise stated all reagents were supplied by Sigma Aldrich, Poole, UK.

## 2.2 Cell culture medium

**A10** – alpha minimal essential medium (MEMα) (ThermoFisher Scientific, Paisley, UK) supplemented with 10% foetal bovine serum (ThermoFisher Scientific, Paisley, UK), 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (ThermoFisher Scientific, Paisley, UK), 2mM L-Glutamine and 100 units/ml penicillin, 0.1mg/ml streptomycin (supplied as penicillin/streptomycin solution).

**D10** – Dulbecco's modified Eagle medium (DMEM) (ThermoFisher Scientific, Paisley, UK) supplemented as for A10

**R10** – RPMI 164 medium (ThermoFisher Scientific, Paisley, UK) supplemented as for A10.

**MSC proliferation medium**– MesenCult® Proliferation Kit (Human) (STEMCELL Technologies, Grenoble, FR) comprising 450ml MesenCult basal medium with 50ml MesenCult™ Stem Cell Stimulatory Supplements, further supplemented with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (ThermoFisher

Scientific, Paisley, UK), 2mM L-Glutamine and 100 units/ml penicillin, 0.1mg/ml streptomycin (supplied as penicillin/streptomycin solution).

**A10-OB** – A10 supplemented with 5mM β-glycerophosphate disodium salt hydrate, 200µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 10nM dexamethasone.

**D10-OB** – D10 supplemented with 4mM β-glycerophosphate disodium salt hydrate and 100µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate.

**R10-OC** – R10 supplemented with 25ng/ml recombinant human macrophage colony stimulating factor (MCSF) (Cambridge Bioscience, Cambridge, UK) and 30ng/ml recombinant human receptor activator of NFκB ligand (RANKL) (Peprotech EC, London, UK).

**A10-triple culture medium** – A10-OB supplemented with 25ng/ml human MCSF, 30ng/ml human RANKL.

**D10-triple culture medium** – D10-OB supplemented with 25ng/ml human MCSF, 30ng/ml human RANKL.

## 2.3 Gaucher disease related reagent preparation.

**(a) Inhibitors.**

100mM stock solutions of conduritol β-epoxide (CBE) (Merck chemicals ltd, Nottingham, UK) and isofagomine D-tartrate (Cambridge bioscience, Cambridge, UK) were made by addition of pure solid to sterile distilled $H_2O$. Stocks were stored in 100µl aliquots at -20°C until required.

**(b) Therapies.**

200 units/ml stock solutions of Imiglucerase (Genzyme, Massachusetts, USA) and velaglucerase alpha (Shire Pharmaceuticals Group Plc, St Helier, Jersey) were prepared by addition of provided solid in sterile PBS. Stocks were stored in 100µl aliquots at -20°C until required.

10mM stock solutions of Miglustat (a kind gift of Actelion Pharmaceuticals, Basel, Switzerland) were prepared by addition of the pure solid to sterile PBS. Stocks were store in 100µl aliquots at -20°C until required.
10mM stock solution of ambroxol hydrochloride was made by addition of pure solid to sterile PBS. Stocks were stored in 100µl aliquots at -20°C until required.

**(c) Sphingolipids.**

Sphingosine (Enzo life sciences, Exeter, UK), sphingosine-1-phosphate, glucosylsphingosine (Insight Biolotechnology, Wembley, UK), ceramide (Oxford Bioscience, Oxford, UK), lactosylceramide (Universal Biologicals, Cambridge, UK), glucosylceramide (Universal Biologicals, Cambridge, UK).
10mM stock solutions were made for all above sphingolipids by addition of 100% methanol to lyophilised solid. In the cases of ceramide, lactosylceramide and glucosylceramide the mixture required heating to 50°C in a water bath to dissolve the sphingolipid. Stocks were stored in 100µl aliquots at -20°C until required. When added to medium a minimum of 10ml working concentration was made i.e. 1µl of stock was added to 10ml medium.

## 2.4 Bone disc preparation.

Bone discs were made from confiscated elephant tusk kindly donated for research by the customs department at Heathrow Airport.

Discs were made in three stages – firstly the distal (narrow) end of the elephant tusk was sawn off to make a 1-1.5cm thick disc using a hacksaw. The disc was then cut in half width ways with a hacksaw to create two semi-circular sections. The second stage involved placing one of the sections on an Isomet low speed, figure 2-1, (Buehler, Dusseldorf, DE) by using a provided chuck and screwing the section in to ensure it was firmly secured and that the flat end of the section was parallel to the diamond edged circular saw (Buehler, series 15 LC) once the chuck was

attached to the support arm. Weights provided were added to the counterbalance to a total of 150g and the speed was set to level 3. Wafers of the bone section 0.55mm thick were made and cleaned with 70% ethanol to remove lubricating fluid. Finally, a heavy duty leather punch (Draper tools Ltd, Hampshire, UK) was used to punch 6mm discs out of the bone wafer by gradually increasing pressure. As the section could be cut in three different planes the direction which produced a bone surface optimal for osteoclast activity i.e. the surface which generated the most bone pits during osteoclast culture was chosen. This process is shown in diagrammatic form on the next page, figure 2.2.

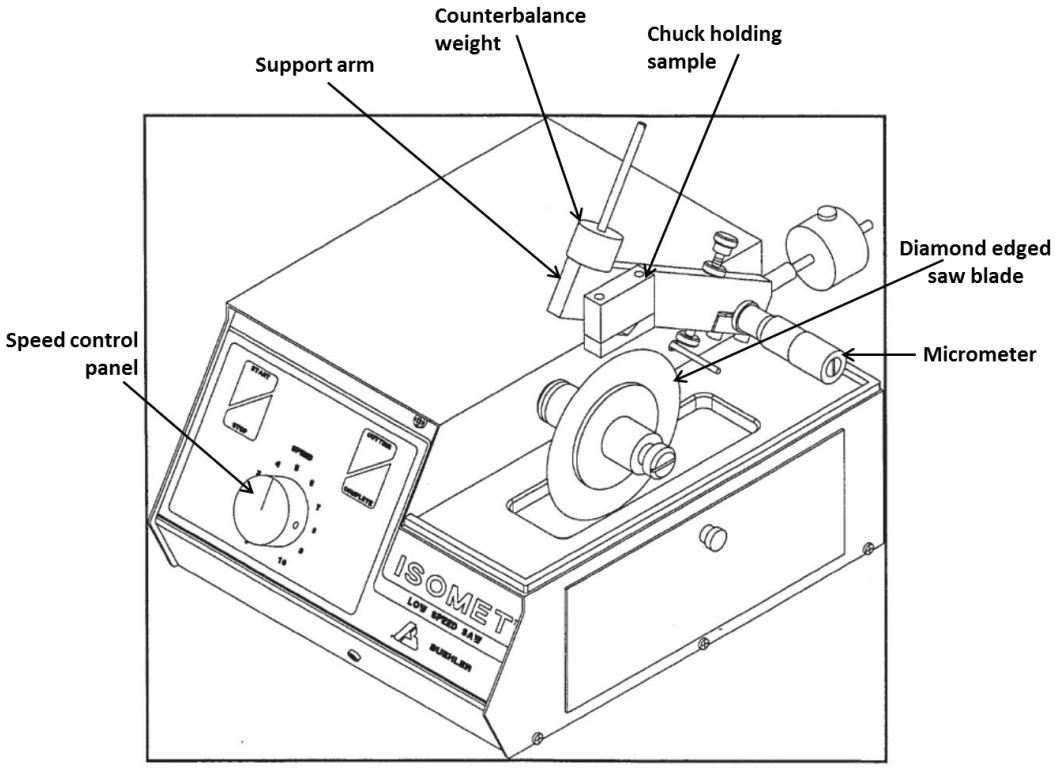



**Figure 2.1** Diagram of Buehler isomet low speed saw (unlabelled diagram obtained from operation and maintenance instruction manual).

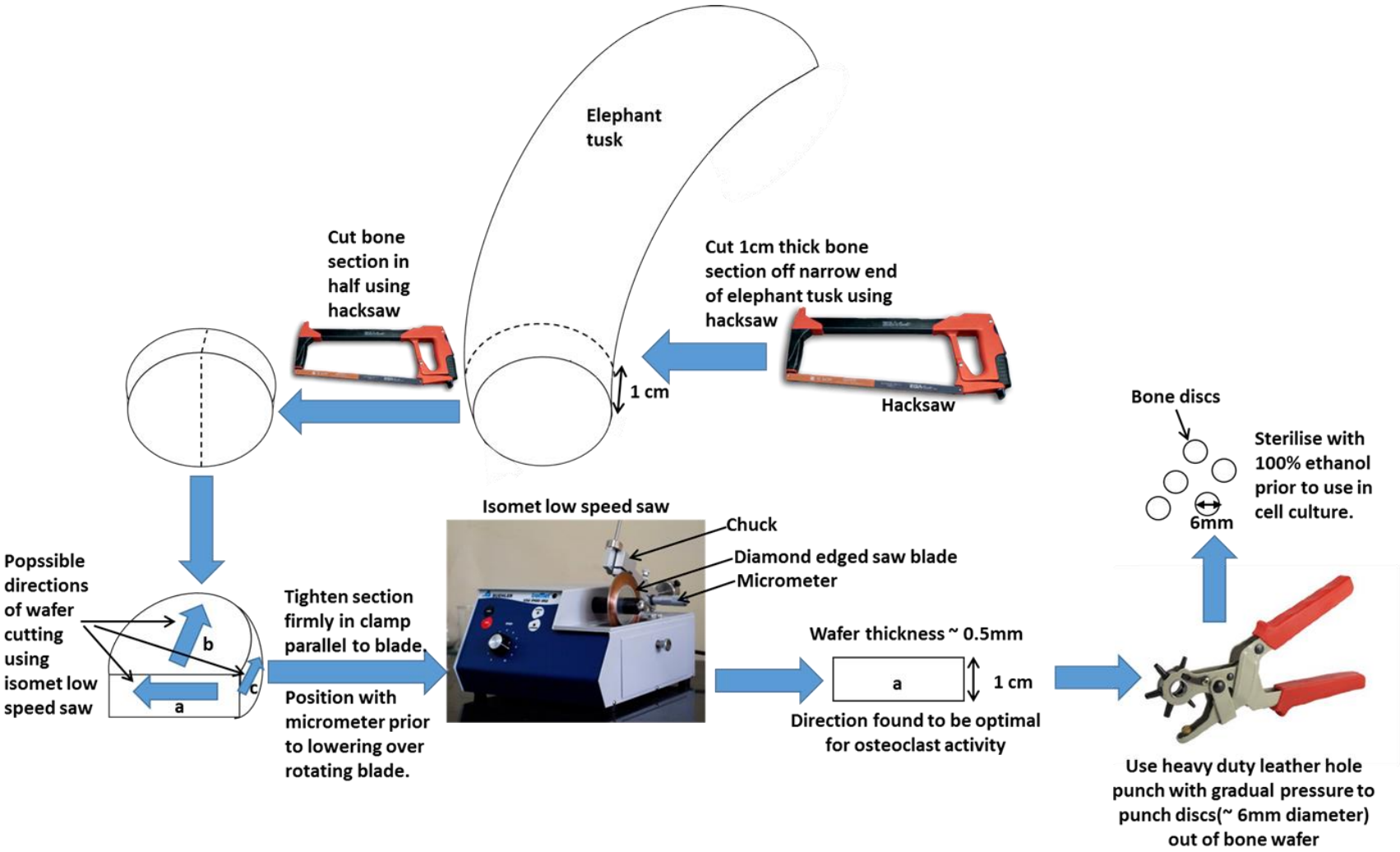



**Figure 2-2.** Diagram showing the process of making 6mm bone discs from an elephant tusk.

# 2.5 Cell culture methods

## 2.5.1 General cell culture.

All primary cells and cell lines were incubated in a humidified atmosphere with 5% $CO_2$ at 37°C (Heraeus Instruments, Michigan, US). To minimise possible incubator contamination sterile water, added to maintain a humidified atmosphere, also contained 1g/l copper (II) sulphate pentahydrate (Fisher Scientific, Loughborough, UK).

## 2.5.2 Freezing and liquid nitrogen storage of cells.

For long term storage cells were pelleted and re-suspended at 1-3x$10^6$cells/ml in freezing medium comprising 90% fetal bovine serum (FBS) (ThermoFisher Scientific, Paisley, UK) and 10% dimethylsulphoxide (DMSO). The resultant cell suspension was pipetted into cryovials, 1ml per vial, prior to being transferred to a propan-2-ol freezing box and gradually cooled at 1°C/minute in a -80°C freezer. The following day the cryovials were transferred to liquid nitrogen dewars.

## 2.5.3 Thawing cells from liquid nitrogen storage.

Cryovials containing frozen cell suspension were taken from a liquid nitrogen dewar and immediately transferred to a pre-warmed 37°C water bath with gentle shaking until thawed. The 1ml cell suspension was then slowly pipetted into 11ml of warmed relevant growth medium e.g. 37°C D10 for SaOs-2, and centrifuged at 250g for 5 minutes at room temperature. Cells were re-suspended in 6ml of relevant medium and pipetted into a 25$cm^2$ tissue culture flask (Fisher Scientific, Loughborough, UK) and placed in an incubator.

## 2.5.4 Cell lines.

**(a) NCI-H929** – Generously provided by Dr Ellie Necheva, Cytogenetics laboratory, academic haematology, Royal Free hospital, UK. A non-adherent human multiple myeloma cell line stated to be positive for plasma cell antigen 1 (PCA-1) and CD38. Maintained in R10 with a doubling time of approximately 70 hours, cells were sub-cultured twice per week maintaining a density between 0.5 and $1x10^6$ cells/ml.

**(b) SaOs-2** (Sigma Aldrich, Poole, UK) – An adherent human osteoblast-like cell line isolated from a primary osteosarcoma. Maintained in D10 with an approximate doubling time of 43 hours, cells were sub-cultured twice per week. Briefly, ~80% confluent cells were detached using 0.25% trypsin-0.2g/l Ethylenediaminetetraacetic acid (EDTA) tetrasodium salt solution, counted by haemocytometer and re-seeded at $1x10^6$ cells per $75cm^2$ in new tissue culture flasks.

## 2.5.5 Primary cells.

**(a) Isolation of peripheral blood mononuclear cells (PBMCs).**

20mls of blood was collected in a sterile 30ml universal container with 1000 units preservative free heparin (200µl of 5,000U/ml, Wockhardt UK Ltd, Wrexham, UK) and inverted several times to ensure adequate mixing to prevent sample clotting. Heparinised blood was transferred to 50ml conical centrifuge tubes, mixed with 10ml of sterile 3% dextran (dextran-500, Fisher Scientific, Loughborough, UK) in phosphate buffered saline (supplied as PBS tablets) and incubated in a 37°C water bath for 45 minutes. The two resultant fractions (supernatant which was predominantly plasma, lymphocytes and monocytes and non-supernatant which was predominantly red blood cells and neutrophils) were transferred to two separate 50ml centrifuge tubes. The total volume in each tube was made up to 20mls by addition of P.E.G., a solution made up of sterile PBS with 1mM EDTA

tetrasodium salt and 10mM D-glucose both added by filtering through sterile 0.2µM pore syringe filters (Fisher Scientific, Loughborough, UK). Both cell suspensions were then carefully layered onto 12mls each of ficoll-paque plus (VWR international, Lutterworth, UK) in separate 50ml centrifuge tubes. Samples were centrifuged at 650g at 4°C for 25 minutes with minimum acceleration and deceleration. The resultant white cell layer, immediately above the ficoll layer, was carefully removed by sterile pipette from both tubes, combined and brought to a total volume of 50ml with P.E.G and centrifuged at 400g at 4°C for 10 minutes with full acceleration and braking. The supernatant was discarded and the remaining cell pellet was re-suspended in 50ml P.E.G, centrifuged at 400g at 4°C for 10 minutes and re-suspended in 5ml of R10.

**(b) Isolation of mesenchymal stem cells from bone marrow.**

5ml of bone marrow aspirate was collected in a sterile 30ml universal container with 9ml of A10 and 1000 units of preservative free heparin. The total volume was made up to 25ml with A10. The resultant cell suspension was carefully layered onto 25ml of ficoll-paque plus in a sterile 50ml centrifuge tube and centrifuged at 650g at 4°C for 25 minutes with minimum acceleration and deceleration. The white cell layer immediately above the ficoll-paque layer was carefully pipetted off and transferred to another sterile 50ml centrifuge tube. The total volume of white cells was made up to 50ml with A10 and centrifuged at 400g at 4°C for 5 minutes with full acceleration and braking. The supernatant was discarded and the remaining cell pellet was re-suspended in 12ml MSC proliferation medium. This cell suspension was pipetted into a 75cm$^2$ tissue culture flask and placed in an incubator. After 24 hours all medium, containing non-adherent cells, was removed and replaced with 10ml PBS. The flask was gently shaken, PBS was removed and replaced with 10ml PBS. Again, the flask was gently shaken and PBS was removed to be replaced with 12ml MSC proliferation medium. The flask was placed in an incubator for expansion until MSC were ~80% confluent at which point MSC's were sub-cultured by

using 0.25% trypsin-EDTA and re-seeded into three 75cm$^2$ tissue culture flasks for further expansion.

## 2.5.6 NCI-H929 total cell counting.

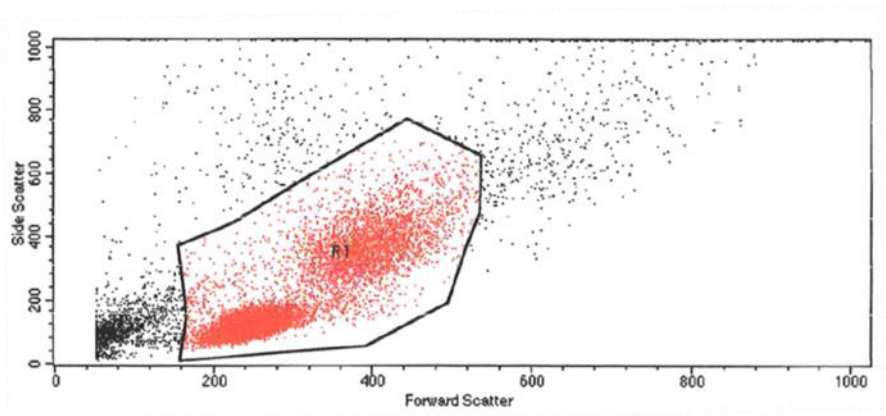
To assess the effect of sphingolipids on NCI-H929 total cell number over time 1ml of 1x10$^5$cells/ml in R10 were placed in triplicate in wells of 24 well plates for each sphingolipid condition and placed in an incubator. At each time point, a plate was removed from the incubator, cells were re-suspended by repeated pipetting prior to removal of 50µl of cells to be counted on a haemocytometer under a Nikon Eclipse E400 bright field microscope and plates were returned to the incubator until the next time point.

## 2.5.7 Cell differentiation cultures.

**(a) Osteoclast culture.**

Peripheral blood mononuclear cells (PBMC's) were separated from whole blood as described above in 2.5.5(a). Total cell number was counted using a phase contrast microscope and haemocytometer. Percentage monocytes was determined by staining between 0.5-1.0x10$^6$ PBMC's with CD14FITC/CD64PE (detailed description in section 2.6 and table 2.1) before undergoing flow cytometry analysis using a FACSCalibur flow cytometer (Becton Disckinson, Oxford, UK) as shown in figure 2.3.

PBMC's were re-suspended in R10 at a cell density of 5x10$^5$ monocytes/ml and cultured in either a 24 well plate on ethanol sterilised 6mm round glass coverslips (VWR international, Lutterworth, UK), 4 coverslips per well or in a 96 well plate on ethanol sterilised 6mm bone discs, 1 disc per well. 500µl of cell suspension was added per well of a 24 well plate and 100µl of cell suspension was added per well of a 96 well plate. Once the cell suspension was added to the plate it was transferred to an incubator. After 2 hours incubation non-adherent cells were washed off by removal of the medium




| Quad | Events | % Gated | % Total |
|---|---|---|---|
| UL | 50 | 0.50 | 0.41 |
| UR | 2815 | 28.31 | 23.32 |
| LL | 6997 | 70.36 | 57.97 |
| LR | 83 | 0.83 | 0.69 |

**Figure 2.3** Flow cytometry of CD14/64. The percentage of monocytes, CD14+/CD64+ in the upper right quadrant, shown in the table as % Gated, Quad UR, highlighted with a red box.

containing non-adherent cells and addition of sterile PBS (1ml for 24 well, 200µl for 96 well). After gentle repeated pipetting PBS was removed and replaced with PBS and the wash step was repeated. Finally, the PBS was removed and replaced with R10-OC medium (1ml for 24 well, 200µl for 96 well) and the plate was transferred to an incubator. Medium was replaced twice per week for up to 21 days.

**(b) SaOs-2 osteoblast culture optimisation**

To determine the optimal conditions for calcium deposition by SaOs-2 cells 48 well plates were seeded with SaOs-2 cells at a density of $1x10^4$ cells/$cm^2$ with a range of concentrations of β-glycerophosphate disodium salt hydrate (β-Gly) and L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A-2-P) in D10, each combination in duplicate as shown below in figure 2.4a. In addition to using a range of stimulatory concentrations a time course was also carried out whereby cultures were fixed and stained for calcium with the alizarin red method after 14, 21 and 28 days. An example of the staining, prior to solubilisation is shown in figure 2.4b for day 21.

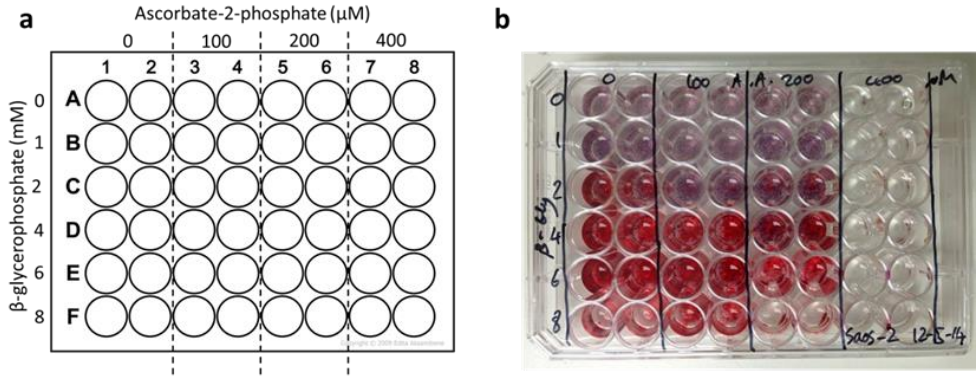



**Figure 2.4 (a)** Diagram of the range of conditions used to determine optimal conditions for calcium deposition by SaOs-2 cell line. **(b)** Example image of degree of calcium deposition by SaOs-2 cells after 21 days of culture. Calcium stained with alizarin red.

**(c) SaOs-2 osteoblast culture.**

When SaOs-2 cells had reached ~80% confluence in a $75cm^2$ tissue culture flask cells were washed by replacing growth medium with 10ml PBS followed by gentle shaking. The PBS was removed and the wash step repeated to remove extraneous protein. The PBS was removed and cells were incubated with 6ml 0.25% trypsin-EDTA for 5-10 minutes followed by repeated tapping of the side of the flask to which the cells were attached until all cells were completely detached, confirmed by viewing with an Olympus CK40 phase contrast microscope. 10ml of D10 was added to the

cell suspension prior to being transferred to a sterile 50ml centrifuge tube which was then centrifuged at 400g at 4°C for 10 minutes with full acceleration and braking. The supernatant was discarded and the remaining cell pellet was re-suspended in 5ml D10. To determine the number of viable cells 10µl of cell suspension was mixed with 10µl of trypan blue. 15µl of this mixture was pipetted onto a haemocytometer and the number of unstained cells was counted using a Nikon Eclipse E400 bright field microscope. Cell density was adjusted to $2x10^4$ cells/ml and were plated at $1x10^4$ cells/$cm^2$ of culture surface (24 well = $2x10^4$ cells per well, 96 well = $0.64x10^4$ cells per well). The tissue culture plate was then transferred to an incubator and the cells were allowed to attach to the tissue culture plastic for 24 hours after which time D10 was removed and replaced with D10-OB (1ml for 24 well, 200µl for 96 well) and placed back into the incubator. Medium was changed twice per week for 21 days.

**(d) MSC osteoblast culture optimisation.**

To determine the optimal conditions for calcium deposition by MSC's, 24 well plates were seeded with MSC's at a density of $2x10^4$ cells/$cm^2$ with a range of concentrations of β-glycerophosphate disodium salt hydrate (β-Gly), dexamethasone and L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A-2-P) in A10. Each combination was cultured in a well of three separate 24 well plates resulting in 3 data points for each condition as shown in figure 2.5. These plates were cultured for 35 days prior to calcium quantification by alizarin red staining. A separate time course was also performed once optimal conditions were determined.

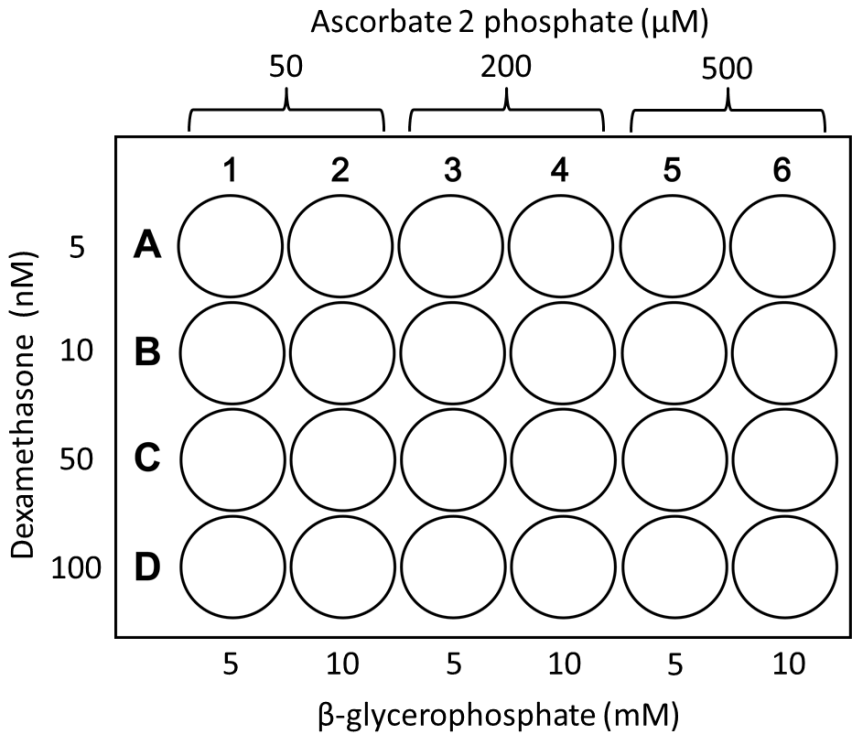



**Figure 2.5** Diagram showing the range of conditions used to determine optimal conditions for calcium deposition by osteoblasts differentiated from MSC.

**(e) MSC osteoblast culture.**

When MSC's had reached ~80% confluence in a 75cm$^2$ tissue culture flask cells were detached by washing with PBS, incubating with 0.25% trypsin-EDTA and cells were counted using trypan blue as in the SaOs-2 osteoblast culture protocol except that the medium used was A10 instead of D10. Once counted cell density was adjusted to 4x10$^4$ cells/ml and were plated at 2x10$^4$ cells/cm$^2$ of culture surface (24 well = 4x10$^4$ cells per well, 96 well = 1.28x10$^4$ cells per well). The tissue culture plate was then transferred to an incubator and the cells were allowed to attach to the tissue culture plastic for 24 hours after which time A10 was removed and replaced with A10-OB (1ml for 24 well, 200µl for 96 well) and placed back into the incubator. Medium was changed once per week for 35 days.

**(f) Osteoclast-plasma cell co-culture**

Osteoclast cultures were set-up as previously described. After 14 days of culture medium in all wells was replaced with 1ml R10-OC prior to addition of hanging cell culture inserts (referred to as transwells henceforth) with a 0.4µm pore size polyethylene terephthalate (PET) membrane (Millipore LTD, Watford, UK) into the relevant wells. For wells which included plasma cells, 500µl of NCI-H929 cell suspension at a density of $4x10^4$ cells/ml was added either directly to wells (when no transwell was present) or to the transwell. For wells which did not receive plasma cells 500µl of R10-OC was added either directly to the well (when no transwell was present) or to the transwell resulting in a final volume of 1.5ml of medium in all wells. The plates were transferred to an incubator for a further 4 days at which time medium was replaced by removal of 1ml of medium from the well of the 24 well plate, not the transwell, with care taken not to disturb either the adhered cells in the well or the transwell. The plates were returned to the incubator for a final 3 days.

**(g) Osteoblast-osteoclast-plasma cell co-culture**

Prior to the triple culture set-up either SaOs-2 cells or MSC's were differentiated into mature osteoblasts and stimulated to deposit calcium matrix by culture in either D10-OB for SaOs-2 for 21 days or A10-OB for MSC for 35 days in wells of 24 well plates as previously described. At this point PBMC's were isolated and cell density was adjusted to $5x10^5$ monocytes/ml, as previously described for osteoclast culture, in either D10-triple culture medium (for SaOs-2) or A10-triple culture medium (for MSC). 500µl of PBMC's suspension was added per well of a 24 well plate, each well containing either 4 glass coverslips, osteoblasts or a transwell with osteoblasts attached to the surface of the 24 well plate. In wells containing osteoblasts all culture medium was removed prior to the addition of the PBMC suspension. Once the cell suspension was added to the plate it was transferred to an incubator. After 2 hours incubation non-adherent cells were washed off by removal of the medium containing non-adherent cells

and addition of sterile PBS. After gentle repeated pipetting PBS was removed and replaced with PBS and the wash step was repeated. Finally, the PBS was removed and replaced with D10-triple culture medium (SaOs-2) or A10-triple culture medium (MSC), 1ml per well, and the plate was transferred to an incubator. Medium was replaced twice per week for 14 days. At this point, medium was removed from all wells containing cells including from transwell and replaced with 1ml of D10-triple culture medium (SaOs-2) or A10-triple culture medium (MSC) directly to the well of the 24 well plate. For wells which included plasma cells, 500µl of NCI-H929 cell suspension at a density of $4x10^4$ cells/ml was added either directly to wells (when no transwell was present) or to the transwell. For wells which did not receive plasma cells 500µl of the appropriate medium was added either directly to the well (when no transwell was present) or to the transwell resulting in a final volume of 1.5ml of medium in all wells. The plates were transferred to an incubator for a further 4 days at which time medium was replaced by removal of 1ml of medium from the well of the 24 well plate, not the transwell, with care taken not to disturb either the adhered cells in the well or the transwell. The plates were returned to the incubator for a final 3 days. All combinations of cells with and without transwell are shown in figure 2.6.

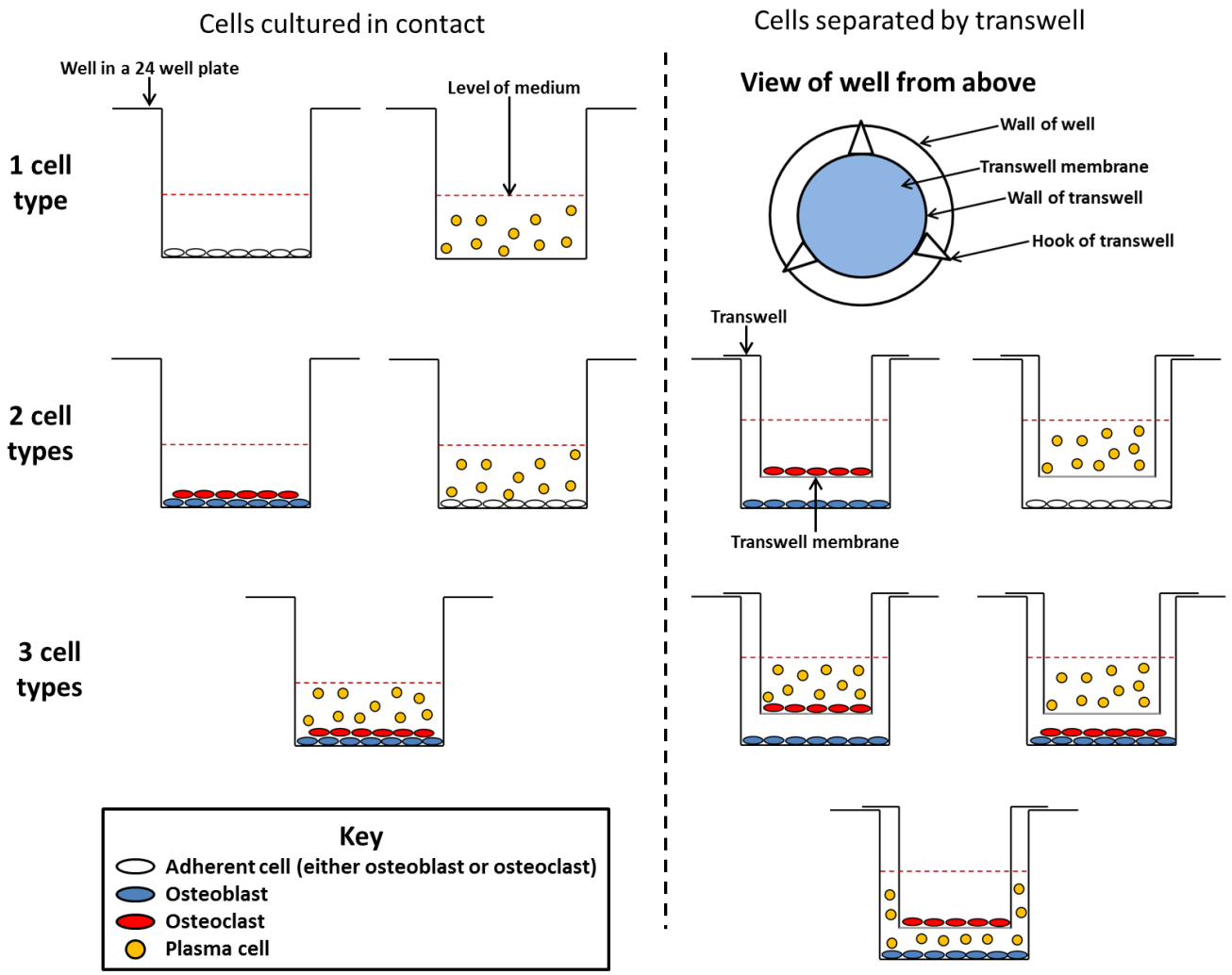



**Figure 2-6.** Diagram of combinations of cells used in triple co-culture experiments

## 2.6 Flow cytometry antibodies

### General flow cytometry.

Throughout this project monoclonal antibodies were used as direct conjugates for flow cytometry. Generally, 0.1-1x$10^6$cell/100µl were incubated in the dark with an antibody cocktail for 15-30 minutes. Cells were washed in PBS twice by addition of 1ml PBS followed by centrifugation at 400g for 5 minutes at 4°C and removal of supernatant. Cells were re-suspended in PBS (except for annexin V/Pi assay – see method below) or FACSflow (Becton Disckinson, Oxford, UK) prior to analysis.

| Target | Species | Ig Class | Product code | Company | Clone | Conjugate | Dilution/ incubation time |
|---|---|---|---|---|---|---|---|
| Human CD14/64 | Mouse | IgG2bκ, IgG2κ | 333179 | BD | MψP9, MD22 | FITC/PE | 1:20 15 minutes |
| Annexin V | - | - | V13242 | Thermo Fisher | - | FITC | 1:20 15 minutes |
| Human CD105 | Mouse | IgGκ | 560839 | BD | 266 | PE | 1:30 30 minutes |
| Human CD90 | Mouse | IgG1κ | 559869 | BD | 5E10 | APC | 1:30 30 minutes |
| Human CD73 | Mouse | IgG1κ | 561254 | BD | AD2 | FITC | 1:30 30 minutes |
| Human CD44 | Mouse | IgG2bκ | 560532 | BD | G44-26 | APC-H7 | 1:30 30 minutes |
| Human CD19 | Mouse | IgG1κ | 557835 | BD | SJ25C1 | PE-CY7 | 1:30 30 minutes |
| Human HLA-DR | Mouse | IgG2a, κ | 307615 | Biolegend | L243 | PE/Cy7 | 1:30 30 minutes |
| Human CD45 | Mouse | IgG1, κ | 560274 | BD | 2D1 | APC-H7 | 1:30 30 minutes |

**Table 2.1** Antibodies used in flow cytometry. Continued on the next page.

| Target | Species | Ig Class | Product Code | Company | Clone | Conjugate | Dilution/ incubation time |
|---|---|---|---|---|---|---|---|
| Human CD14 | Mouse | IgG2a, κ | 561116 | BD | M5E2 | PerCP-Cy5.5 | 1:30 |
| Human CD34 | Mouse | IgG1, κ | 347222 | BD | 8G12 | PerCP-Cy5.5 | 1:30 |
| Isotype control | Mouse | IgG1, κ | 641401 | BD | X40 | APC-H7 | 1:30 |
| Isotype control | Mouse | IgG1κ | 557646 | BD | MOPC-21 | PE-CY7 | 1:30 |
| Isotype control | Mouse | IgG1κ | 554681 | BD | MOPC-21 | APC | 1:30 |
| Isotype control | Mouse | IgG1κ | 552834 | BD | MOPC-21 | PERCP CY5.5 | 1:30 |

**Table 2.1 continued.** Antibodies used in flow cytometry.

Stained samples were kept on ice if there was a delay for analysis. Samples were acquired on a FACSCalibur and analysed with CellQuest™ (Becton Dickinson, Oxford, UK) or FlowJo 7.6.1 software (Tree Star Inc, Oregon, US). Typically between 10,000 and 50,000 events were collected for analysis.

# 2.7 Cell viability assays

## 2.7.1 Annexin V/Propidium iodide flow cytometry assay.

Annexin V is a calcium-dependent phospholipid binding protein which preferentially binds to phosphatidylserine (PS). In healthy cells, PS is normally found on the cytoplasmic side of the plasma membrane bi-layer however during early apoptosis PS is translocated to the extracellular surface. By using Annexin V FITC as a probe in a calcium rich buffer in conjunction with propidium iodide (PI), which cannot be excluded by cells which are either dead or in a late phase of apoptosis and binds to DNA, both the degree and stages of apoptosis can be determined in a single assay by flow cytometry. Cells in early apoptosis are Annexin $V^{+ve}$, $PI^{-ve}$, necrotic cells which are dead or in late phase apoptosis are Annexin $V^{+ve}$, $PI^{+ve}$. The flow plots showing the gated populations are shown below in figure 2.7.

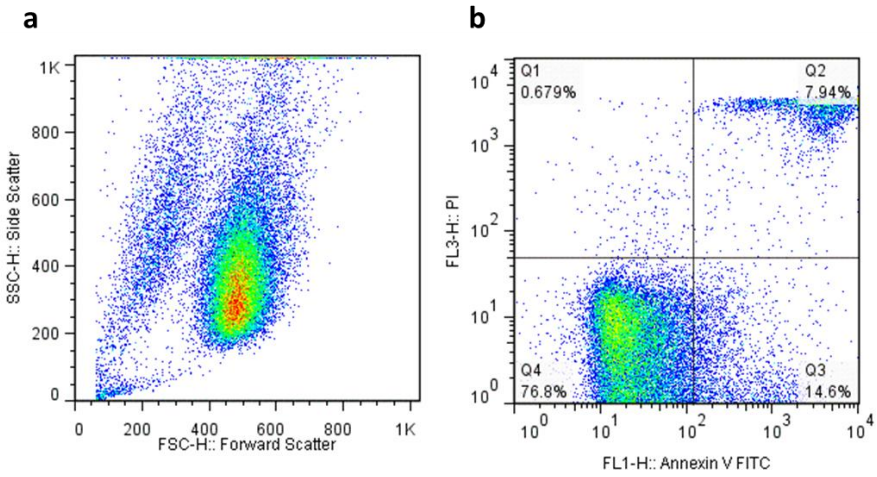



**Figure 2.7** Example flow plots of annexin V/PI staining of NCI-H929 cells. **(a)** Plot of total cell population. **(b)** Plot of total cell population with annexin V FITC on the horizontal axis versus propidium iodide on the vertical axis.

An annexin V FITC/PI staining kit, including buffer and stains, was obtained from ThermoFisher Scientific and staining was performed in accordance with manufacturers' instructions. Briefly, between 0.5-1x$10^5$ NCI-H929 cells were gently re-suspended through repeated pipetting and transferred to 15ml centrifuge tubes

and centrifuged at 400g for 5 minutes at 4°C. After which supernatant was removed and the cell pellet was re-suspended in 5ml PBS, centrifuged at 400g for 5 minutes at 4°C. This PBS wash step was repeated once more after which the cell pellet was re-suspended in 100µl annexin V binding buffer and transferred to a FACS tube (Becton Disckinson, Oxford, UK). 5µl FITC labelled annexin V (100µg/ml) was added to the cell suspension followed by 10µl of propidium iodide solution (10µg/ml). Samples were incubated at room temperature in the dark for 15 minutes after which time 400µl annexin V binding buffer was added and the cell suspension was gently vortexed prior to analysis with a FACSCalibur flow cytometer (Becton Disckinson, Oxford, UK). PI emission maxima is 638nm and can be measured in FL2, FITC emission maxima is 520nm and can be measured in FL1 on the flow cytometer.

### 2.7.2 MTS dye reduction assay.

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is a tetrazolium salt which is converted to a water soluble formazan which can be detected at absorbance of 490-500nm, this reaction is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in the mitochondria.

Cells were seeded in triplicate in 96 well plates at the density used under standard differentiation culture conditions i.e. monocytes for osteoclast cultures were seeded at $1.25x10^5$, SaOs-2 at $1x10^4$ and MSC's at $2x10^4$ cells/$cm^2$ for osteoblast cultures in their respective optimised medium. Cells were cultured with the reagent of interest e.g CBE at relevant concentrations in 200µl of the respective medium and assessed for cell viability at either the first medium change i.e. day 4 for osteoclast and SaOs-2 and day 7 for MSC or as a time course over the duration of the culture. At the chosen time point medium was removed and replaced with 100µl of the respective medium which was also added to three empty wells to serve as blanks. To all wells containing medium 20µl of the supplied MTS solution (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega UK Ltd, Southampton, UK) was added and placed in an incubator for between 1 and 4 hours as according to manufacturers' instructions. Once sufficient colour change was

observed, changing from yellow to brown, all wells containing medium and MTS were carefully re-suspended by pipetting to ensure the formazan product was fully solubilised while avoiding the creation of bubbles. Absorbance was then measured at 490nm using a BMG FluoStar Galaxy plate reader (reference wavelength 650nm).

## 2.8 Staining protocols.

### 2.8.1 Tartrate resistant acid phosphatase (TRAP) staining.

TRAP is an enzyme highly expressed by osteoclasts. Fast garnet GBC salt in the presence of a naphthol is converted into a highly insoluble azo dye by TRAP allowing the visualisation of TRAP activity and thus identification of osteoclasts.

TRAP assay kits were obtained from Sigma Aldrich and staining was performed according to manufacturers' instructions. Briefly, each 6mm glass coverslip on which PBMC's were attached and had been cultured in R10-OC for between 7 and 21 days were carefully lifted out of a well of a 24 well plate using ethanol sterilised #7 watchmaker forceps (SLS LTD, Hessle, UK), washed once in PBS prior to fixing by being submerged in a mixture of acetone, citrate and distilled water for 30 seconds. The glass coverslip was then washed in distilled water, placed in a clean well of a 24 well plate and allowed to air dry for a minimum of 30 minutes during which time the TRAP stain was prepared. This preparation involved pre-heating 8.8ml distilled water in a centrifuge tube to 37°C in a water bath prior to the addition of 3mg fast garnet powder, followed by vigorous vortexing to ensure fast garnet was in solution. Once achieved 400µl tartrate solution, 400µl acetate and 400µl naphthol bi-phosphoric acid was added in order. The solution was again vortexed, placed in a water bath to return the solution to 37°C, filtered into a new centrifuge tube with a 0.2µM pore syringe filter. 500µl of TRAP stain was added to each well containing 2 coverslips and incubated for 1 hour at 37°C in the dark after which time the stain was removed and the coverslips were washed in 1ml distilled water 5 times and air dried overnight prior to mounting on a glass slide, cell side facing upwards, with

DPX mountant. Osteoclasts were defined as cells with 3 or more nuclei with a red/pink staining indicative of TRAP activity as shown in figure 2.8.

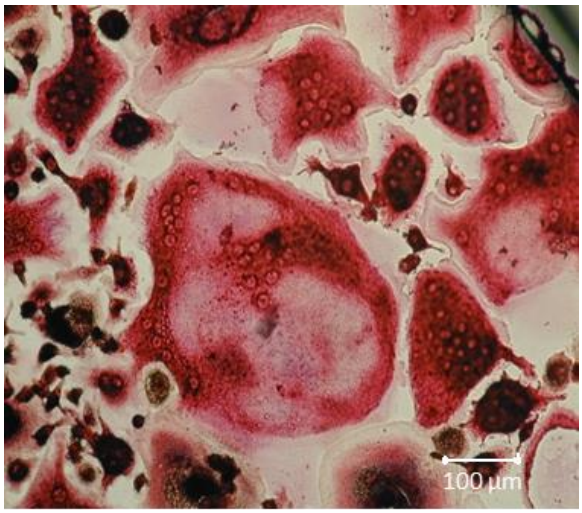



**Figure 2.8** Example image of TRAP staining of osteoclasts generated from a GD subject culture showing large multinucleate cells with distinct red/pink intracellular staining.

### 2.8.2 Vitronectin receptor (CD51/61) Alexa Fluor 488/ texas red phalloidin staining.

The α5β3 vitronectin receptor (VNR) is a member of the integrin superfamily and is highly expressed on the cell surface of osteoclasts. To facilitate the focal release of bone matrix degrading proteins osteoclasts form filamentous (F) actin ring structures. Phalloidin is a phallotoxin which binds monomerically to F actin therefore fluorescent conjugates of phalloidin when used in conjunction with antibody mediated fluorescent staining of VNR allows for specific identification of osteoclasts i.e. cells which are strongly positive for VNR and have an actin ring/s are counted as osteoclasts as shown in figure 2.9.

A 6mm glass coverslip or bone disc on which PBMC's were attached and had been cultured in R10-OC for between 7 and 21 days were carefully lifted out of a well of a 24 well plate (96 well plate for bone discs) using ethanol sterilised #7 watchmaker forceps and transferred to a well of a 24 well plate containing 1ml PBS and incubated for 5 minutes at room temperature. PBS was removed and replaced with

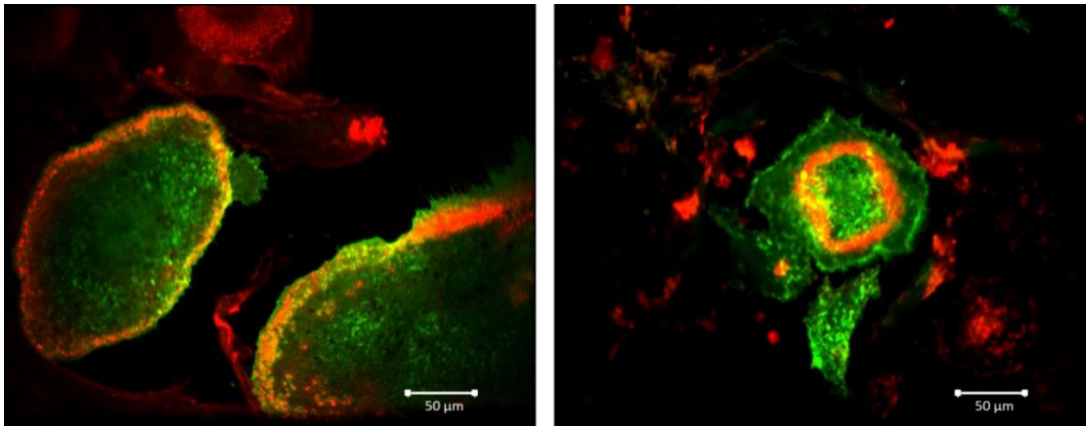



**Figure 2.9** Example images of fluorescent staining of vitronectin receptor (green) and F-actin (red). **(a)** Gaucher subject osteoclast culture on glass. **(b)** Control subject osteoclast culture on bone.

1ml PBS repeating the previous wash step. PBS was removed and replaced with 500µl 4% paraformaldehyde in PBS pH7.0 and incubated at room temperature for 20 minutes. The paraformaldehyde was removed and the wash step with 1ml PBS was repeated twice. PBS was removed and replaced with 500µl 1% BSA in PBS and incubated for 20 minutes at room temperature. 1% BSA was removed and replaced with 200µl vitronectin receptor (CD51/61) 1°mouse anti-human antibody (BD biosciences, Oxford, UK) solution comprising a 1:50 dilution (10µg/ml) of antibody in 1% BSA in PBS and incubated for 75 minutes at room temperature in the dark. Vitronectin receptor solution was removed and the wash step with 1ml PBS was repeated three times, incubated in the dark for this and all subsequent wash steps. PBS was removed and replaced with a 200µl Alexa Fluor 488 $F(ab')_2$ fragment of rabbit anti-mouse IgG (ThermoFisher Scientific, Paisley, UK) solution comprising a 1:200 dilution of F(ab')2 fragment (10µg/ml) in PBS. Alexa Fluor 488 solution was removed and the wash step with 1ml PBS was repeated three times. PBS was removed and replaced with 500µl 0.1% Triton X-100 (VWR, Lutterworth, UK) in PBS and incubated for 10 minutes at room temperature. The 0.1% triton was removed and the wash step with 1ml PBS was repeated twice. PBS was removed and replaced with 200µl Texas Red-X phalloidin solution (5µl Texas Red-X phalloidin stock solution = 1 unit (ThermoFisher Scientific, Paisley, UK) in 200µl 1% BSA in PBS) and incubated for 30 minutes at room temperature in the dark. Texas Red-X

phalloidin solution was removed and the wash step with 1ml PBS was repeated three times. Coverslips were removed from PBS with #7 watchmaker forceps and mounted onto a glass slide with Prolong Gold anti-fade mountant (ThermoFisher Scientific, Paisley, UK) and kept in the dark until visualisation with a fluorescence microscope (either an Olympus IX70 confocal fluorescence microscope with an Olympus LSR Ultraview camera or a Zeiss LSM510meta confocal microscope).

### 2.8.3 Toluidine blue staining.

A 6mm bone disc on which PBMC's were attached and had been cultured in R10-OC for 21 days was carefully lifted out of a well of a 96 well plate using ethanol sterilised #7 watchmaker forceps and transferred to a well of a 24 well plate containing 1ml distilled water. Water was removed and replaced with 1ml distilled water. This wash step was repeated once more followed by the removal of water and addition of 200µl mammalian cell lysis buffer and incubation at room temperature for a minimum of 30 minutes with shaking. The cell lysis buffer was then removed and wash steps with 1ml distilled water were repeated 3 times. Water was replaced with 500µl 1% toluidine blue in 0.5% sodium tetraborate solution made with distilled and incubated at room temperature with gentle shaking for between 30 and 60 seconds. The toluidine blue solution was removed and wash steps with 1ml distilled water were repeated 5 times. Distilled water was removed and the bone disc was air dried overnight. Pit formation was determined by counting individual pits using a phase contrast microscope.

### 2.8.4 Alizarin red staining.

Alizarin red S is a water soluble anthraquinone derivative which chelates calcium in a stoichiometric ratio of 1:1 at pH 4.1-4.3 to form a brick red dye which can subsequently be solubilised in acetic acid and quantified by colourimetry.

Medium was removed from wells of a 24 well plate of SaOs-2 cell line or MSC derived osteoblast cultures and replaced with 1ml PBS lacking $Ca2^{+}$ and Mg2+

(ThermoFisher Scientific, Paisley, UK). PBS was removed and replaced with 1ml PBS lacking $Ca2^{+}$ and Mg2+ as a second wash step. PBS was replaced with 500µl 4% paraformaldehyde in PBS lacking $Ca2^{+}$ and Mg2+, pH7.0 and incubated at room temperature for 15 minutes. Paraformaldehyde was removed and replaced with 1ml distilled water. The water was removed and again replaced with 1ml distilled water. The water was removed and replaced with 500µl 40mM alizarin red S solution which had been adjusted to pH4.1 with 10% ammonium hydroxide in distilled water and filtered with a 0.2µM pore syringe filter. The 24 well plate was incubated at room temperature for 20 minutes with gentle shaking. Alizarin red was removed and replaced with 1ml distilled water and the plate was gently shaken for 5 minutes prior to removal of water to be replaced with 1ml distilled water. This wash step was repeated a total of 5 times. At the final wash step water was removed and replaced with 600µl per well 10% acetic acid in distilled water and incubated at room temperature for 30 minutes with gentle shaking. Cells were detached with a cell scraper and the resultant slurry was transferred to a 1.5ml microcentrifuge tube, vortexed for 30 seconds, overlaid with 500µl mineral oil and transferred to a water bath pre-heated to 85°C and incubated for 10 minutes. After which the microcentrifuge tube was cooled in ice before centrifugation at 2000g for 15 minutes in a microcentrifuge. 500µl of supernatant was transferred to a clean 1.5ml microcentrifuge tube to which was added 200µl 10% ammonium hydroxide and briefly vortexed. 150µl of resultant solution was transferred to each of 3 wells of a 96 well plate. For quantification a range of alizarin red concentrations were added to the 96 well plate to produce a standard curve, each concentration in triplicate. These concentrations were 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0mM alizarin red S prepared in a diluent of 10% acetic acid and 10% ammonium hydroxide (ratio of 5:2).

## 2.9 β-glucocerebrosidase activity fluorimetric assay.

Cells were seeded in triplicate in 96 well plates at the density used under standard differentiation culture conditions in their respective optimised medium as in the MTS method (NCI-H929 at a density of $4x10^4$ cells/ml). Cells were cultured with the β-glucocerebrosidase inhibitor of interest i.e. conduritol β-epoxide (CBE) or isofagomine tartrate, at relevant concentrations in 200µl of the respective medium and assessed for cell viability after 4 days of culture. For adherent cells, after 4 days, medium was removed and replaced with 200µl PBS which was again removed and replaced with PBS as second wash step. PBS was removed and replaced with 50µl distilled $H_20$. Cells were detached by scraping with a 200µl pipette tip until no cells were observed to be attached when viewed under an Olympus CK40 phase contrast microscope. The 50µl cell suspension of each replicate was transferred to a 0.65ml microcentrifuge tube so each tube contained a total of 150µl of cell suspension. Non-adherent cells (NCI-H929) were re-suspended by repeated pipetting prior to transfer of replicates to a 15ml centrifuge tube i.e. 1 tube per condition. Cells were centrifuged at 400g at 4°C for 10 minutes with full acceleration and braking. Supernatant was removed and replaced with 1ml PBS, again centrifuged at 400g at 4°C for 10 minutes as a wash step. PBS was removed and the wash step was repeated after which PBS was removed and replaced with 150µl distilled $H_20$. The cell pellet was re-suspended by repeated pipetting prior to transfer to a 0.65ml microcentrifuge tube. The suspension of either adherent or non-adherent cells was freeze thawed by placing the tube in a -80°C freezer for a minimum of 30 minutes followed by thawing at room temperature. The resultant lysate was vortexed prior to being centrifuged at 2000g for 5 minutes. Supernatant was transferred to a 0.65ml microcentrifuge tube. As enzyme activity was quantified as nmol/hr/mg protein, total protein was determined using a bicinchoninic acid protein assay kit (Sigma Aldrich, Poole, UK) and was performed according to manufacturers' instructions. Briefly, protein concentration was measured against a standard curve of BSA dilution from 0-1000µg/ml. 25µl of the diluted standard or cell lysate was transferred to a well of a 96 well plate, in duplicate. 200µl of a 10:1 solution of bicinchoninic acid : Copper(II) Sulfate Pentahydrate 4% solution was added to each

well and placed in an incubator for 30 minutes. Absorbance was then measured at 562nm using a BMG FluoStar Galaxy plate reader (reference wavelength 650nm).

The following solutions were prepared for the β-glucocerebrosidase activity assay.

**Buffer:** 0.15M McIlvaine citrate phosphate buffer, pH 5.2. 100ml was prepared by combining 46.9ml 0.1M citric acid with 53.1ml 0.2M $Na_2HPO_4.2H_2O$ solution. Stored at room temperature.

**Standard:** A stock solution of 4-methylumbelliferone was prepared by dissolving 176.9mg in 1L distilled $H_20$ warmed to 80°C. 100µl of stock solution was added to 19.9ml distilled $H_2O$ to give a working solution of 5nmol/ml. Stock and working solutions were stored at -20°C until required.

**Glycine stop solution:** 55.7ml of solution A (75g glycine and 58g NaCl in 1L distilled $H_20$ was combined with 44.3ml of solution B (1M NaOH). Stored at room temperature.

**4-methylumbelliferylglucopyranoside (4-MUG) substrate+inhibitor.**
For a 1L solution – 9.6g of competitive inhibitor sodium taurocholate was added to a 0.2mM solution of 4-MUG in McIlvaine citrate phosphate buffer. Stored at -20°C until required.

The β-glucocerebrosidase activity assay was then performed in a 96 well plate as shown below, figure 2.10.

| Standard | Substrate blank | Sample blank | Sample 1 |
|---|---|---|---|
| " | " | " | Sample 2 |
| " | " | " | Sample 3 |

**Key**

= 3 wells

**Figure 2.10** 96 well plate layout for β-glucocerebrosidase activity fluorimetric assay.

20µl of sample supernatant was added to sample wells e.g. Sample 1. Once all samples were added 20µl of 4-MUG substrate+inhibitor was added to the substrate blank and sample wells. The 96 well plate was then incubated at 37°C for 2 hours in the dark. The enzyme reaction was terminated by addition of 200µl glycine stop solution to sample wells and substrate blank. 20µl sample supernatant was added

to standard, substrate blank and sample blank followed by 20µl $H_2O$ to sample blank. 20µl standard was added to standard wells followed by 200µl glycine stop solution to standard and sample blank wells. Fluorescence was measured using a BMG FluoStar Galaxy plate reader (excitation 365nm, emission 450nm). Enzyme activity was calculated using the formula below.

((Average of sample-substrate blank)/(Standard-sample blank)*(60/120)*(1000/µg protein)*(1000/20))

Note: Fluorescence reading for standard must be multiplied by 10 at the start of the calculation as 20µl of 5nmol/ml standard is added to wells = 0.1nmoles whereas the units of the calculation are nmol/hr/mg.

## 2.10 Statistics.

All experimental data was analysed by either Microsoft Excel versions 2012-2016 (Microsoft Corporation, California, US) or GraphPad Prism 5.0™ (GraphPad Software Inc, California, US). Standard deviation (SD), standard error of the mean (SEM) and mean were used to summarise the data. The D'Agostino-Pearson omnibus normality test was used to determine whether data fitted a Gaussian (normal) distribution. Unpaired t-tests were used for unpaired parametric data and Mann-Whitney U tests were used for unpaired non-parametric data. Paired t-tests were used for paired parametric data sets while Wilcoxon signed rank tests were used for non-parametric paired data sets. All tests were two tailed and a p value of $< 0.05$ was regarded as statistically significant.

# 3 In vitro osteoclast cultures

## 3.1 Introduction

In type 1 Gaucher disease (GD) one of the most common and clinically significant features is bone disease with 75-90% of patients suffering skeletal symptoms which in some patients can be severely debilitating (506,528). The most common bone features are osteonecrosis with 43% of patients having a history of osteonecrosis in a UK cohort (122) and osteopenia/osteoporosis with one study from the Gaucher registry suggesting that 76% of Gaucher patients between the ages of 12 and 20 had low bone mineral density and an increased risk of fracture (529). Although bone disease can respond to ERT it can take several years to show improvement (84,507) with considerable residual skeletal disease after several years of ERT (84). Potential challenges for effective treatment of skeletal aspects of type 1 GD are that the understanding of the bone microenvironment and the mechanisms underlying the bone disease including the effect of therapies on the cells in this environment are still poorly understood.

The main storage cell in type 1 GD is the macrophage primarily due to its role of phagocytosis in particular of effete red blood cells (530) resulting in foamy macrophages commonly referred to as Gaucher cells. These cells are present in Gaucher patient bone marrow and are thought to be an important contributing factor to bone disease as they have been shown to secrete several cytokines including IL6 which is known to affect several cell types in the bone microenvironment. However, while the number of these cells diminish over time with therapy the bone disease is much slower to respond suggesting other cells and factors in the bone microenvironment are also contributing to the skeletal disease. Osteoclasts, cells which degrade bone matrix, are terminally differentiated from the same lineage as macrophages, namely monocytes, and are also phagocytic (531). Due to this shared lineage and phagocytic activity it may be possible that osteoclasts are also affected by the altered sphingolipid profile of Gaucher patients resulting in a change in their numbers and/or activity.

The relatively recent development over the last 15 or so years of in vitro techniques for differentiating osteoclasts from peripheral blood by culturing with RANKL and MCSF, negating the need to co-culture with osteoblasts (532,533), has led to a rapid increase in our understanding of the origins and functions of osteoclasts. Use of this established and relatively non-invasive technique to study osteoclasts from Gaucher patients may therefore provide insight into the bone microenvironment of these patients.

However, true osteoclasts are traditionally defined by their ability to degrade and resorb bone matrix resulting in the creation of pits, usually circular, in the bone surface. Historically, de-vitalized bone, dentine or ivory slices have been used as a substrate on which to culture osteoclasts. Recent developments have led to the use of calcium phosphate coated glass to act as a substrate due to the limited availability of the other substrates however this substrate lacks the proteins normally present in the bone matrix. While de-vitalised bone is probably the most biologically relevant substrate the presence of pre-existing bone pits makes identification of new pits problematic. Therefore, if available, dentine or ivory is probably the most applicable substrate for assessment of osteoclast activity as neither have pre-existing bone pits due to the mode of their formation.

Recent publications have shown that several sphingolipids are involved in osteoclast migration, differentiation and activity. In particular sphingosine-1-phosphate has been shown to play a critical role in regulating the migration of osteoclast precursors to and from the peripheral blood and the bone marrow compartment (380). Lactosylceramide has been linked to RANKL mediated osteoclastogenesis (534) while ceramide has been suggested to increase osteoclast survival but reduce their resorptive activity (535).

Taken together these findings suggest the altered sphingolipid profile found in Gaucher patients may markedly affect the bone microenvironment and directly affect osteoclasts potentially explaining at least in part the bone disease observed in GD.

## 3.2 Aims and Hypotheses

**Aim:** To understand the roles of osteoclasts in Gaucher patient bone disease.

**Objectives:**

(1) To characterise GD patient osteoclast generation, size and activity in vitro compared to control subjects.

(2) To determine the effect of Gaucher-specific therapies on GD osteoclast generation and activity in vitro.

(3) To determine the effect of sphingolipids on both control and GD osteoclast generation and activity in vitro.

**Hypothesis 1:** Circulating GD monocytes have an increased potential to form osteoclasts compared to control monocytes.

**Rationale:** The majority of GD patients have low BMD with an average of 75% presenting with osteopenia. This bone loss could result from increased osteoclast number and/or activity, decreased osteoblast number and/or activity or a combination thereof. Several groups have found increased pro-osteoclastic cytokines in the plasma and serum of GD patients. Sphingolipids including ceramide and sphingosine-1-phosphate, both found to be elevated in GD serum, have been reported to affect osteoclast migration and maturation.

**Methods:** Differentiation of monocytes isolated from the peripheral blood of GD patients and control subjects into functional osteoclasts by culture with established osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

**Hypothesis 2a:** Increased osteoclastogenesis is directly related to β-glucocerebrosidase deficiency rather than protein misfolding.

**Rationale:** GD results from pathogenic mutations in the GBA gene causing either a decrease in β-glucocerebrosidase activity or misfolding of the protein resulting in degradation by the ER associated degradation pathway (ERAD). Data generated by our group prior to this project and research published during this project by Mucci et al with the irreversible inhibitor of β-glucocerebrosidase CBE in cell lines showed inhibition resulted in increased osteoclastogenesis suggesting it is directly related to a decrease in to β-glucocerebrosidase activity.

**Methods:** Culture of control subject monocytes with CBE in the presence of osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

**Hypothesis 2b:** Addition of GD-specific therapies to GD osteoclast cultures reduces osteoclastogenesis.

**Rationale:** If increased osteoclastogenesis is directly related to β-glucocerebrosidase deficiency, replacement of the deficient enzyme or reduction of the substrate should reverse this effect.

**Methods:** Culture of GD or control subject monocytes with ERT or SRT in the presence of osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

**Hypothesis 3:** The altered sphingolipid profile found in GD patients causes increased osteoclastogenesis.

**Rationale:** A number of sphingolipids concentrations have been found to be elevated in GD serum and plasma including ceramide, sphingosine, S1P, glucosylsphingosine and glucosylceramide. Research has shown roles for sphingolipids such as ceramide, sphingosine-1-phosphate and lactosylceramide in the formation and function of osteoclasts. The elevated concentrations of these sphingolipids, possibly including glucosylsphingosine and glucosylceramide - the roles of which in relation to osteoclasts have yet to be elucidated, may cause increased osteoclastogenesis.

**Methods:** Culture of GD or control subject monocytes with a number of different sphingolipids added exogenously in the presence of osteoclastogenic cytokines RANKL and MCSF. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

## 3.3 Results

### 3.3.1 Increased generation, activity and nucleation of Gaucher derived osteoclasts.

Monocytes isolated from the peripheral blood of control and Gaucher subjects ($1.25x10^5/cm^2$) were cultured in osteoclastogenic medium for up to 21 days on glass coverslips. At either day 14 or 21 these cells were fixed and stained for tartrate resistant acid phosphatase activity (TRAP), highly expressed in osteoclasts, resulting in pink deposits within osteoclasts and thus allowing their identification by direct light microscopy, as shown by arrows in figure 3.1a. At both time points osteoclast numbers were significantly higher in Gaucher subject cultures compared to control (day 14 p = 0.0008, day 21 p = 0.0016, figure 3.1b). When these monocytes were cultured in osteoclastogenic medium for 21 days on bone discs, active osteoclasts resorbed the bone creating pits and furrows in the bone surface. After 21 days the cells were lysed and washed from the bone surface which was then stained with toluidine blue to allow visualisation of the pits as indicated by arrows in figure 3.1c. Enumeration by direct light microscopy found Gaucher subject cultures generated significantly more pits than control cultures (p = 0.02, figure 3.1d), suggesting the osteoclasts were either more active or more numerous in Gaucher subject cultures than in control. It has been previously shown that osteoclasts with more nuclei have higher resorptive activity (536). TRAP staining of osteoclasts cultured on glass enabled visualisation by direct light microscopy of nuclei within the osteoclasts, as shown by arrows in figure 3.1e. By averaging the number of nuclei per osteoclast in different control and Gaucher subject cultures a significantly higher number of nuclei were present in Gaucher subject osteoclasts compared to control cultures (p = 0.0001, figure 3.1f) suggesting that Gaucher subject derived osteoclasts are both more numerous and more active in comparison to control subject derived osteoclasts.

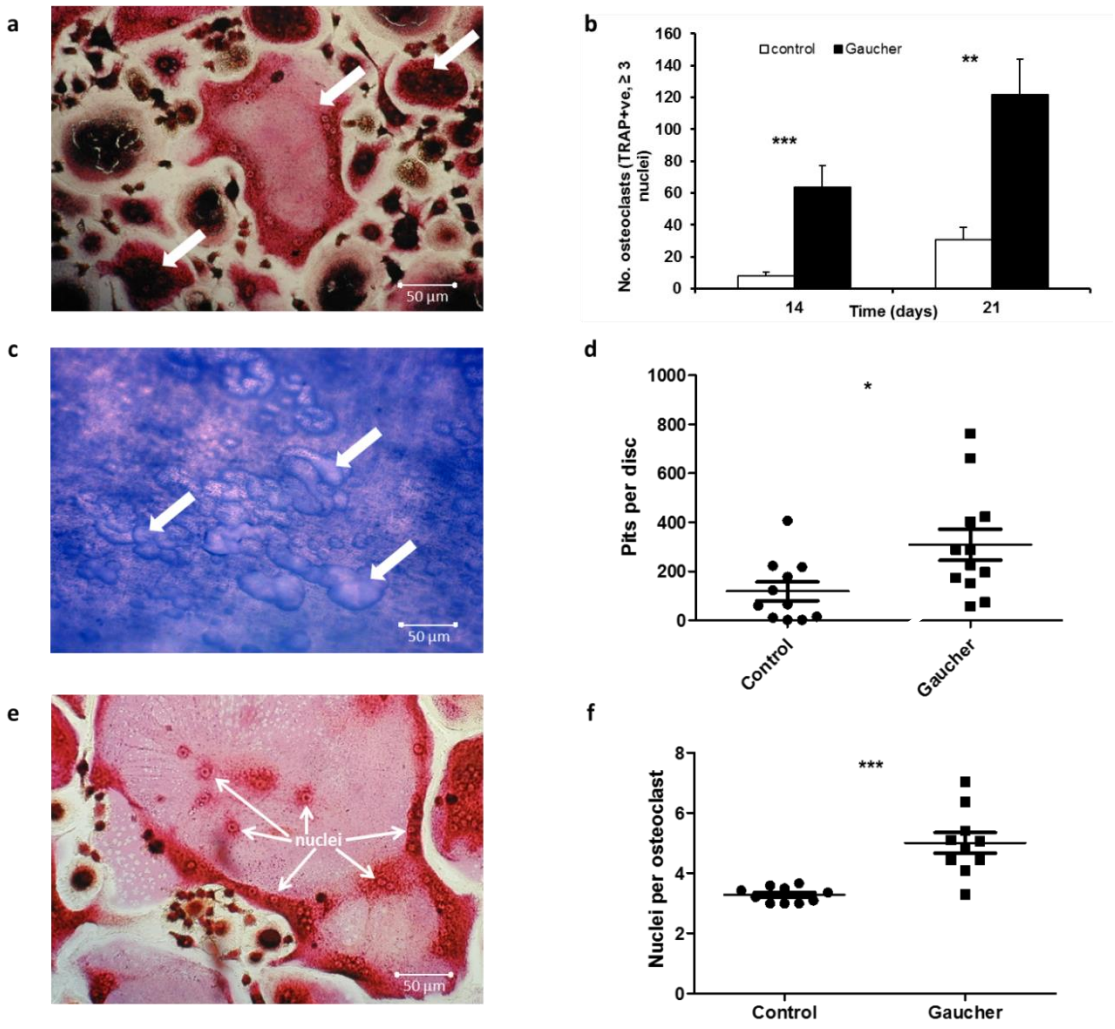



**Figure 3.1** Increased generation, activity and nucleation of Gaucher derived osteoclasts. **(a)** Representative TRAP staining of Gaucher derived osteoclasts, indicated by arrows, cultured for 21 days on glass. **(b)** Number of osteoclasts generated from adherent mononuclear cells after 14 and 21 days of culture on glass. Control n = 25, Gaucher n = 38, mean ± SEM plotted. **(c)** Representative image of bone pits generated in b, toluidine blue staining, pits indicated by arrows. **(d)** Bone pits resorbed by osteoclasts after 21 days of culture on bone, mean shown by horizontal line. **(e)** Representative image of a TRAP stained Gaucher derived osteoclast, nuclei indicated by arrows. **(f)** Averaged nuclei per osteoclast of TRAP stained osteoclast cultures, mean ± SEM shown by horizontal lines. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, unpaired t-tests.

### 3.3.2 Gaucher derived osteoclast generation occurs earlier than control derived osteoclasts.

To determine whether the significantly higher number of osteoclasts generated in Gaucher subject cultures and nuclei present in Gaucher subject osteoclasts was gender specific the data was divided into groups of male and female for both control and Gaucher subject cultures. Osteoclast number and nuclei per osteoclast was significantly higher for Gaucher subject osteoclast cultures compared to control for both genders (osteoclast number: female $p = 0.028$, male $= 0.034$, nuclei number: female $p = 0.036$, male $p = 0.012$, figure 3.2a and b) and no significant differences were found between either control male and female or Gaucher male and female groups.

The finding of significantly higher osteoclast generation at day 14 in Gaucher subject cultures, figure 3.1a, suggested osteoclasts were being generated earlier in comparison to control cultures. To confirm this finding mononuclear cells cultured in osteoclastogenic medium were fixed at days 7, 10, 14 and 21 to create a time course of generation. Significantly higher osteoclast generation in Gaucher subject cultures was found from day 10 (day 7 $p = 0.09$, day 10 $p = 0.007$, figure 3.2c) confirming the hypothesis that Gaucher subject osteoclasts form more quickly in comparison to controls.

To investigate whether the increased rate and total number of osteoclasts generated in Gaucher subject cultures was dependent on the presence of the osteoclastogenic cytokines RANKL and MCSF, mononuclear cells were cultured with either one or both of these cytokines excluded for 21 days on glass. Significantly more osteoclasts were formed in Gaucher subject cultures when RANKL was excluded compared to control subjects ($p = 0.041$, figure 3.2d). However numerically this difference is very small and does not account for the highly significant differences previously observed in figures 3.1a and 3.2c.

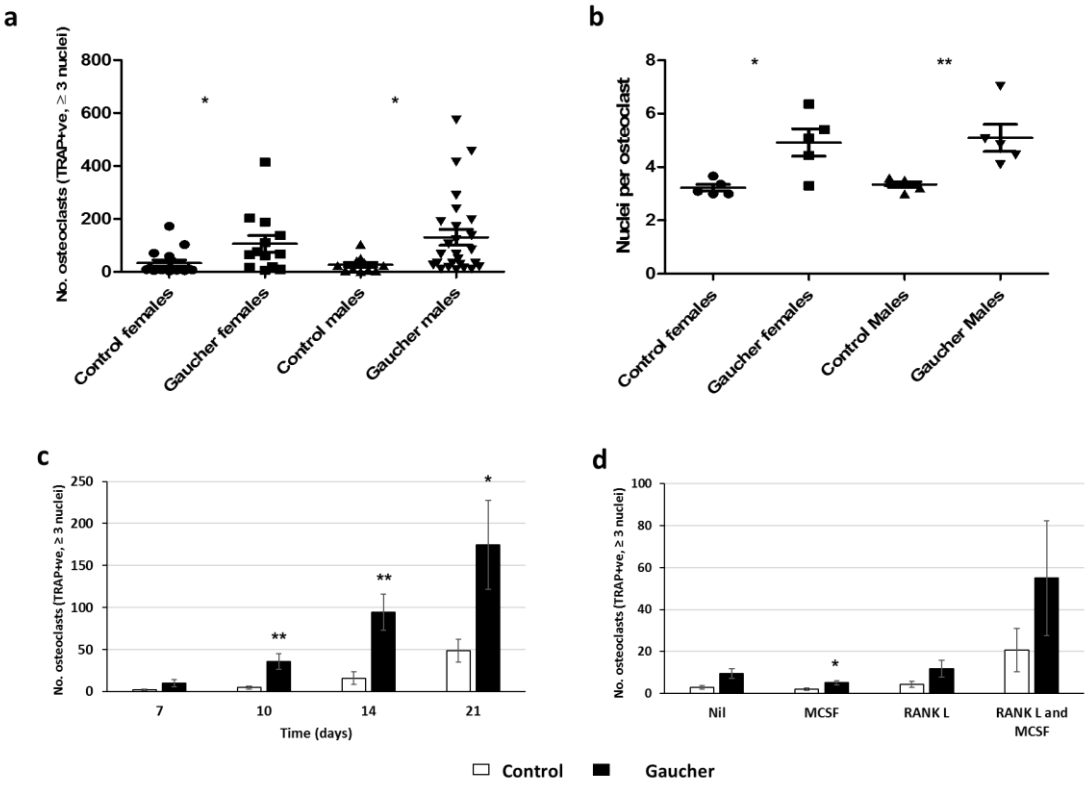



**Figure 3.2** Characterisation of Gaucher derived osteoclasts. **(a)** Number of osteoclasts generated from adherent mononuclear cells on glass, determined by TRAP staining, after 21 days of culture. **(b)** Averaged nuclei per osteoclast of TRAP stained osteoclast cultures, mean shown by horizontal line. **(c)** Number of osteoclasts generated from adherent mononuclear cells on glass, determined by TRAP staining, at several time point over the 21-day culture. N = 5 for control and Gaucher subject at each time point, mean ± SEM plotted. **(d)** Number of osteoclasts generated after 21 days of culture in R10 with the cytokine indicated. A minimum of 8 subjects were cultured for each condition. Mean ± SEM is plotted. * $p \leq 0.05$, ** $p \leq 0.01$, unpaired t-tests.

### 3.3.3 Gaucher subject mononuclear cells lack an inhibitory signal for osteoclastogenesis.

To determine whether the increase in osteoclastogenesis was caused by stimulation or a lack of inhibition and also whether this was mediated by physical contact or soluble factors, control and Gaucher subject mononuclear cells were cultured in osteoclastogenic medium for 21 days in each other's presence. Control and Gaucher subject cells were either adhered onto separate glass coverslips and

subsequently transferred to the same well so that they were sharing the same medium but physically separated, figures 3.3a and 3.3b, or mixed together prior to being adhered onto glass coverslips so that the cells were both in physical contact and sharing the same medium, figures 3.3c and 3.3d. To assess whether osteoclast generation had been affected in the contact experiments the number of osteoclasts generated by control and Gaucher subjects when cultured on their own was added together and halved to provide an expected number of osteoclasts in the mixed culture assuming osteoclast generation was unaffected, referred to as Control+Gaucher expected, with the actual osteoclast number generated from the mixed culture labelled as Control+Gaucher actual in figures 3.3c and 3.3d. After 14 days Gaucher subject derived osteoclast numbers in both separate and contact co-cultures were not significantly different to those of control subjects and were significantly lower than Gaucher subject cultured on their own when separated (Gaucher vs Gaucher co-culture, $p = 0.0133$, figure 3.3a) and the actual value when cultured in contact (Gaucher vs control+Gaucher actual, $p = 0.0101$, figure 3.3c). However, by day 21 the number of osteoclasts for Gaucher subjects in the co-cultures for both separated and contact conditions were significantly higher than for control subjects (Control vs Gaucher co-culture $p = 0.0402$, figure 3.3b. Control vs control+Gaucher actual $p = 0.0491$, figure 3.3d). A significantly lower number of Gaucher subject derived osteoclasts in co-culture was still observed when compared to Gaucher subjects alone when physically separated (Gaucher vs Gaucher co-culture $p = 0.0064$, figure 3.3b) and when compared to the actual value in the contact cultures (Gaucher vs control+Gaucher actual $p = 0.0057$, figure 3.3d). These findings suggest Gaucher subject mononuclear cells lack an inhibitory signal for osteoclastogenesis as a stimulatory factor generated by the Gaucher subject cells would have significantly increased the number in the control cultures. These results also suggest that this signal may be soluble although this experiment does not exclude the possibility that inhibition is mediated through both soluble and contact dependent factors.

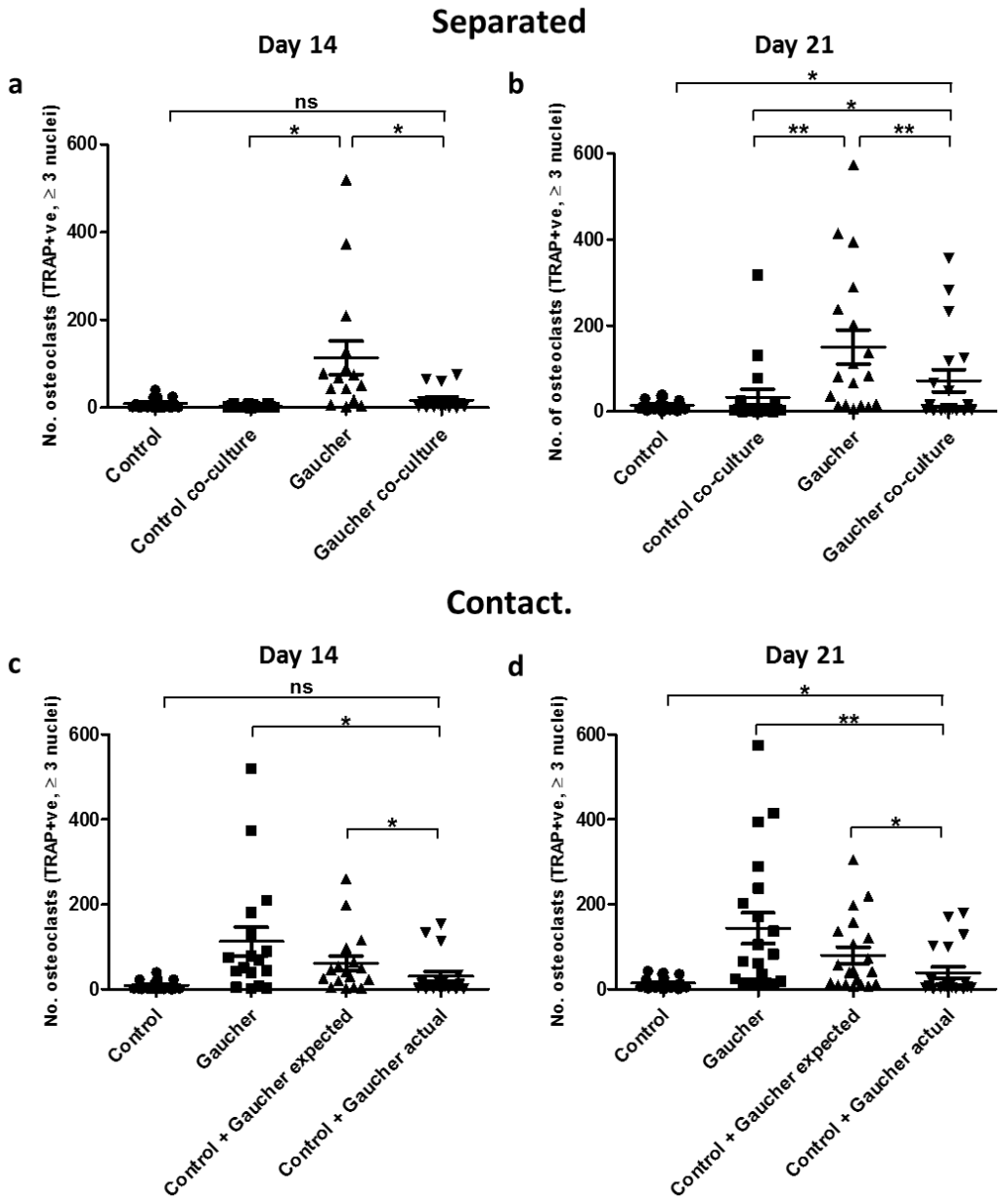



**Figure 3.3** Co-culture of control and Gaucher subject osteoclasts. **(a, b)** Number of osteoclasts generated after **(a)** 14 days and **(b)** 21 days of culture in which control and Gaucher subject mononuclear cells were adhered onto separate glass coverslips overnight and transferred to wells of 24 well plates, 4 coverslips per well. Combinations included 4 control coverslips, 4 Gaucher coverslips or 2 control coverslips with 2 Gaucher coverslips. Control or Gaucher co-culture indicates the number of osteoclasts generated by control or Gaucher subjects in the final combination previously stated. **(c, d)** Number of osteoclasts generated when mononuclear cells isolated from control and Gaucher subjects were mixed in equal numbers, adhered onto glass coverslips and cultured for **(c)** 14 days and **(d)** 21 days, labelled as control + Gaucher actual. Control + Gaucher expected is a calculation of control plus Gaucher osteoclasts divided by 2. Mean shown by horizontal line. * $p \leq 0.05$, ** $p \leq 0.01$, paired t-tests.

### 3.3.4 Potential cross correction

An alternative possibility to a lack of an inhibitory signal is cross correction of the deficient enzyme, β-glucocerebrosidase, from the control to the Gaucher subject cells via secretion of the enzyme into the culture medium. To assess this possibility β-glucocerebrosidase activity was measured in the supernatant of control and Gaucher subject osteoclast cultures after 21 days of culture. β-glucocerebrosidase activity was significantly higher in control than Gaucher subject supernatant ($p = 0.04$, figure 3.4) however activity was very low for both cohorts.

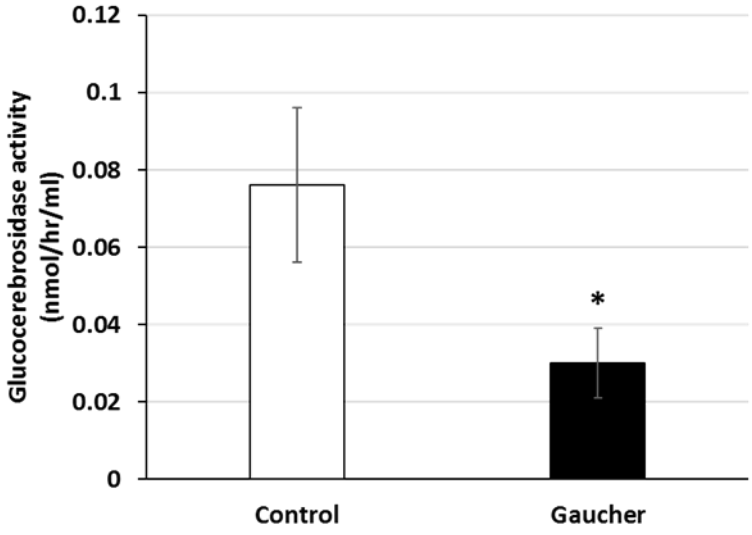



**Figure 3.4** Glucocerebrosidase activity measured in control and Gaucher subject supernatant from 21-day old osteoclast cultures. Control n = 18, Gaucher n = 19. Mean ± SEM plotted. * $p \leq 0.05$, unpaired t-test.

### 3.3.5 β-glucocerebrosidase inhibition by CBE increased generation, size and nucleation of control subject derived osteoclasts.

To investigate the role of reduced β-glucocerebrosidase activity in osteoclast generation, control subject mononuclear cells were cultured on glass in osteoclastogenic medium with an irreversible inhibitor of β-glucocerebrosidase, conduritol-β-epoxide (CBE), at a concentration which reduced the activity of β-glucocerebrosidase by up to 99% as shown in figure 3.5c ($p = 0.0003$). Medium (including inhibitor) was replaced twice per week. As the binding of the inhibitor to the enzyme is irreversible it was necessary to prove that the inhibitor was added at a high enough concentration (100μM) to ensure inhibition was still sufficient after the maximum period between medium changes, hence the time point of 4 days stated in figure 3.5c. Addition of CBE to the control subject osteoclast cultures for 21 days resulted in the generation of larger, more nucleated cells as can be seen in figure 3.5a (without CBE) compared to figure 3.5b (with CBE). Osteoclast generation in control subject cultures was also significantly increased in the presence of CBE ($p = 0.001$, figure 3.5d). However, this increase only partially recapitulates the degree of osteoclast generation observed in Gaucher subject cultures suggesting additional factors may also affect the rate of osteoclastogenesis of Gaucher mononuclear cells. In addition, although CBE inhibition resulted in a significant reduction of β-glucocerebrosidase activity in Gaucher subject cultures ($p = 0.05$) no significant increase in osteoclast numbers was observed again suggesting other factors are responsible for the osteoclast generation by Gaucher subject cultures being higher than inhibited control cultures.

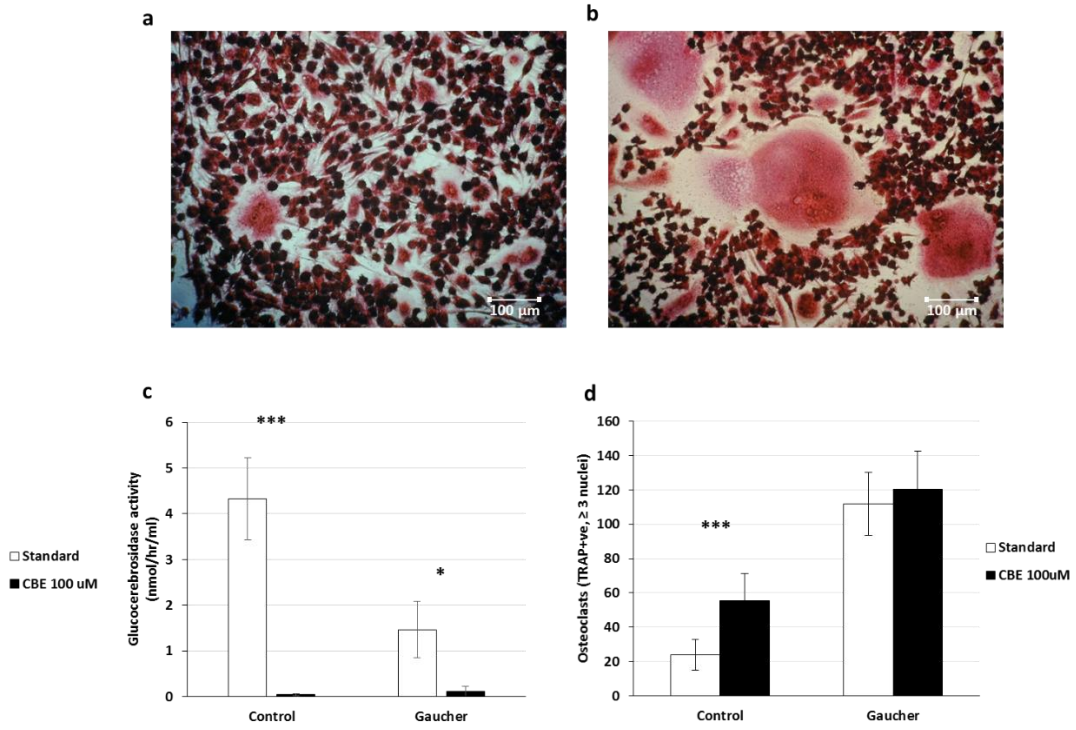



**Figure 3.5** Effect of CBE on osteoclast generation. **(a, b)** Representative TRAP stained images of osteoclasts generated from control subject adherent mononuclear cells after 21 days of culture in the **(a)** absence or **(b)** presence of 100µM CBE. **(c)** Glucocerebrosidase activity measured in whole cell lysates of adherent mononuclear cells cultured for 4 days with CBE, n = 8 for control and Gaucher per condition. **(d)** Number of osteoclasts generated from adherent mononuclear cells cultured for 21 days on glass. Control n = 17 per condition, Gaucher n = 22 per condition. Mean ± SEM plotted. * $p \leq 0.05$, *** $p \leq 0.001$. Paired t-tests.

### 3.3.6 Gaucher disease specific therapies reduce osteoclast generation in Gaucher patient derived cultures in vitro.

To assess the effect of Gaucher specific therapies on osteoclast generation, control (figure 3.6) and Gaucher (figure 3.7) subject mononuclear cells were cultured in osteoclastogenic medium with enzyme replacement therapies imiglucerase (1 unit/ml), velaglucerase (1unit/ml), substrate reduction therapy miglustat (50µM) or a combination of imiglucerase (1unit/ml) and miglustat (50µM) for 21 days with medium (including therapies) replaced twice per week. In control subject osteoclast

cultures only the addition of imiglucerase significantly reduced the number of osteoclasts generated (p = 0.0015, figure 3.6). In contrast, a significant reduction in osteoclastogenesis was observed in Gaucher subject cultures when in the presence of any of the therapies. However, the most statistically significant reduction was again attained by addition of imiglucerase (imiglucerase p = 0.001, velaglucerase p = 0.013, miglustat p = 0.023, imiglucerase+miglustat p = 0.05, figure 3.7). Suggesting the increase in osteoclast generation in Gaucher subject cultures is directly related to this disease and possibly the reduced activity of β-glucocerebrosidase.

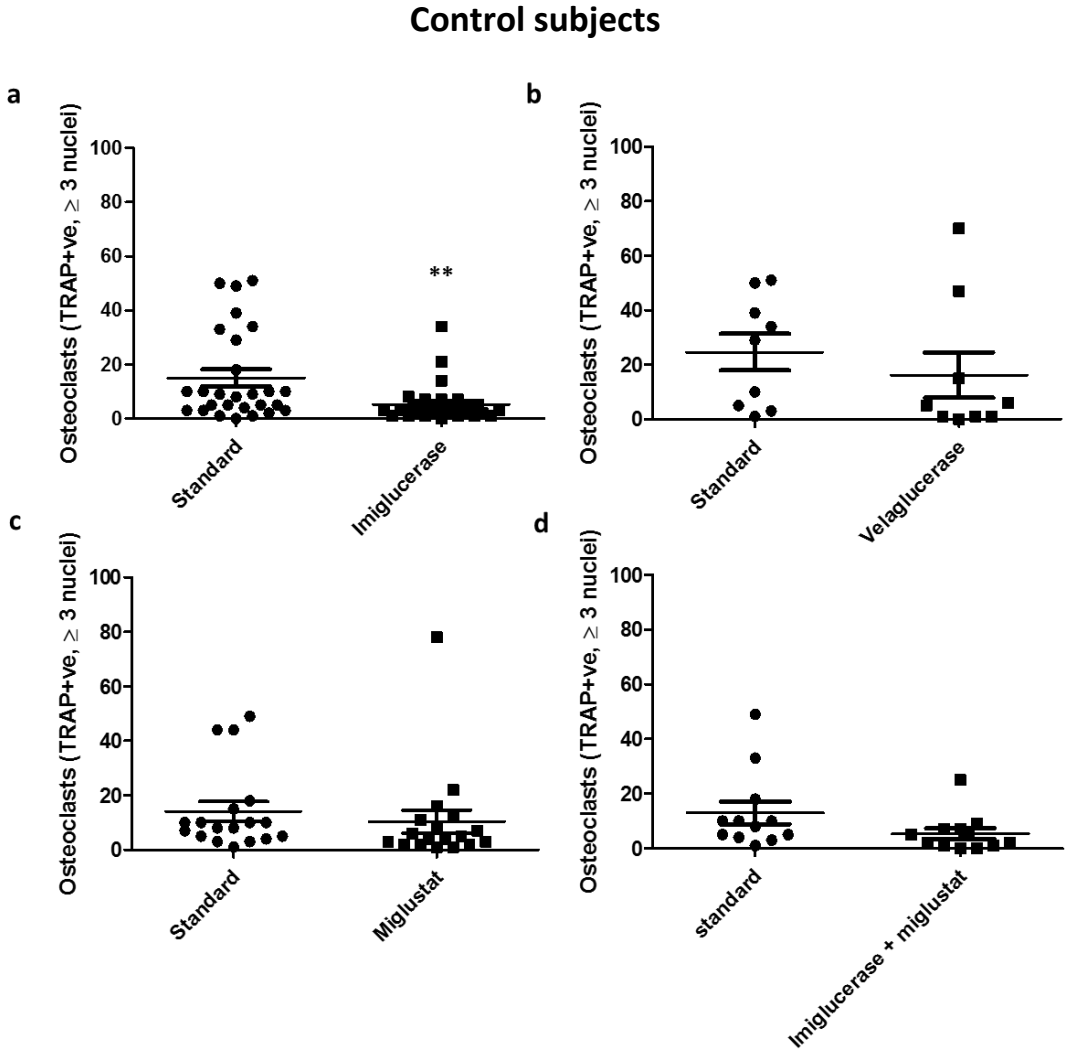



**Figure 3.6** Effect of therapy on control osteoclast generation in vitro. Number of osteoclasts generated from control subjects adherent mononuclear cells cultured for 21 days on glass in the presence of **(a)** imiglucerase (1 unit/ml), **(b)** velaglucerase (1 unit/ml), **(c)** miglustat (50μM) or **(d)** imiglucerase (1 unit/ml) + miglustat (50μM). ** p ≤ 0.01, paired t-tests.

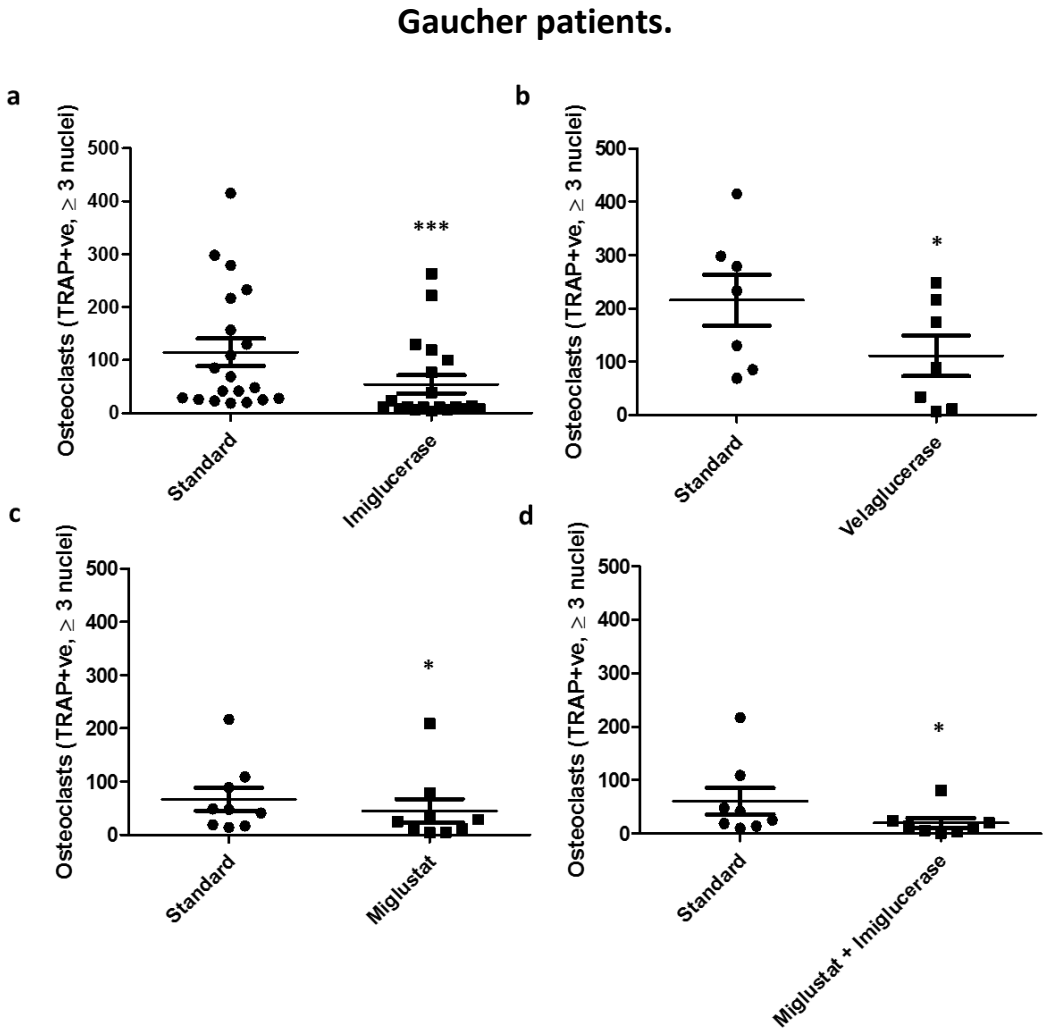



**Figure 3.7** Effect of therapy on Gaucher osteoclast generation in vitro. Number of osteoclasts generated from Gaucher subjects adherent mononuclear cells cultured for 21 days on glass in the presence of **(a)** imiglucerase (1 unit/ml), **(b)** velaglucerase (1 unit/ml), **(c)** miglustat (50µM) or **(d)** imiglucerase (1 unit/ml) + miglustat (50µM). * $p \leq 0.05$, *** $p \leq 0.001$, paired t-tests.

### 3.3.7 Ambroxol hydrochloride reduces osteoclast generation and activity in both control and Gaucher patient derived cultures.

Recent publications have shown that ambroxol hydrochloride, currently licensed as an expectorant and used as a treatment for several respiratory diseases (233,537), has the molecular structure to act as a pharmacological chaperone for β-glucocerebrosidase, increasing its lysosomal fraction and activity (538,539). It is therefore regarded as a potential therapy for Gaucher disease. In order to study the effect of this potential chaperone therapy on in vitro Gaucher subject osteoclast generation and activity, control and Gaucher subject mononuclear cells were cultured on glass or bone for 21 days in osteoclastogenic medium in the presence of 20μM ambroxol hydrochloride, medium replaced twice per week. In control cultures the addition of ambroxol significantly reduced both osteoclast generation ($p = 0.008$, figure 3.8a) and resorptive activity (pit formation) $p = 0.005$, figure 3.8b. A similar effect was observed for Gaucher subject cultures in which again osteoclast number ($p = 0.005$, figure 3.8c) and activity ($p = 0.006$, figure 3.8d) were significantly reduced. While these effects suggest increased osteoclastogenesis is at least in part related to β-glucocerebrosidase activity, ambroxol's marked effect of reducing osteoclast number and activity in control cultures suggests this compound may be affecting osteoclast formation, maturation and activity through a mechanism independent of β-glucocerebrosidase.

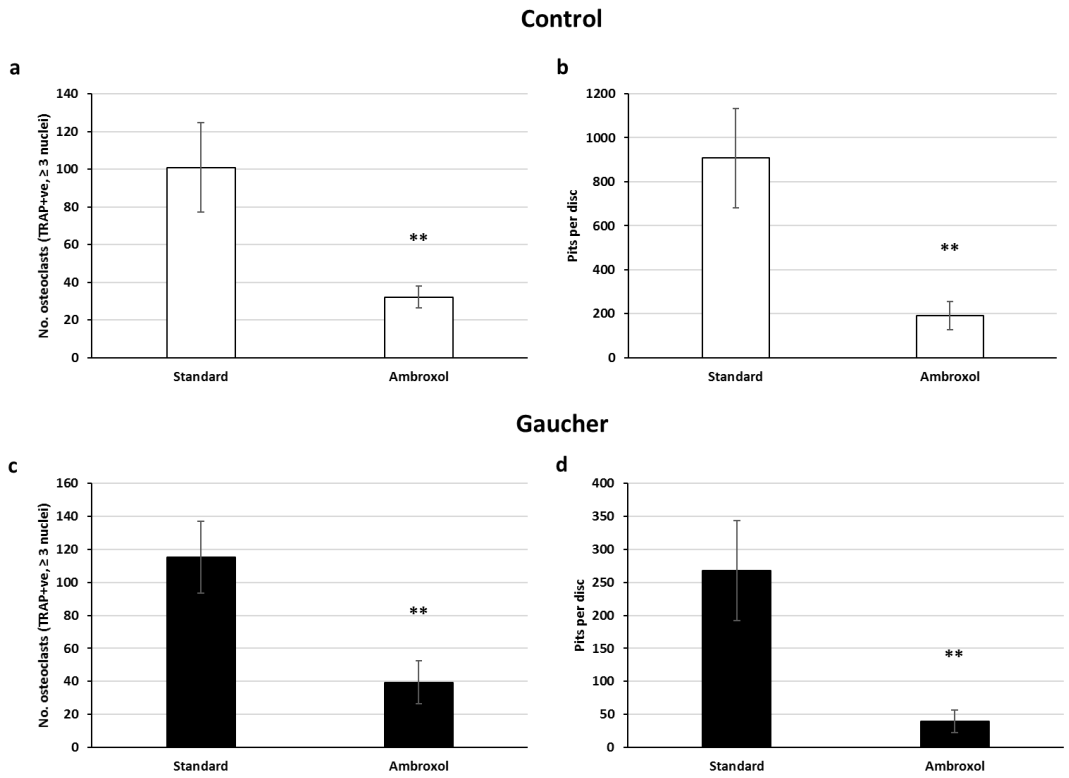



**Figure 3.8** Ambroxol reduces osteoclastogenesis and osteoclast activity. **(a, c)** Number of osteoclasts generated from adherent mononuclear cells after 21 days of culture on glass in the absence or presence of 20uM ambroxol hydrochloride, determined by TRAP staining. **(a)** Control n = 6, **(c)** Gaucher n = 7, mean ± SEM plotted, paired t-tests. **(b, d)** Bone pits resorbed by osteoclasts after 21 days of culture on bone. **(b)** Control n = 6, **(d)** Gaucher n = 7, mean ± SEM plotted, unpaired t-tests. Control shown as white bars, Gaucher shown as black bars. ** $p \leq 0.01$

## 3.3.8 Blocking TNF-α reduces control subject but not Gaucher patient derived osteoclast generation.

TNFα, a cytokine mainly produced by monocytes (392), shown to induce osteoclastogenesis (540) has been found to be significantly higher in the serum of Gaucher patients (143) and has been suggested to be a factor for the observed increase in osteoclastogenesis in other cellular models of GD (525,541). To elucidate a potential mechanism for the increased osteoclast generation in the GD osteoclast cultures TNF-α signalling was either stimulated by addition of TNF-α or inhibited by addition of a TNF-α blocking antibody to the osteoclast cultures from

initiation of the culture, replaced twice per week for the duration of the culture. Addition of a TNFα neutralising antibody (2ng/ml) to control or Gaucher subject mononuclear cells cultured in osteoclastogenic medium for 21 days on glass resulted in a significant decrease in osteoclast number in control cultures ($p = 0.03$, figure 3.9a). A decrease in osteoclast numbers was also observed in Gaucher subject cultures however this difference was not significant (figure 3.9c). Addition of TNFα to similar cultures did not significantly affect osteoclast generation for either control or Gaucher subjects (figures 3.9b and 3.9d respectively).

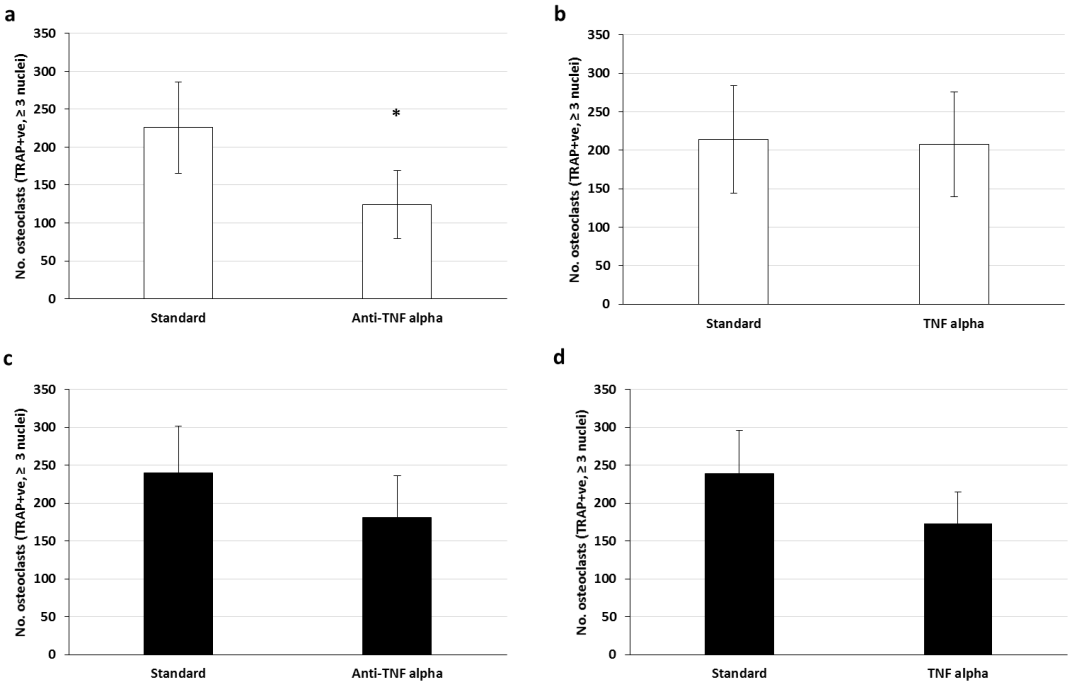



**Figure 3.9** Effect of TNF on osteoclast generation. Number of osteoclasts generated from adherent mononuclear cells after 21 days of culture on glass in the absence or presence of **(a, c)** 2ng/ml anti-TNFα, **(b, d)** 10ng/ml TNFα, determined by TRAP staining. Control (white bars) n = 6, Gaucher (black bars) n = 7, mean ± SEM plotted, paired t-tests. * $p \leq 0.05$

### 3.3.9 Specific sphingolipids affect osteoclastogenesis in vitro.

Gaucher patient serum has been shown to have elevated concentrations of glucosylsphingosine, glucosylceramide, sphingosine and sphingosine-1-phosphate. To investigate the exogenous effect of these lipids and others present in the lipid synthesis pathway on osteoclast generation and activity, control and Gaucher subject mononuclear cells were cultured in osteoclastogenic medium for 21 days on glass in the presence of a sphingolipid (1μM) or of the solvent used, methanol, at a dilution of 1:10,000 methanol (or lipid):medium. Medium and thus sphingolipid was replaced twice per week. Addition of sphingosine to control subject cultures led to a significant decrease in osteoclast generation ($p = 0.029$, figure 3.10a) in comparison to solvent alone while addition of lactosylceramide or glucosylceramide resulted in significant increases in osteoclast generation (lactosylceramide $p = 0.043$, glucosylceramide $p = 0.006$, figure 3.10a). However, while the significant increases in osteoclast generation were accompanied by respective increases in resorptive activity these increases did not attain statistical significance (figure 3.10b). Interestingly, the addition of sphingosine-1-phosphate to control subject cultures had the opposite outcome with osteoclast numbers being increased but not reaching significance ($p = 0.08$) while activity was significantly increased ($p = 0.011$, figure 3.10b). Sphingolipid addition to Gaucher subject cultures did not result in significant changes in either osteoclast number (figure 3.11) or activity (figure 3.12) with the exception of glucosylceramide. As in control cultures addition of exogenous glucosylceramide resulted in a significant increase in osteoclast generation after 21 days ($p = 0.028$) (figure 3.11f) however this increase did not lead to an increase in resorption pits (figure 3.12).

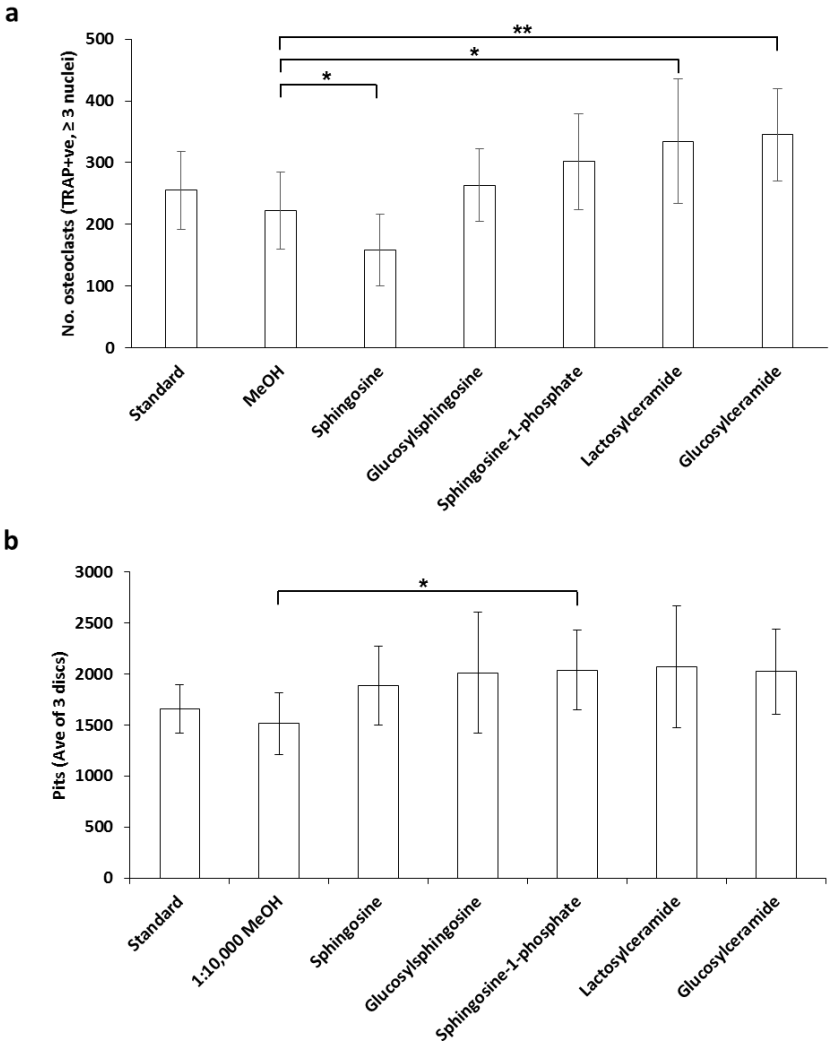



**Figure 3.10** Addition of sphingolipids to control osteoclast cultures. **(a)** Number of osteoclasts generated from control subjects adherent mononuclear cells after 21 days of culture on glass in the absence or presence of 1µM of the lipid indicated. **(b)** Bone pits resorbed by osteoclasts after 21 days of culture on bone in the absence or presence of 1µM of the lipid indicated. Lipids added in a methanol solution at a dilution factor of 1:10,000. N = 6 per condition, mean ± SEM plotted. * $p \leq 0.05$, ** $p \leq 0.01$, paired t-tests.

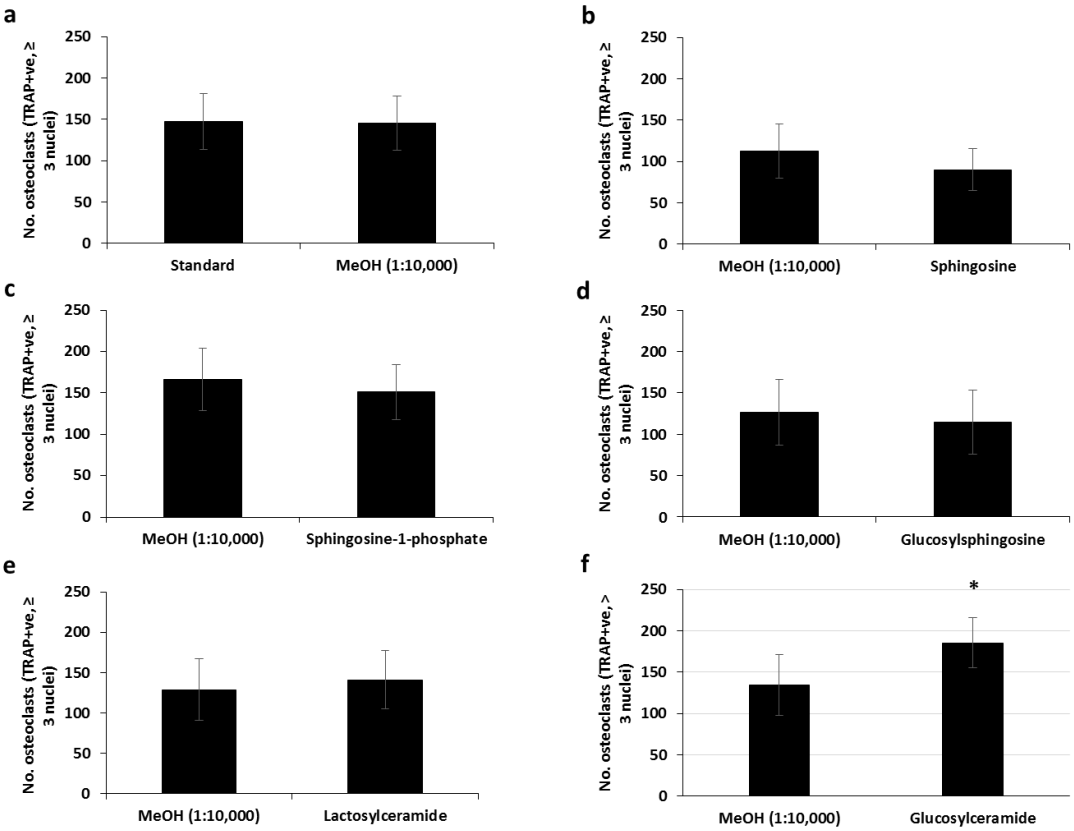



**Figure 3.11** Addition of sphingolipids to Gaucher osteoclast cultures. Number of osteoclasts generated from Gaucher subjects adherent mononuclear cells after 21 days of culture on glass in the absence or presence of 1µM of the lipid indicated. N per condition = **(a)** 16, **(b)** 10, **(c)** 8, **(d)** 13, **(e)** 11, **(f)** 11. Lipids added in a methanol solution at a dilution factor of 1:10,000. Mean ± SEM plotted. *p ≤ 0.05. Unpaired t-tests.

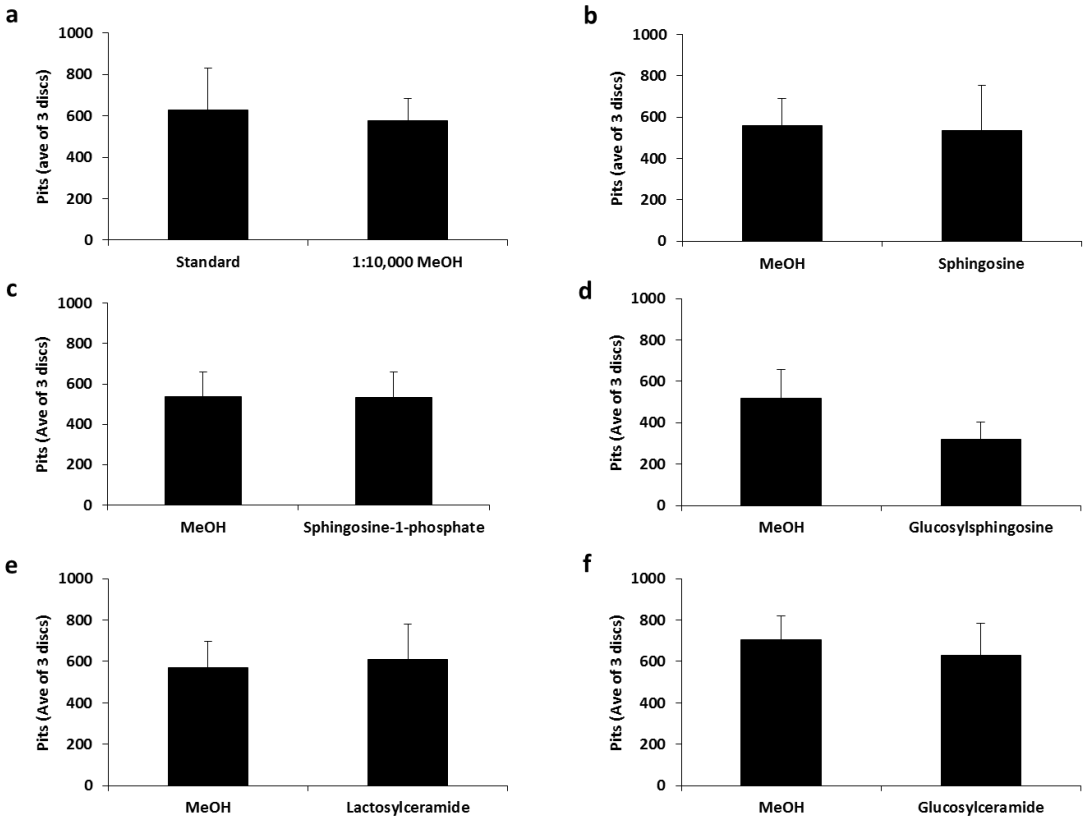



**Figure 3.12** Addition of sphingolipids to Gaucher osteoclast cultures. Bone pits resorbed by osteoclasts after 21 days of culture on bone in the absence or presence of 1μM of the sphingolipid indicated. N per condition = **(a)** 16, **(b)** 11, **(c)** 13, **(d)** 10, **(e)** 10, **(f)** 8. Lipids added in a methanol solution at a dilution factor of 1:10,000. Mean ± SEM plotted.

## 3.3.10 Osteoclasts can be generated from myeloma patient peripheral blood and bone marrow.

To determine whether in vitro osteoclast generation of multiple myeloma subjects was similar to control or Gaucher subjects, mononuclear cells were isolated from peripheral blood and bone marrow of multiple myeloma subjects. Peripheral blood mononuclear cells were cultured on glass in osteoclastogenic medium for 21 days in the presence of β-glucocerebrosidase inhibitors CBE or isofagomine or Gaucher disease specific therapies imiglucerase or miglustat. While effects on osteoclast generation could be discerned statistical significance could not be attained due to an insufficient number of subjects. Mononuclear cells were also isolated from bone marrow samples subsequent to stromal cell removal by an additional adherence

step. The mononuclear cells were adhered onto glass coverslips or bone discs and cultured in osteoclastogenic medium for 21 days, representative images of TRAP stained osteoclasts are shown for cultures on glass (figures 3.14a and b) and on bone (figures 3.14c and d, white arrows indicate TRAP+ve osteoclasts, black arrows indicate resorption pits). The representative images on glass show that the osteoclasts generated from bone marrow isolates were very large, highly nucleated and numerous. Images on bone disc show a very high density of pits indicating that mononuclear cells isolated from bone marrow may have a high propensity to form osteoclasts with substantial resorptive activity. Osteoclast generation was significantly greater in cultures of mononuclear cells isolated from bone marrow compared to those isolated from peripheral blood of multiple myeloma subjects ($p = 0.027$), control subjects ($p < 0.0001$) and Gaucher subjects ($p = 0.0002$), data not shown. However, this observation is based on bone marrow samples from only 3 multiple myeloma subjects, this cohort should be increased to confirm this observation. Mononuclear cells cultured in osteoclastogenic medium in the presence of 50µM Miglustat for 21 days on glass generated significantly fewer osteoclasts in comparison to standard conditions ($p = 0.02$, figure 3.14f). However, no difference in osteoclast activity (pit number) was observed when mononuclear cells were cultured in osteoclastogenic medium in the presence of miglustat on bone discs (figure 3.14h). Similarly, no significant differences were observed for addition of CBE, imiglucerase or ambroxol hydrochloride for either osteoclast generation or activity (figures 3.14g and h). Again the cohort is very small and will need to be expanded to determine the veracity of these observations.

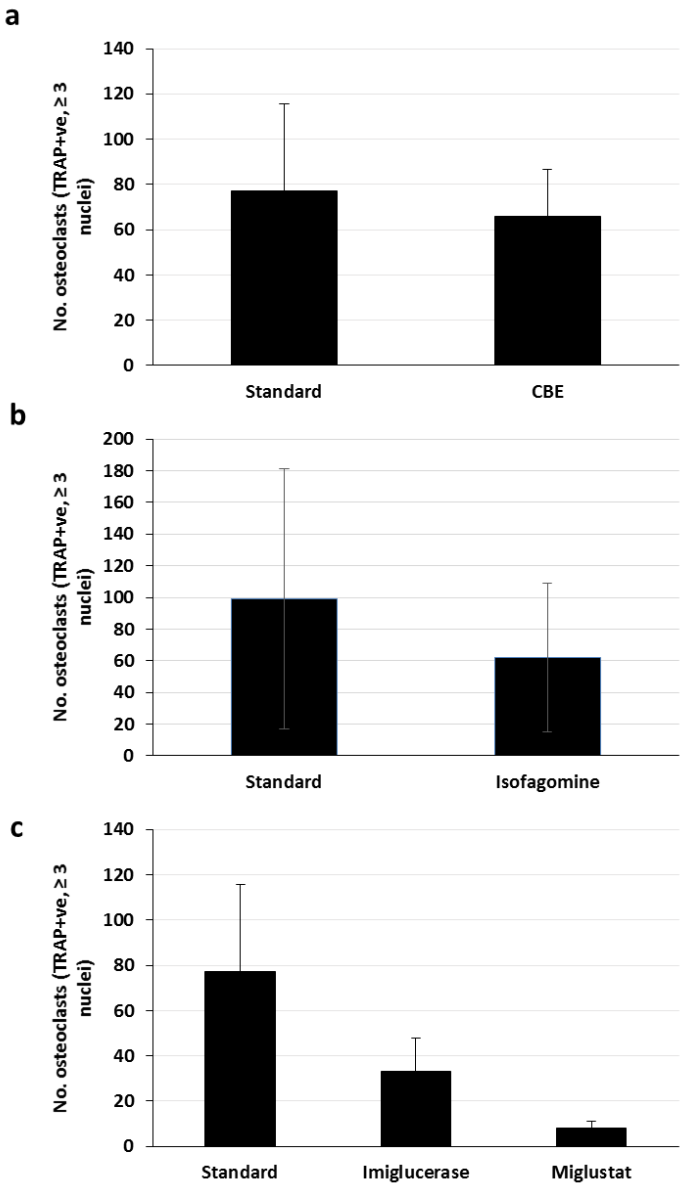



**Figure 3.13** Multiple myeloma peripheral blood osteoclast cultures. Number of osteoclasts generated from adherent mononuclear cells after 21 days of culture on glass. **(a)** Addition of 100uM CBE, n= 4. **(b)** Addition of 100uM isofagomine, n = 2 **(c)** Addition of 1 unit/ml imiglucerase or 50uM miglustat, n = 4. Mean ± SEM plotted. Paired t-tests, no significances.

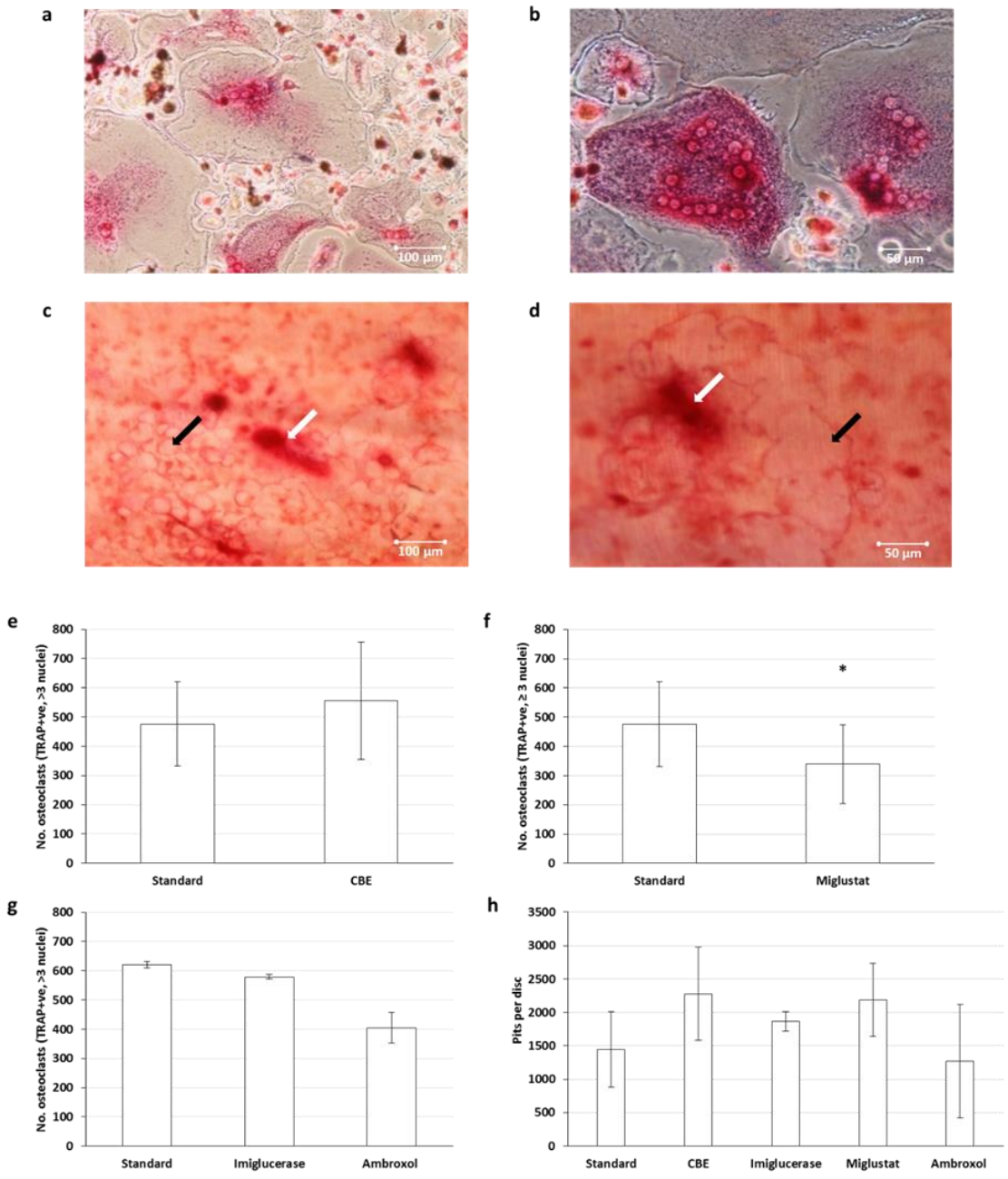



**Figure 3.14** Multiple myeloma bone marrow osteoclast cultures.
**(a, b)** Representative images of TRAP stained osteoclasts cultured for 21 days on glass. **(c, d)** Representative images of TRAP stained osteoclasts and pits cultured on bone for 21 days. Osteoclasts indicated with white arrows, pits indicated with black arrows. **(e, f, g)** Number of osteoclasts generated from adherent mononuclear cells after 21 days of culture on glass. **(e)** Addition of 100µM CBE, n = 3, **(f)** Addition of 50µM miglustat, n = 3, **(g)** Addition of 1 unit/ml imiglucerase or 20µM ambroxol hydrochloride, n = 2, **(h)** Bone pits resorbed by osteoclasts after 21 days of culture on bone. Conditions at concentrations as stated for culture on glass, isofagomine added at 100µM, n = 2 per condition. Mean ± SEM plotted. *p ≤ 0.05, paired t-test

## 3.4 Discussion

Bone disease is a very common and debilitating feature of GD. As detailed in table 1.2 several pro-osteoclastogenic proteins have been found to be elevated in the plasma and serum of type 1 GD patients including IL-1β, IL-8, MIP-1α, MIP-1β and TNF-α. Systemic regulation may also be affected as two studies have shown that GD patients have a deficiency in 1,25(OH)2 vitamin D3 which is known to stimulate osteoblast differentiation (521,522). Taken together, these findings suggest GD bone marrow may have a highly pro-osteoclastogenic environment which may in part explain the bone loss observed in GD patients. In addition, osteoclasts are terminally differentiated from monocytes, as are the main storage cells in GD, macrophages. Therefore, our initial studies focused on characterising osteoclasts differentiated from GD monocytes in vitro.

The results from this chapter demonstrate for the first time that GD patient derived osteoclasts are abnormal and osteoclasts may play a central role in the pathology of bone disease in GD, many of these results being published by our group in October 2013 (526). This data supports our preliminary findings presented as posters from 2005 to 2008 and the finding by Mucci et al published in 2012 that treatment of osteoclast precursors with conditioned medium from CBE treated peripheral blood mononuclear cells (PBMC's) resulted in increased osteoclast differentiation (525).

**Increased osteoclast generation in GD cultures**

From our studies we have found significantly more osteoclasts were generated at days 14 and 21 of osteoclast culture from GD monocytes than control monocytes suggesting either that a larger proportion of GD monocytes are osteoclast precursors or that GD monocytes have an increased capacity for differentiating into osteoclasts and may be more sensitive to pro-osteoclastogenic cytokines.

Functional assessment of osteoclast activity involving osteoclast generation on bone followed by counting the number of pits resorbed by active osteoclasts resulted in GD osteoclast cultures producing significantly more resorption pits than

control cultures. This increased resorption could simply be a direct consequence of increased osteoclast number but could also indicate these osteoclasts have higher individual activity.

Light microscopy counting of nuclei per osteoclast showed GD osteoclasts had more nuclei and were consequently larger when cultured on glass. The combination of increased number and size may explain the increased activity as previously published research demonstrated a direct correlation between the number of nuclei and the resorptive activity of osteoclasts (536). However, it should be noted that this study assessed the activity of osteoclasts generated from normal subjects and therefore this correlation may not be the case for GD derived osteoclasts.

No difference was found for gender in relation to number of osteoclasts generated and nuclei per osteoclast. Both male and female GD patients had significantly higher numbers of osteoclast and nuclei than control male and females respectively suggesting gender may not be a factor in GD specific bone disease however, it does not preclude the possibility of low oestrogen levels due to menopause further increasing osteoclast generation (315,542) in female GD patients exacerbating the bone complications commonly seen in GD.

Osteoclasts were generated much earlier in GD cultures compared to controls suggesting monocytes could also be pre-stimulated or pre-committed to form osteoclasts in addition to more precursors as control osteoclast generation did not 'catch up' but might if cultured for longer. However, human osteoclasts have a relatively short lifespan of about 2 weeks (543) therefore extending the duration of the culture beyond 21 days may not lead to an overall increase in osteoclast number as osteoclast loss by apoptosis may be equivalent to formation.

To investigate whether GD osteoclast precursors are more sensitive to pro-osteoclastogenic cytokines MCSF and RANKL than control cells cultures were set up with neither cytokine, MCSF only, RANKL only or both. From these experiments a significant difference in osteoclast generation in the presence of MCSF only was found. Numerically this difference is very small and appears to have a minimal effect which does not account for the increases observed under standard culture

conditions however it may also indicate that GD osteoclast precursors are more sensitive to stimulation than control osteoclast precursors.

To determine whether the increase in osteoclast generation in GD cultures is due to a stimulatory signal or a lack of inhibitory signal and whether this/these signals are secreted or require cellular contact GD and control monocytes were cultured in the same well either physically separated or mixed together to enable direct cellular contact. Culture of GD and control monocytes in osteoclastogenic conditions in the same well but adhered to separate coverslips resulted in significantly fewer osteoclasts generated compared to Gaucher in isolation after both 14 and 21 days suggests control monocytes secrete a factor, either not present or expressed at much lower levels by GD monocytes, which inhibits osteoclastogenesis, acting as a negative regulator. This data indicates a lack of inhibitory signal as if there was a stimulatory factor produced by GD monocytes the number of control osteoclasts would have increased. Evidence that GD patient monocytes/osteoclast precursors can still respond to this factor suggests therapies designed to inhibit osteoclast formation may be effective.

However, while at day 14 there was no statistically significant difference between control and Gaucher osteoclast generation (when cultured in the presence of controls) a significant difference was noted at day 21 with GD cultures generating more osteoclasts. Therefore, this negative regulator may only slow osteoclast formation but does not reduce the eventual number of osteoclasts generated suggesting there may be a higher percentage of osteoclast precursors in the monocyte population of GD patients.

A very similar effect was observed when GD and control monocytes were mixed and cultured in contact as for when cells were separated. However, while evidence from cultures in which the cells were physically separated clearly suggests a role of a secreted factor/s it does not exclude the role of contact dependent factors involved in osteoclast precursor fusion proteins such as DC-STAMP and OC-STAMP which if overexpressed may lead to the earlier osteoclast development observed in GD osteoclast cultures as both are essential for osteoclast precursor cellular fusion with DC-STAMP considered a master regulator of osteoclastogenesis (331). It is also

possible that the inhibitory factor may simply be cross correction of the deficient enzyme, secreted from control monocytes and taken up by GD monocytes. However, uptake of exogenous native β-glucocerebrosidase may be limited as a publication by Takasaki et al in 1984 found human placental β-glucocerebrosidase contained a high percentage of galactose-terminated oligosaccharide side chains which targeted the enzyme to hepatocytes (197) and Brady et al in 1991 demonstrated that efficient uptake of β-glucocerebrosidase by monocytes and macrophages required modification of the oligosaccharide side chains from terminating in sialic acid or galactose to terminating in mannose residues to take advantage of the expression of mannose receptors on the cell surface of monocytes and macrophages, also found to be expressed on the cell surface of osteoclasts (544). To address this β-glucocerebrosidase activity was measured in the supernatant of cultures. Activity in control and Gaucher supernatants was very low when compared to activity measured in cell lysate (normal range of 5-15nmol/hr/mg in standard leukocyte assay) therefore it is unlikely that the reduction in osteoclast generation was due to cross-correction of secreted enzyme as with little activity in the supernatant and potentially limited cellular uptake only a very small increase in cellular of β-glucocerebrosidase is likely. However, this may be an additional factor as small increases in functional enzyme may be sufficient to reduce the rate of osteoclast generation observed.

**Increased osteoclastogenesis is directly related to β-glucocerebrosidase deficiency.**

Conduritol β-epoxide (CBE) is an irreversible inhibitor of β-glucocerebrosidase, binding to its active site, preventing it from binding to and catalysing its substrate glucosylceramide. This inhibitor has been used by a number of groups such as Mucci et al, Lecourt et al and Berger et al (141,524,525) to create cellular models of GD.

Culture of control monocytes with CBE to create a model of GD resulted in much larger osteoclasts with a higher averaged number of nuclei per osteoclast than non-

inhibited control osteoclast cultures. CBE inhibited controls also generated significantly more osteoclasts but not as many as GD osteoclasts. These findings demonstrate increased osteoclastogenesis and activity is at least in part related to reduced GBA activity but also suggest that either there are other osteoclast specific factors involved, that GD monocytes and osteoclast precursors already have sphingolipid storage or that there is a larger pool of osteoclast precursors in the mononuclear cell population which CBE cannot mimic/create. However, 21 days of culture with CBE may not be sufficient time to result in substantial glucosylceramide accumulation although previous experiments using CBE have demonstrated the accumulation of glucosylceramide at 72 hours in the THP1 cell line and by day 15 in fibroblast cultures (545). It may also indicate that long term exposure to glucosylceramide is required to create a larger pool of osteoclast precursors.

**Addition of GD-specific therapies to GD osteoclast cultures reduces osteoclastogenesis.**

Since the advent of enzyme replacement therapy (ERT) several long term studies have shown significant increases in bone mineral density and reduction of bone crises in type 1 GD (506–510). However, a number of studies have also shown some patients continue to suffer bone events while on ERT (84,505,511). To investigate the effects of GD-specific therapies including ERT (imiglucerase, velaglucerase) and SRT (miglustat) on control and GD osteoclast generation and activity and to further test the hypothesis that increased osteoclastogenesis is directly related to a deficiency in β-glucocerebrosidase activity these therapies were added to osteoclast culture medium for the duration of culture.

Addition of GD specific therapy to GD osteoclast cultures resulted in significant reduction in osteoclast generation for all therapies. Previous results such as CBE inhibition indicated that the effect was specifically related to a reduction in β-glucocerebrosidase activity therefore addition of functional enzyme or reduction in substrate burden should reverse this effect. These findings therefore provide

further support for the theory that the increased osteoclastogenesis is directly related to a reduction in enzyme activity resulting in sphingolipid accumulation/dysregulation/altered ratios which in turn may lead to altered signalling either directly by lipids or indirectly by cytokines.

Addition of GD-specific therapy to control osteoclast cultures resulted in a reduction in osteoclastogenesis in general but only reached significance for imiglucerase. This may be due to the low numbers of osteoclasts generated by control cells in this culture system. To determine whether the effect of therapy is specific to GD osteoclast cultures it would be necessary to alter the conditions in control cultures to enhance osteoclast generation by increasing the pro-osteoclastic cytokines MCSF and RANKL to enable the observation of a reduction in osteoclast numbers in the presence of GD-specific therapies. If this is not the case it would suggest that the factors are specific to GD and therefore we would not expect to see a significant reduction in controls.

Another therapeutic approach is the use of pharmacological chaperone therapies (PCT). These are low molecular weight compounds which stabilise or aid correct protein folding and are designed to bind to the active site at neutral pH and dissociate at the acidic pH present in the lysosome. One such PCT is ambroxol hydrochloride which has been shown to increase GC activity and protein levels in human primary skin fibroblasts (538). However, although ambroxol may be a promising therapy for GD it is not currently approved as a therapy for GD.

Addition of ambroxol to osteoclast culture medium resulted in a similar effect as observed for ERT and SRT in relation to the GD osteoclast numbers however, a significant decrease was also observed for control osteoclasts. The clear effect on control osteoclast generation would suggest that β-glucocerebrosidase regulates osteoclast generation directly through an inverse ratio i.e. the more β-glucocerebrosidase, the fewer osteoclasts. However, this could be for a number of possible reasons (a) β-glucocerebrosidase is directly involved in the signalling cascades involved in osteoclast fusion and formation, (b) glucosylceramide induces osteoclast generation therefore an increase in β-glucocerebrosidase would lower the amount of glucosylceramide reducing the pro-osteoclastogenic signal, (c)

ceramide, shown to be a bioactive lipid and a breakdown product of glucosylceramide, inhibits osteoclast differentiation, (d) ambroxol hydrochloride has off-target effects, it is also used as an expectorant and is known to affect other enzymes (232,546) which may also affect osteoclastogenesis. This is possibly the case as ambroxol is a pharmacological chaperone it may not affect cells with normal lysosomal β-glucocerebrosidase activity as trafficking to the lysosome is already optimal and therefore further enhancement by ambroxol may be minimal.

Reduced osteoclast numbers were reflected in a reduction in osteoclast activity for both control and GD cultures. Again, ambroxol appears to affect osteoclast activity, however whether this is due to a decrease in osteoclast number or an effect on osteoclast function is yet to be determined. The fact that percentage reduction for both number and activity is very similar may support the former.

TNF-α is a pro-osteoclastic cytokine which plays a major role in the regulation of bone homeostasis (392). A recent publication by Mucci et al in 2012 suggests that TNF-α may be an important cytokine in mediating the increased osteoclastogenesis in their CBE model of GD (525). To investigate this effect on control and GD osteoclast generation TNF-α signalling was either blocked by addition of a TNF-α blocking antibody or stimulated by addition of TNF-α to osteoclast culture medium.

Addition of TNF-α blocking antibody significantly reduced the number of osteoclasts generated in control cultures but not in GD cultures. Although a reduction is observed in the GD cohort this did not reach significance ($p = 0.16$). Based on this preliminary data of 8 samples a power calculation based on a paired t-test, with significance level of 5% and power of 90% the estimated subject size is 36. Therefore, more samples will be required to determine whether GD osteoclast generation is affected by blocking TNF-α signalling.

TNF-α did not increase osteoclast numbers for either control or GD cultures. This may be due to the inclusion of RANKL in the cultures superseding the effect of the TNF as both cytokines are members of the TNF superfamily and use many of the same TNF Receptor-Associated Factor (TRAF) family of adaptor proteins for intracellular signalling (392,547). To determine this, it would be necessary to set up

cultures lacking RANKL however publications have shown that RANKL is the primary pro-osteoclastogenic cytokine (547,548), in addition to data shown in figure 3.2d in which very few osteoclasts were generated when GD subject osteoclast cultures did not include RANKL such conditions may not be deemed physiologically relevant.

**The altered sphingolipid profile found in GD patients causes increased osteoclastogenesis.**

Several sphingolipids have been found to modulate osteoclast formation and function. These include ceramide which has been shown to reduce osteoclast activity by inhibiting actin ring formation (375), lactosylceramide which has been shown to increase RANK expression in osteoclasts (376) and sphingosine-1-phosphate (S1P) which has been found to be a chemotactic factor, regulating precursor osteoclast migration between the bone marrow and the blood (378). To investigate the potential roles of sphingolipids in osteoclastogenesis several sphingolipids in the synthesis and degradation pathway of glucosylceramide, shown in figure 1.2, were added to osteoclast medium for the duration of the cultures. Glucosylsphingosine, not shown in figure 1.2, was also added to osteoclast medium. Glucosylsphingosine is not present in figure 1.2 as the source of this sphingolipid, found to be in very high concentrations in GD plasma, remains unclear and is theorised to be formed from the glucosylation of sphingosine and from the deacylation of glucosylceramide (549).

Addition of sphingosine to control osteoclast cultures resulted in a significant decrease in osteoclast generation. Sphingosine has been reported to be a pro-apoptotic lipid for other cell types (550) and therefore may be reducing osteoclastogenesis non-specifically via this mechanism by inducing apoptosis in the osteoclast precursors or mature osteoclasts or in both. In addition, this may be exacerbated as it has also been shown that cells which undergo apoptosis can also release pro-apoptotic signals inducing apoptosis in other cells in the culture (551,552). However, if inhibiting osteoclast generation is a function of sphingosine, shown to be elevated in GD serum, previous results in relation to osteoclast

generation in this chapter would suggest the effect is either minimal in vivo or is negated by the over-abundance of other pro-osteoclastic signalling molecules including other sphingolipids.

In contrast, addition of lactosylceramide to control cultures resulted in a significant increase in osteoclast generation suggesting lactosylceramide may be a pro-osteoclastic factor however reports suggest lactosylceramide concentrations are lower in GD serum than control subject serum (154,553) which therefore may suggest an overall negative effect on osteoclastogenesis. However, publications which measured lactosylceramide in GD serum and GD storage cells are very few. Further analysis of GD serum, bone marrow aspirate, monocytes and osteoclasts may be necessary to clarify its concentration in comparison to the normal population.

Addition of glucosylceramide to control cultures also resulted in a significant increase in osteoclast generation. Again, this result suggests it may be a pro-osteoclastic factor. As it is one of the main storage lipid in GD with levels up to 3 fold higher than controls when measured in GD serum and plasma (155) this effect may be clinically significant as although in this culture system the increase is modest and does not account for the large differences observed in osteoclast generation between control and GD these cells are only exposed to the sphingolipid for 21 days while in vivo the osteoclast precursors, circulating in the blood prior to migrating to the bone marrow, may be exposed to this sphingolipid for much longer.

However, an increase in osteoclast number was not accompanied by an increase in resorption, with the exception of S1P. This may be due to the osteoclasts being less active or it may be due to the limitations of the resorption assay as there was substantial variation both between replicates and patients in this set of experiments. Therefore, a larger cohort is required to determine whether this is a true result or a statistical anomaly.

In comparison, addition of sphingosine to GD osteoclast cultures did not affect osteoclast generation nor did the addition of lactosylceramide. This may suggest

that GD osteoclast precursors respond differently to stimulus. If this is the case the elevated levels of sphingosine in GD serum will not inhibit osteoclastogenesis while the lower concentration of lactosylceramide will not have an effect on osteoclast numbers. It may also be that if GD osteoclast precursors are pre-stimulated the negative and positive effects of sphingosine and lactosylceramide respectively could be negated depending on what stage these sphingolipids affect osteoclast development.

Addition of glucosylceramide further enhanced osteoclastogenesis in GD cultures, increasing osteoclast generation by 37.9 % (figure 3.11f), providing further evidence that glucosylceramide has a pro-osteoclastic function and possibly that it has a strong effect as it can drive osteoclastogenesis in a culture already producing high osteoclast numbers.

However, as before this difference is not observed in regards to activity suggesting glucosylceramide may affect osteoclast numbers but not their activity indicating additional factors may be required.

**Comparison of MM and GD in vitro osteoclast generation.**

A number of studies have shown an increased risk of multiple myeloma (MM) for type 1 GD patients (146,157,241,242,266). Bone disease is common to GD and MM with clinical features of bone disease present in more than 80% of both disorders (101,470). To investigate the characteristics of MM osteoclasts for comparison to GD osteoclasts peripheral blood monocytes and bone marrow osteoclast precursors were cultured in osteoclast medium alone, in the presence of CBE or GD-specific therapies.

Interestingly, culture of MM peripheral blood mononuclear cells with CBE did not result in the increase in osteoclast numbers observed in control cultures suggesting MM monocytes either do not respond to the same factors or are being stimulated through a different pathway.

Osteoclasts obtained from the peripheral blood of MM patients showed reduced generation in the presence of GD-specific therapies however the cohort size was too small for any definite conclusions to be reached. Further patient samples are required to determine whether the finding with CBE is true and whether GD-specific therapies reduce osteoclast generation. If the latter is the case it may suggest the effect of therapies on lipid composition may be critical for influencing osteoclast formation.

Generation of osteoclasts from MM bone marrow led to a markedly higher number of osteoclasts than generated from peripheral blood. This may be as expected as peripheral blood only contains osteoclast precursors while bone marrow contains migrated osteoclast precursors and mature osteoclasts therefore the density of osteoclasts isolated and differentiated is likely to be much higher. It may also be due to the MM as MM patients are known to suffer bone events indicative of increased osteoclast activity.

Osteoclasts isolated from MM bone marrow also exhibit high resorptive activity creating over 4000 pits per disc in some case, completely covering the surface of the bone disc. As noted in previous findings this may simply reflect the increased number of osteoclasts however it may also suggest that MM patient BM derived osteoclasts are hyperactive. Either result supports the evidence that increased bone resorption is responsible for the bone disease in MM patients (474).

**Limitations.**

All of the above findings were generated from in vitro culture which has several limitations compared to in vivo studies. Firstly, the osteoclasts were generated in isolation which does not take into account the potential effects of other cells in the bone marrow such as mesenchymal stem cells, osteoblasts, osteocytes, plasma cells, and T-cells. Secondly, these cells were cultured as a monolayer which does not replicate the three-dimensional environment present in vivo. Thirdly, cell cultures were performed in static medium. This method can lead to a depletion of

nutrients and osteogenic factors and a build-up of waste products between medium changes.

**Summary.**

In summary, GD subject osteoclast cultures generated osteoclasts earlier and in greater numbers than control cultures. These osteoclasts were larger with more nuclei and had increased resorptive activity and may be due to a lack or reduction of signals inhibitory for osteoclast generation. These results were partially recreated by the inhibition of β-glucocerebrosidase using CBE indicating deficient β-glucocerebrosidase activity is directly related to osteoclast development and function. This conclusion was supported by findings that addition of GD-specific therapy reduced osteoclast generation in GD cultures and that glucosylceramide increased osteoclast generation in both control and GD cultures.

# 4 In vitro osteoclast cultures – clinical correlations

## 4.1 Introduction

The findings from chapter 3 that Gaucher patient PBMC's generate greater numbers of osteoclasts compared to controls and that these cells are larger, have more nuclei and are more active suggests that osteoclasts may play an important role in the skeletal disease of GD. Additionally, the reduction of osteoclast generation in the presence of ERT or SRT suggests there is a direct effect by these therapies on osteoclast differentiation and activity as suggested in other recent publications (134,540). This may suggest the substrate, glucosylceramide, is a key factor as data shown in chapter 3 demonstrated addition of exogenous glucosylceramide increases osteoclast generation in vitro therefore reduction of this sphingolipid either by preventing its synthesis by SRT or degrading it by ERT would result in a reduction in osteoclast generation.

However, these findings may be of limited value due to the fact that these are observations of osteoclast generation in isolation and therefore cannot take into account the effects of other cell types such as osteoblasts and osteocytes, present in the bone microenvironment and known to modulate osteoclastogenesis and activity (315). While these interactions can be explored with animal models such as knock out mice, of which there are several for GD (271,554,555) there may still be ambiguity as to how they translate to the human setting due to a number of differences in bone biology, for example in murine models bones have continuous growth versus growth plate closure in humans and different remodelling activities (556).

As the osteoclasts characterised in chapter 3 are primary cells isolated from Gaucher patients this may allow us to test the validity of this data by comparison with human physiology and correlation with the Gaucher patients in this cohort for example in relation to bone disease and therapy. This data may also be useful in testing clinical correlations such as a recent finding that, in the Gaucher population,

anaemia is a risk factor for avascular necrosis (AVN) as anaemic patients were 60% more likely to develop AVN with an adjusted odds ratio of 1.56 (529).

In Gaucher patients, bone disease is generally assessed by dual X-ray absorbiometry (DEXA) scans in conjunction with magnetic resonance imaging (MRI), the latter is also used to assess bone marrow burden (BMB) to assess the degree of Gaucher cell infiltration (557). Both DEXA and MRI are required to assess bone mineral density (BMD) as DEXA alone cannot compensate for local changes caused by AVN or sclerosis resulting in artificially low or high bone densities respectively (123). MRI is regarded as the most sensitive method for the detection of bone marrow infiltration of Gaucher cells in GD and is used to assess the severity of marrow involvement and response to GD specific treatment (558).

Several studies have been conducted to investigate the usefulness of biochemical markers of bone formation including pro-collagen carboxyterminal propeptide (PICP), osteocalcin and bone resorption including cross-linked type I collagen C-terminal telopeptide (CTX) and d-pyridinolines in assessing bone disease in GD. However, there appears to be substantial variability and little consensus in the findings from the different studies. While some studies found reduced bone formation markers and elevated resorption markers, suggesting bone loss due to increased osteoclast activity (512,513) another study found reduced bone formation markers but no change in resorption markers (515) suggesting reduced osteoblast activity. In addition, studies following response to ERT and SRT either found no significant changes in bone formation markers over 4.5 years on ERT (514) or conflicting data in which two studies found significantly increased osteocalcin (508,516) while a third study found a decrease in osteocalcin, although this did not reach statistical significance (509).

As a consequence, bone markers are not generally used to assess bone disease in GD. Investigating the roles of cells such as osteoclasts in the bone microenvironment may shed some light on the reasons for the observed variability in biochemical bone markers and clinical correlation of the osteoclast data may lead to a means of following skeletal response to therapy in conjunction with MRI and DEXA.

## 4.2 Aims and Hypotheses

**Aim:** To understand the relationship between in vitro findings and clinical features of bone disease in GD patients.

**Objectives:**

(1) To validate in vitro osteoclast data by correlation with MRI findings of active bone disease in GD patients.

(2) To investigate whether in vitro osteoclast generation correlates with anaemia in GD patients providing a potential link to anaemia being a risk factor for AVN.

(3) To determine whether in vivo treatment of GD patients with Gaucher-specific therapies affects osteoclast generation in vitro.

**Hypothesis 1:** GD patients with active bone disease generate more osteoclasts in vitro.

**Rationale:** A number of bone diseases such as osteoporosis can be caused by increased osteoclast generation leading to increased bone resorption. The number of nuclei per osteoclast has also been shown to correlate to osteoclast activity.

**Methods:** Correlation of MRI based evidence of active bone disease with contemporaneous in vitro osteoclast generation and size.

**Hypothesis 2:** GD patients with anaemia generate more osteoclasts in vitro.

**Rationale:** A recent publication by Khan et al in 2012 has shown anaemia to be a risk factor for avascular necrosis in GD patients with anaemic patients 60% more likely to develop AVN with an adjusted odds ratio of 1.56.

**Methods:** Correlation of patients with levels of haemoglobin indicative of anaemia with in vitro osteoclast generation.

**Hypothesis 3:** Treatment of GD patients with GD-specific therapies will reduce in vitro osteoclast generation.

**Rationale:** Previous data in this thesis has shown increased osteoclast generation is directly related to a decrease in β-glucocerebrosidase activity and that addition of GD-specific therapy to GD in vitro cultures reduced osteoclast generation. Therefore, GD-specific treatments such as ERT and SRT designed to replace the enzyme or reduce the impact of its decreased activity will probably ameliorate the effect on osteoclastogenesis in vivo.

**Methods:** Compare osteoclast generation of naïve patients with patients receiving therapy. Longitudinal study of individual patients by taking blood samples for osteoclast culture at each patient visit. Osteoclasts will be identified either by being histochemically positive for TRAP or high surface expression of vitronectin receptor in conjunction with actin ring formation labelled with fluorochromes.

## 4.3 Results

### 4.3.1 In vitro osteoclast generation, nucleation and activity correlate with active bone disease and osseous manifestation in Gaucher subjects.

To investigate the clinical relevance of our findings that Gaucher subjects in vitro osteoclast cultures generated greater numbers of osteoclasts which were larger, had more nuclei and higher resorptive activity compared to control subject cultures we correlated several clinical parameters with osteoclast generation in our Gaucher cohort. Firstly, we compared Gaucher subjects classified as having active vs inactive bone disease based on annual MRI assessment performed as part of routine assessment for Gaucher patients. Gaucher subjects with active bone disease had significantly higher osteoclast generation compared to control ($p < 0.0001$) or Gaucher subjects with inactive bone disease ($p = 0.0019$), figure 4.1a. The number of nuclei per osteoclast were also significantly greater in cultures from subjects with active bone disease compared to control ($p = 0.015$) or Gaucher subjects with inactive bone disease ($p = 0.0093$), figure 4.1b. In addition, Gaucher subjects with inactive bone disease did not generate significantly more osteoclasts than control subjects nor did the osteoclasts contain a significantly greater number of nuclei compared to the control cohort.

Correlation of osteoclast generation and activity with osseous manifestations of individual Gaucher subjects showed that, in general, subjects with relatively high osteoclast generation or activity correlated with a higher number and severity of osseous manifestations as shown in figure 4.2. The correlations observed of osteoclast number and activity with active bone disease and osseous manifestations suggest high osteoclast generation and activity in vitro indicate more severe bone disease in vivo.

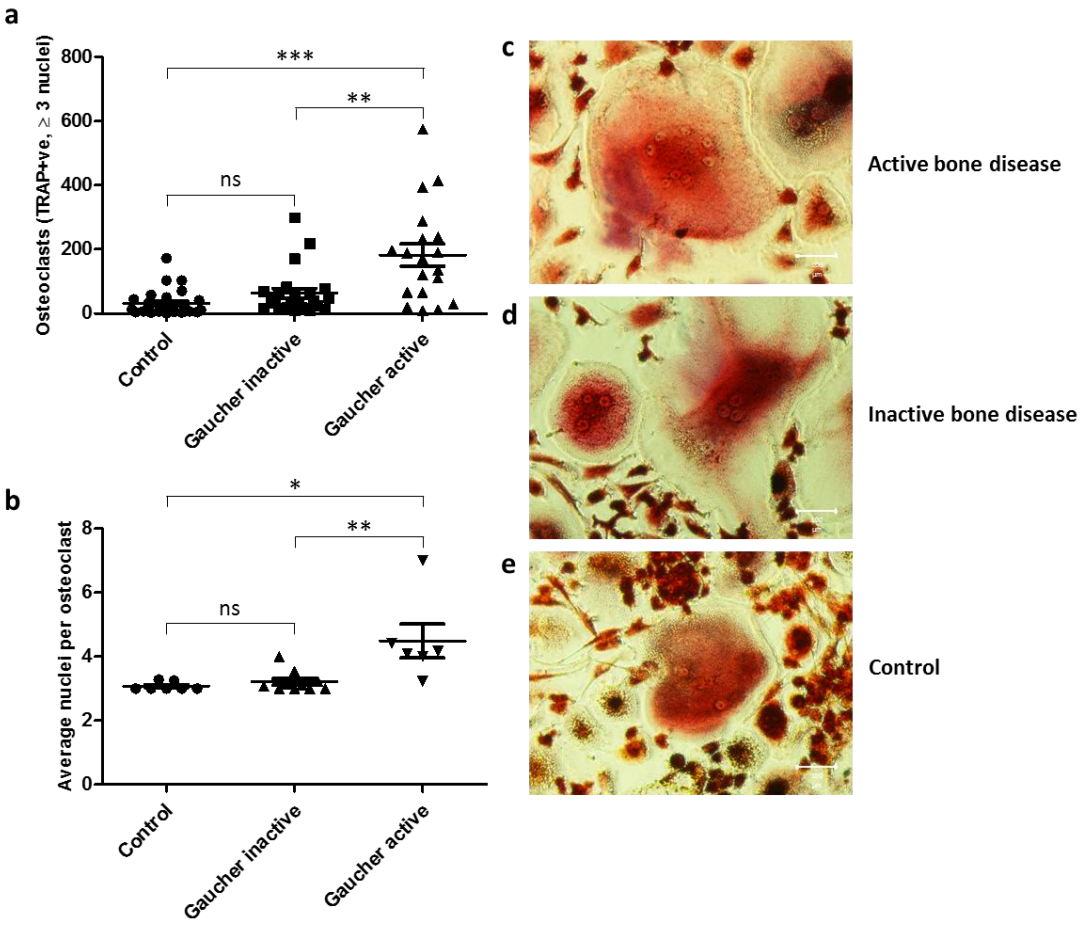



**Figure 4.1** Correlation of active bone disease with osteoclast generation and size. **(a)** Number of osteoclasts generated from control and Gaucher subject adherent mononuclear cells after 21 days of culture on glass. Active and inactive bone disease in Gaucher subjects determined by annual MRI assessment. **(b)** Averaged nuclei per osteoclast of TRAP stained osteoclast cultures, mean ± SEM shown by horizontal lines. **(c, d, e)** Representative TRAP staining of Gaucher and control subject derived osteoclasts cultured for 21 days on glass. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, unpaired t-tests.

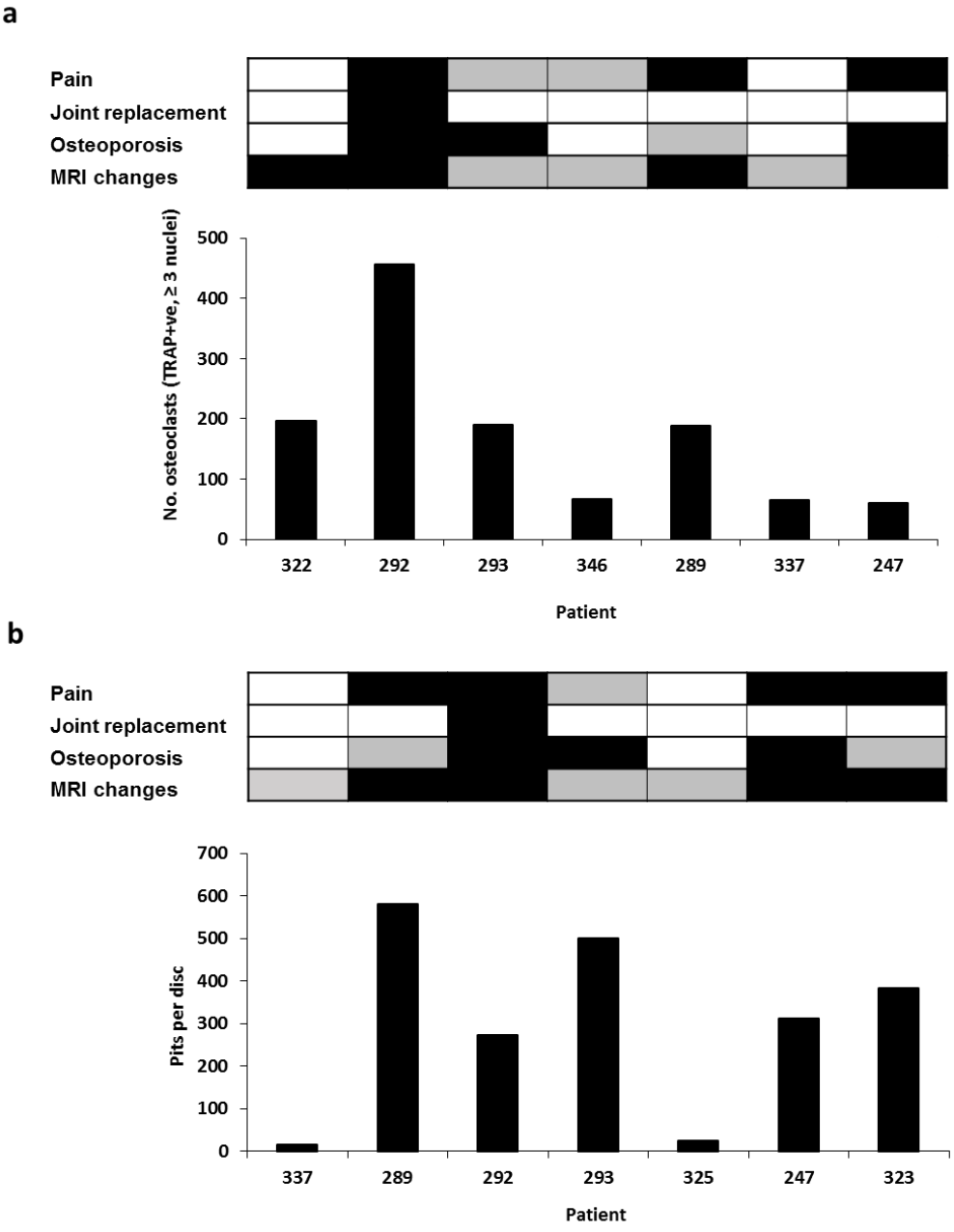



**Figure 4.2** Correlation of osseous manifestations with osteoclast generation and activity. **(a)** Number of osteoclasts generated from adherent mononuclear cells of individual Gaucher subjects after 21 days of culture on glass correlated with clinical markers of bone disease. **(b)** Bone pits resorbed by osteoclasts of individual Gaucher subjects after 21 days of culture on bone correlated with clinical markers of bone disease. Clinical parameters are coded DEXA scanning (white: normal bone density, grey: osteopenia, black: osteoporosis); skeletal MRI (white: no evidence of bone infiltration, grey: mild patchy infiltration, black: severe, diffuse infiltration); joint replacement (white: no joints replaced, grey: 1 joint replaced, black: joint revision or multiple replacements) and pain (white: no pain, grey: mild intermittent pain, black: severe or chronic pain).

### 4.3.2 In vitro osteoclast generation correlates with bone pain and anaemia in Gaucher subjects.

In addition to quantification of bone disease, data obtained from the ICGG Gaucher registry was also used to assess which Gaucher subjects were experiencing bone pain and whether this also correlated with increased osteoclast generation. This was shown to be the case in this cohort as subjects reporting bone pain had significantly higher osteoclast numbers ($p = 0.014$, figure 4.3a) compared to those without bone pain.

Anaemia was recently reported as being associated with an increased risk for avascular necrosis in type 1 Gaucher patients (531). To evaluate this in the Gaucher cohort, subjects were categorised as being anaemic if a male had haemoglobin of less than 130g/l or a female had haemoglobin of less than 115g/l and osteoclast generation was compared between the anaemic vs non-anaemic groups. Osteoclast generation in the anaemic cohort was significantly higher ($p = 0.0051$, figure 4.3b) in comparison to the non-anaemic cohort. Clinical data for bone pain and anaemia collected by Yehudit Bauerfreund and Niamh Cunningham.

Chitotriosidase activity is commonly used as a surrogate marker to follow overall disease progression in Gaucher subjects. To determine whether chitotriosidase activity measured as part of routine assessment for Gaucher patients in the Royal Free LSDU correlated with osteoclast generation, data for both parameters obtained from the same patient visit were plotted. Linear regression analysis showed no correlation between chitotriosidase activity and osteoclast number ($r = 0.15$, figure 4.3c).

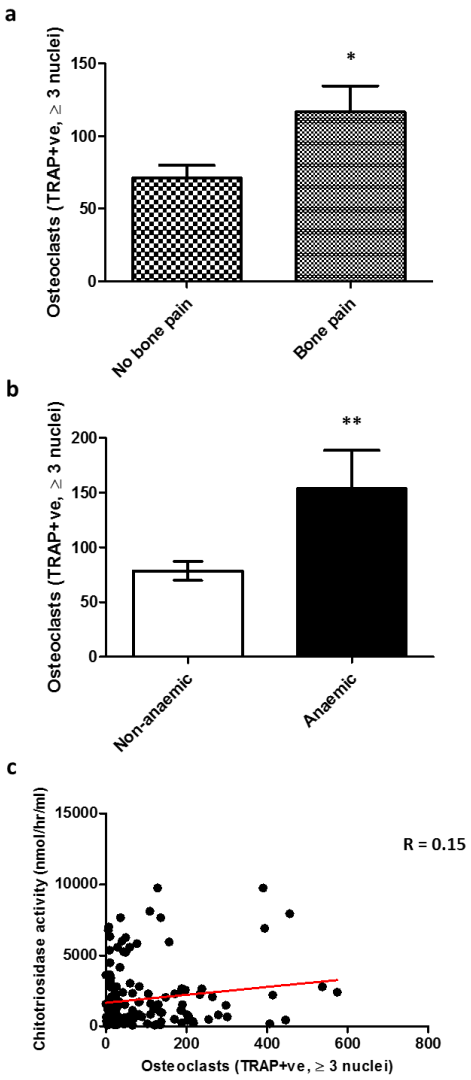



**Figure 4.3** Correlation of osteoclast generation with clinical parameters and a surrogate marker of disease. **(a, b, c)** Number of osteoclasts generated from adherent mononuclear cells after 21 days of culture on glass. **(a)** Correlation of osteoclast generation with bone pain. No bone pain n = 92, with bone pain n = 62, mean ± SEM plotted. **(b)** Correlation of osteoclast generation with anaemia, defined as haemoglobin < 13g/dl for males and < 11.5g/dl for females. Non-anaemic n = 135, anaemic n = 20, mean ± SEM plotted. **(c)** Correlation of osteoclast generation with chitotriosidase activity determined at the same patient visit, n = 155, correlation not significant. *p ≤ 0.05, **p ≤ 0.01, unpaired t-tests. Clinical data for bone pain and anaemia collected by Yehudit Bauerfreund and Niamh Cunningham.

### 4.3.3 In vivo Gaucher specific therapies appear to reduce the number of osteoclasts generated from Gaucher subjects in vitro.

An alternative method of osteoclast identification to TRAP staining was used. This involved immunofluorescent labelling of vitronectin receptor (CD51/61), highly expressed on the osteoclast surface, with fluorescent co-labelling of internal f-actin ring structures unique to osteoclasts with phalloidin. An example of this method is shown in figure 4.4a in which vitronectin was labelled green and f-actin was labelled red. The osteoclast number is assessed by counting the number of double positive cells in the observable field at x400 magnification with a minimum of 10 fields chosen at random. To determine whether Gaucher specific therapies correlated with lower osteoclast numbers in vivo as well as in vitro, subjects were divided into three groups, control, Gaucher subjects who did not receive Gaucher specific therapies and Gaucher subjects who did receive Gaucher specific therapies. The number of osteoclasts generated from in vitro osteoclast cultures of these subjects mononuclear cells were enumerated by fluorescent labelling and correlated with these groups. Both treated and untreated Gaucher subject groups had significantly higher osteoclast numbers when compared to control subjects (treated $p = 0.044$, untreated $p = 0.0005$, figure 4.4b). The untreated Gaucher group also had higher osteoclast numbers than the treated group however this did not reach statistical significance ($p = 0.054$).

To further assess the effect of in vivo therapy on osteoclast generation in vitro, osteoclast cultures were performed for 5 Gaucher subjects on three sequential visits for each subject. Plotting osteoclast numbers for the sequential visits showed that for subjects with relatively high osteoclast numbers at the first visit, labelled Pt 1, Pt 4, Pt 5 in figure 4.4c, treatment with Gaucher specific therapies led to a marked reduction in osteoclast numbers and that those with relatively low osteoclast at the first visit labelled pt 2, pt 3, remained low.

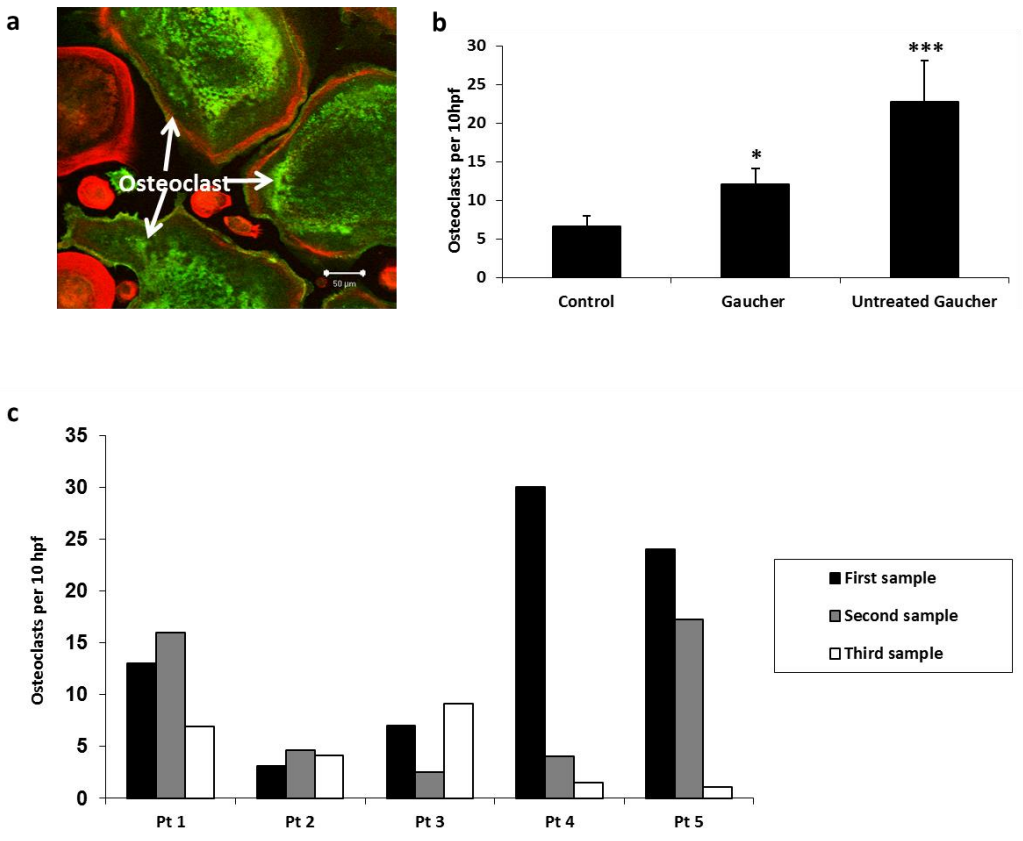



**Figure 4.4** In vivo treatment effect on in vitro osteoclast generation. **(a)** Representative image of immunofluorescent staining of vitronectin receptor (green) highly expressed in osteoclasts and actin rings (red), structures primarily found in osteoclasts, marked with white arrows. **(b, c)** Number of osteoclasts generated from adherent mononuclear cells after 21 days of culture on glass, identified by immunofluorescent staining. **(b)** Correlation of osteoclast generation with in vivo therapy, control n = 16, treated Gaucher subjects n = 20, untreated Gaucher subjects n = 4. Mean ± SEM plotted. **(c)** Number of osteoclasts generated from adherent mononuclear cells of 5 individual Gaucher patients over 3 time points each cultured on glass coverslips for 21 days and identified by immunofluorescent staining. *$p \leq 0.05$, *** $p \leq 0.001$, unpaired t-tests.

## 4.4 Discussion.

Bone disease in GD patients is generally assessed by review of BMD by DEXA in conjunction with analysis of bone marrow infiltration and secondary bone events by MRI (123) however these techniques only provide a snapshot of disease progression and cannot provide information on when bone dysregulation begins or whether a patient is undergoing a period of bone loss. To address this issue several studies have been carried out to assess the efficacy of markers of bone turnover (508,512–519). However, due to conflicting data between studies and the fact that few studies have measured the same analytes in their respective patient cohorts or were consistent in how samples were taken and stored, markers of bone turnover are not routinely used to assess bone disease in GD. To investigate whether our in vitro osteoclast assay could be used as an early indicator of active bone disease and whether it reflects in vivo response to treatment data from in vitro osteoclast cultures have been correlated with clinical features of bone disease and time courses of in vitro osteoclast generation from patients receiving GD-specific therapy.

The results discussed below demonstrate for the first time that GD patient derived osteoclasts correlate with several clinical features including evidence of active bone disease and may indicate a direct causative link between increased osteoclast generation and bone disease in GD patients and provide a possible method for tracking bone disease in GD patients.

**GD patients with active bone disease generate more osteoclasts in vitro.**

GD patients with active bone disease assessed by MRI generated significantly more osteoclasts than the GD cohort with inactive disease and control cohort. This finding suggests the degree of osteoclast generation is indicative of active bone disease in GD patients. However, there is overlap in osteoclast number between the groups. This overlap could occur for several possible reasons (a) the sample could have been taken after the recorded bone event as it is very difficult to know

precisely when the event began and therefore the sample would potentially have lower osteoclast numbers as osteoclast numbers may only have been elevated during the event. (b) Those in the inactive group with higher osteoclast numbers may be entering a bone event or may have ongoing but sub-clinical osteopenia which may not be as yet detectable by MRI. These limitations highlight the need for time courses for individual patients as in regards to these patients we do not have a base line to indicate whether the osteoclast number is abnormally high or low.

Similar results were obtained for the average number of nuclei per osteoclast with the average numbers higher for GD patients with active bone disease than patients with inactive bone disease and the control cohort, suggesting osteoclasts are more active in patients with active bone disease. If this translates to the bone marrow having a higher number of more active osteoclasts this may account for a significant proportion of the bone events observed in GD and provide a direct causative link.

GD patients without active bone disease were not significantly different to the control cohort in either number of osteoclasts generated or number of nuclei per osteoclast. Taken together this data suggests there may be a subset of GD patients which have uncoupling of osteoclasts and osteoblasts while still receiving ERT supporting clinical observations in certain studies which found some GD patients continue to suffer bone events after receiving ERT and in some cases after increasing doses of ERT.

Comparison of individual patient clinical features with in vitro osteoclast generation and activity showed that in the majority of cases higher osteoclast numbers and activity correlated with higher number and severity of osseous manifestations, supporting the hypothesis that higher osteoclast numbers and activity may be a major factor in contributing to the bone disease in GD patients. This finding may also demonstrate that this assay can be patient specific and possibly be used to follow the progress of a patient and indicate if a bone event is ongoing. However, it also suggests that both osteoclast number and activity need to be assessed as in the case of patient 247 assays generated conflicting data with relatively low osteoclast numbers but relatively high resorption. This may be due to assay conditions being less favourable in the enumeration assay as they are cultured in different sized

wells and on a different material i.e. glass rather than bone. As glass is not the natural surface for osteoclast generation it may affect generation. However, as this is not seen for other patients this may be an anomalous result suggesting the assay for this patient for both number and activity needs to be repeated to determine which result if any is anomalous.

Correlation of bone pain recorded in the International Collaborative Gaucher Group (ICGG) Gaucher Registry with osteoclast generation further supports the theory that in vitro osteoclast generation is indicative of bone disease in GD patients. As patients are routinely assessed by MRI if the patient reports bone pain a concurrent osteoclast assay may be useful to support the need for anti-resorptive agents as MRI may not be able to distinguish new bone degradation if the degradation is at an early stage and developing at a site with previous disease. Also, this approach may provide additional data to validate the use of the osteoclast assays for indicating active bone disease and its possible use as an early indicator/marker of a bone event. However, limitations of this approach should be noted during data analysis as the data provided is subjective and may not be sufficiently time specific i.e. patients may not accurately report when the pain was experienced and therefore may not provide a robust correlation with osteoclast number and activity.

**GD patients with anaemia generate more osteoclasts in vitro.**

Correlation of osteoclast generation with anaemia was also found. This may be relevant as a recent finding that, in the Gaucher population, anaemia is a risk factor for avascular necrosis (AVN) as anaemic patients were 60% more likely to develop AVN with an adjusted odds ratio of 1.56 (529). As this study found similar clinical and surrogate markers of disease severity between the groups of patients with and without AVN it is unlikely that this correlation is a reflection of patient severity. AVN is caused by reduction in blood flow to the bone leading to cellular necrosis and localised bone loss however the atraumatic causes are poorly understood (559). Anaemia may also be a consequence of reduced blood supply to the bone as this would have a negative impact on the bone marrow which may affect HSC

number and differentiation. A recent publication using iPSC's as a model of GD showed abnormal hematopoietic progenitor cell (HPC) differentiation in favour of myeloid lineage cells over erythroid lineage cells (140) which may lead to a reduction in red cell production and an increase in monocyte/osteoclast precursor production which, between the two scenarios may in part provide a link between anaemia and AVN.

No correlation was found between chitotriosidase activity and osteoclast number. Chitotriosidase is made by GD macrophages and so is an indirect biomarker and is used as an indicator of general disease burden. Chitotriosidase is used to follow GD patient response to therapy over time as it decreases over time when patients receive GD-specific therapy. While in the normal population its activity range is quite narrow in the GD population its range is very broad with reported ranges of 4–76 nmol/ml/h in controls and 3122–65,349 nmol/ml/h in GD patients (560) therefore a single measurement of chitotriosidase activity is unlikely to correlate with osteoclast number as to assess if the chitotriosidase activity is high or low depends on the previous and naïve activities of the GD patient. This data suggests chitotriosidase cannot be used to indicate bone disease at a single time point and that osteoclast numbers do not reflect overall disease burden but are specific for bone disease at a single time point. To investigate a temporal correlation, it would be necessary to perform a time course of chitotriosidase activity with contemporaneous osteoclast number and activity to determine if osteoclast number also correlates with general disease burden over time.

**Treatment of GD patients with GD-specific therapies will reduce in vitro osteoclast generation.**

Untreated GD patients generate significantly more osteoclasts than treated patients suggesting in vivo therapy is effective for the majority of patients at reducing osteoclast generation and follows clinical observations of several studies which demonstrate that bone events are reduced in the majority of GD patients (506–510). This may be expected as although the plasma half-life of imiglucerase

reportedly ranges from 3.6 to 10.4 minutes its reported half-life in GD patient bone marrow is 14.1 hours (618). Bone marrow monocytes and osteoclast precursor migrate to and from the bone marrow and peripheral blood, in part mediated by S1P concentration gradient (584) and therefore may receive sufficient enzyme to ameliorate the inherent deficiency in β-glucocerebrosidase activity. However, this does not exclude the possibility of the existence of a non-responsive subgroup, to determine this a larger cohort would be required.

Time courses of GD patients show either a decrease or maintenance of osteoclast generation when receiving therapy. This data demonstrates that osteoclast generation in this assay is responsive to changes of in vivo conditions i.e. active bone disease, response to therapy. This responsiveness suggests the osteoclast assay may be useful for following response to therapy and that therapy may be effective in these patients at treating bone disease. However, previous data suggests the existence of a non-responsive subgroup the possibility of which this data does not exclude. To confirm the existence of this subgroup it would be necessary to follow more patients over longer time periods of time. To fully explore the uses of this assay it would be necessary to assess patients who have been found to be refractory in regards to bone disease by clinical parameters to see if therapy is having no effect at the osteoclast level and to follow a relatively young but substantial cohort for several years to determine if a resistant subgroup emerges and whether this could be predicted by the osteoclast assay.

**Limitations.**

In addition to the limitations stated within the above discussion it should be noted that all of the above correlations were with retrospective clinical data which resulted in incomplete data sets for some patients, limiting the size of cohorts for some of the correlations.

**Summary.**

In summary, osteoclast numbers generated from GD cultures and their resorptive activity correlated with active bone disease, as did the number of nuclei per osteoclast. In addition, osteoclast number correlated with bone pain reported by questionnaire and anaemia. Patients not receiving GD-specific therapy generated significantly more osteoclasts in vitro and those receiving therapy generated fewer osteoclasts when cultures were repeated over a period of up to 21 months.

# 5. In vitro osteoblast culture

## 5.1 Introduction

Osteoclasts have been shown to be regulated by an ever increasing number of local and systemic mechanisms ranging from local signalling by osteocytes (306) to signalling by the immune (561,562) and neuropathic systems (563,564). However, the main paradigm for bone homeostasis remains the interaction and signalling between osteoclasts and osteoblasts, commonly referred to as coupling (289,436). Such a close relationship would suggest that changes in osteoclast generation, size and activity may have a direct effect on osteoblast number and activity but it may be the case that osteoblasts are also abnormal in GD due to factors other than osteoclast abnormalities. Murine models of GD have suggested that the bone disease may be caused by reduced osteoblastic activity (271,541). Other publications have shown that mesenchymal stem cells (MSC's) have a decreased capacity to differentiate into osteoblasts when isolated from type 1 Gaucher patients and impaired proliferation with altered secretion of soluble factors when normal MSC were inhibited with CBE (138,524). These findings suggest there may be an imbalance between osteoclasts and osteoblasts in GD both in terms of numbers and activity possibly caused by a reduced source of MSC leading to a reduction in overall osteoblast numbers and abnormal secretion profiles of mature osteoblasts causing increased osteoclast activity resulting in the uncoupling of the osteoclast-osteoblast homeostatic mechanism.

Unlike for in vitro osteoclast culture, isolation of osteoblasts from peripheral blood has remained problematic due to the relative low percentage of osteoblast precursors in PBMC's, estimated to be 1 to 2% (565). As a result, several techniques were developed to isolate MSC from peripheral blood however MSC are a similarly rare population in PBMC's and so these techniques either required large quantities of blood or the subject required a course of GCSF injections to mobilise the MSC (566). Other sources of MSC include adipose tissue (567) and bone marrow (568) of which the latter was chosen for this project. However, due to the invasive nature of

obtaining bone marrow or adipose tissue the use of human osteoblast cell lines is common.

In regards to osteoblast cell lines only two human cell lines capable of producing calcium deposits are described, namely MG-63 (regarded as an osteoblast pre-cursor) and SaOs-2 (regarded as osteoblast-like cells) both originally isolated from osteosarcomas (569). As with most cell lines neither the MG-63 nor SaOs-2 fully recapitulate the characteristics of primary human osteoblasts. MG-63 show poor mineralisation, hence regarded as pre-osteoblastic, and heterogeneity, while SaOs-2 appear to over express alkaline phosphatase over time (570). In addition, both cell lines have a much higher proliferative rate than primary human osteoblasts. However, SaOs-2 have been shown to have many of the expression markers of primary osteoblasts and to form mineral-matrix readily (569) making them a potentially useful model for investigating the possible effects of CBE, GD specific therapies and sphingolipids on the rate of mineralisation when assessed in parallel to primary MSC-derived osteoblast cultures.

## 5.2 Aims and Hypotheses

**Aim:** To understand the role of osteoblasts in GD bone disease.

**Objectives:**

(1) To differentiate control, GD and MM MSC's into functional osteoblasts.

(2) To characterise GD osteoblast activity in comparison to control and MM osteoblasts.

(3) To determine the effects of GD-specific therapies on osteoblast activity in both primary cells and a cell line.

(4) To determine the effects of exogenous sphingolipids on osteoblast activity in both primary cells and a cell line.

**Hypothesis 1:** There is dysregulation between osteoblasts and osteoclasts, commonly referred to as uncoupling, in GD patients.

**Rationale:** Osteoblasts and osteoclasts are normally very tightly regulated primarily by each other. In GD increased osteoclast generation and activity may result in an imbalance between osteoclast and osteoblast number and function leading to bone loss.

**Methods:** Flow cytometry using markers identifying osteoblast precursor percentage in peripheral blood samples is correlated with the number of osteoclasts generated by contemporaneous in vitro osteoclast cultures.

**Hypothesis 2:** Osteoblast mineralisation is reduced when β-glucocerebrosidase activity is inhibited.

**Rationale:** Studies of a GD murine model by Mistry et al (272) showed reduced proliferation of bone marrow stromal cells and reduced osteoblast differentiation and two Zebra fish GD models developed by Zancan et al (523) demonstrated loss

of Gba1 function was associated with impaired canonical Wnt signalling, osteoblast differentiation and reduced bone mineralisation.

**Methods:** Primary and cell line osteoblasts cultured with CBE under mineralising condition. Mineralisation quantified by alizarin red staining and solubilisation.

**Hypothesis 3:** Osteoblast mineralisation is reduced in the presence of exogenous glucosylceramide.

**Rationale:** Previous data in this thesis has shown exogenous glucosylceramide directly affects osteoclast generation by significantly increasing osteoclast numbers in both control and Gaucher cultures indicating that this is a bioactive sphingolipid and therefore may also affect osteoblast function.

**Methods:** Primary and cell line osteoblasts cultured with exogenous glucosylceramide under mineralising condition. Mineralisation quantified by alizarin red staining and solubilisation.

**Hypothesis 4:** Osteoblast function is not directly impacted by GD-specific therapies.

**Rationale:** Previous data in this thesis has shown GD-specific therapies reduce osteoclast generation in vitro both directly and when given to patients. However, a subset of patients have been reported to continue to suffer bone events while receiving GD-specific therapy suggesting a negative impact on osteoblast function is negating the reduction in osteoclast numbers. This may be via the indirect impact of reduced osteoclast activity having the overall effect of reducing bone matrix resorption which in turn means a reduction in osteoblast signalling factors released from the bone matrix.

**Methods:** Primary and cell line osteoblasts cultured with GD-specific therapies in the absence or presence of CBE under mineralising conditions. Mineralisation quantified by alizarin red staining and solubilisation.

# 5.3 Results

## 5.3.1 Uncoupling between Gaucher subject osteoblasts and osteoclasts.

While bone remodelling is influenced by a number of factors both locally and systemically a major factor controlling the rate of bone resorption and formation are the interactions, commonly referred to as coupling, between osteoclasts and osteoblast (289,436). To investigate a possible imbalance between osteoblasts and osteoclasts the numbers of osteoclasts differentiated from peripheral blood mononuclear cells and the percentage of osteoblast precursors in the mononuclear cell fraction isolated from peripheral blood were evaluated. The overall percentage of peripheral blood osteoblast precursors, identified as CD15-ve, osteocalcin+ve, did not significantly differ between control and Gaucher cohorts (figure 5.1a). However, when osteoclast numbers were plotted against osteoblast precursor percentage for each subject the control cohort showed a strong correlation ($r = 0.6$, figure 5.1b, $p = 0.022$) whereas only a very weak correlation was observed in the Gaucher cohort ($r = 0.06$, figure 5.1c, p = not significant) suggesting Gaucher subject osteoblasts and osteoclasts may be uncoupled.

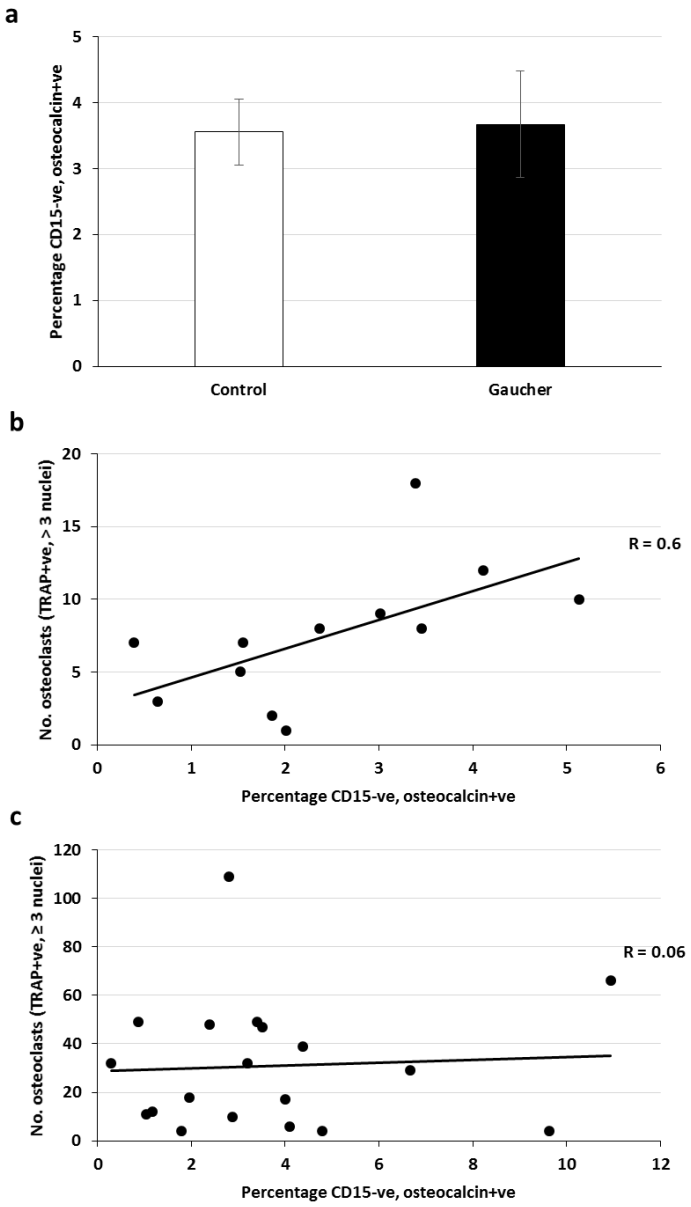



**Figure 5.1** Evidence for uncoupling between osteoclasts and osteoblasts in Gaucher subjects. **(a)** Percentage of osteoblast precursors, CD15-ve osteocalcin+ve, in mononuclear cells isolated from the peripheral blood of control and Gaucher subjects. **(b)** Control osteoblast precursors, CD15-ve osteocalcin+ve, plotted against the number of osteoclasts generated by the same patient in vitro. **(c)** Gaucher osteoblast precursors, CD15-ve osteocalcin+ve, plotted against the number of osteoclasts generated by the same patient in vitro.

### 5.3.2 Confirmation of mesenchymal stem cell markers.

BM-derived MSCs were isolated from freshly obtained healthy BM donor and MM patient samples as detailed in Chapter 2: Methods. To ensure the isolated cells met the criteria specified by the International society for cellular therapy (ISCT) for confirmation of the MSC's phenotype, after expansion MSC's underwent cellular characterization to confirm successful isolation and purity. All MSCs were shown to be adherent to tissue culture plastic under standard growth conditions. MSC samples were successfully differentiated into adipocytes (by Dr Brendan Beaton) and osteoblasts as shown in figure 5.2. All samples were phenotyped with a panel of markers specified by the ISCT using flow cytometry. An example of the histograms obtained are shown on the next page in figure 5.3. It should be noted that due to autofluorescence at the emission wavelengths of FITC and PERCP Cy5.5 a relatively high amount of fluorescence for isotype control was obtained, confirmed as autofluorescence by comparing the fluorescence of unstained cells to isotype control. Flow cytometry staining and analysis was carried out by Dr Brendan Beaton. Image of adipocytes in figure 5.2 and histograms shown in figure 5.3. provided by Dr Brendan Beaton.

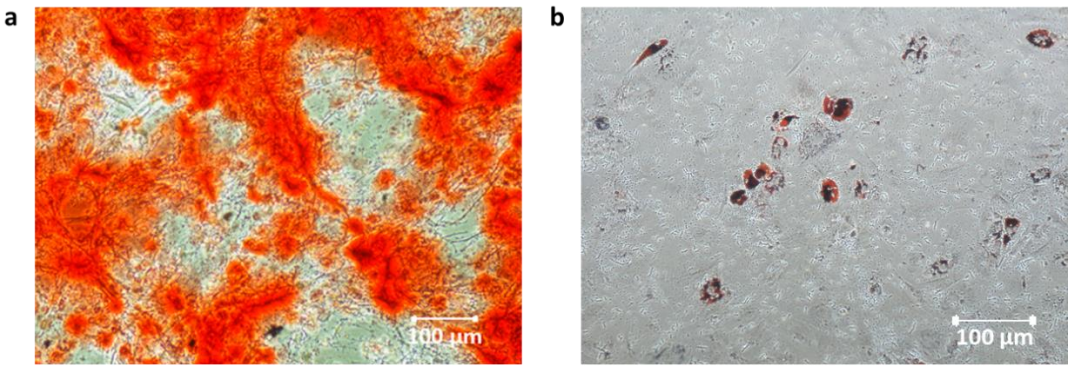



**Figure 5.2** MSC differentiation. Representative photographs demonstrating osteoblast and adipocyte differentiation of bone marrow MSC's, sample 2186. **(a)** Calcium deposition by osteoblasts confirmed by alizarin red staining. **(b)** Lipid storage in adipocytes confirmed by Oil red O staining.

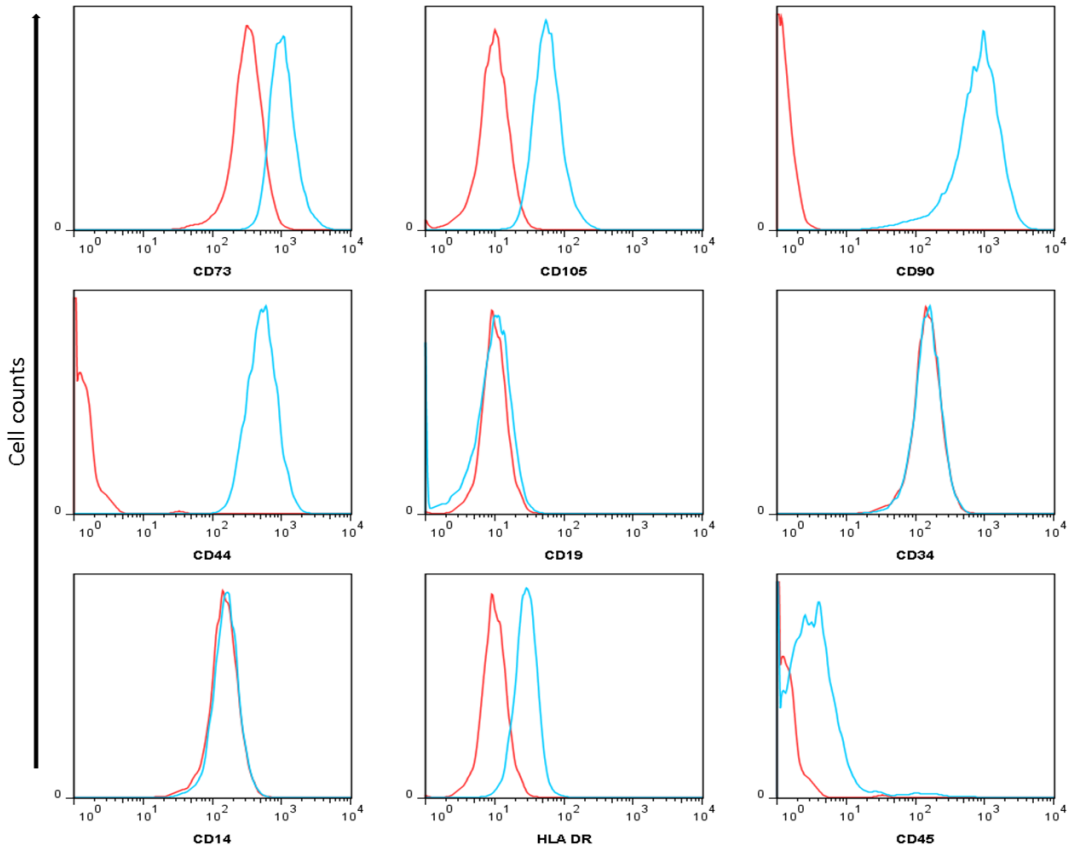



**Figure 5.3** Phenotyping of MSC cells isolated and expanded from bone marrow samples. Expression of markers established to identify MSC. Typical flow cytometry histograms demonstrating cells to be positive for CD73, CD106, CD90, CD44 and negative for CD19, CD34, CD14, CD45. Weakly positive for HLA-DR indicating MSC activation.

### 5.3.3 Differentiation of mesenchymal stem cells into functional osteoblasts.

Several publications have shown that osteoblasts may play a direct role in contributing to bone disease in Gaucher patients (138,272,523,541). To assess osteoblast function in GD a primary osteoblast cell culture model was optimised. Mesenchymal stem cells isolated from subjects bone marrows were cultured for 35 days in alpha minimal essential medium supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES, 100 units/ml penicillin, 0.1mg/ml streptomycin and a range of concentrations of dexamethasone, β-glycerophosphate and ascorbate-2-phosphate to induce differentiation of the mesenchymal stem cells into functional osteoblasts. Mature osteoblasts were defined by their ability to deposit calcium. Alizarin red, a chemical which binds monomerically to calcium, was used to visualise the calcium deposition, staining it red (figures 5.4a, b). This stain was subsequently solubilised and quantified by absorbance spectrometry. In order to permit the detection of increased or decreased calcium deposition due to modifications in culture conditions an intermediate degree of calcium deposition was selected, as indicated by a black arrow in figure 5.4c and a representative image, figure 5.4b (optimised medium referred to as A10-OB). To assess optimal culture duration control subject mesenchymal stem cells were cultured in optimal calcium deposition conditions for a total of 49 days. At 7-day time points 3 wells of a 24 well plate were fixed in 4% paraformaldehyde and stained for calcium with alizarin red, solubilised and quantified by absorbance spectrometry. Under these conditions little calcium deposition was observed for the first 28 days. However, the rate of calcium deposition markedly increased between days 28 and 49. An optimal degree of calcium deposition was determined to be at day 35.

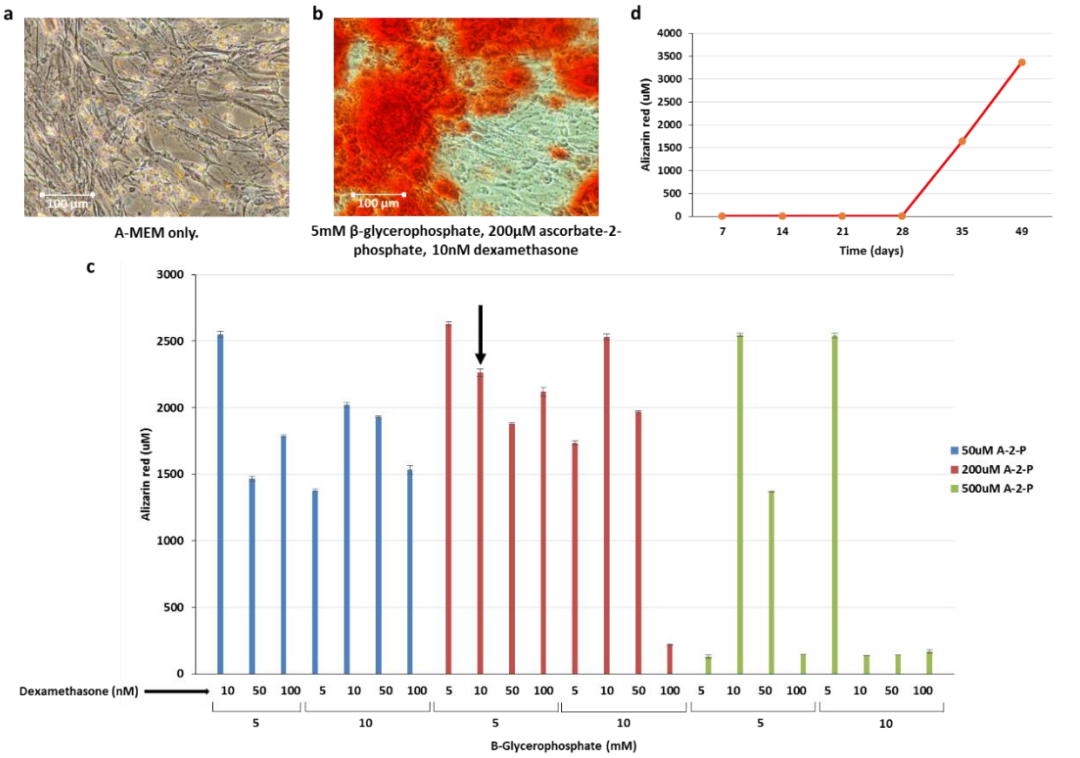



**Figure 5.4** Optimisation of conditions required to differentiate control MSC into osteoblasts and to induce calcium deposition. **(a, b)** Representative images of control MSC cultured **(a)** without and **(b)** with optimised osteogenic conditions for 35 days. **(c)** Control MSC cultured with a range of conditions for 35 days in triplicate, mean ± SEM plotted. Selected conditions for subsequent cultures indicated by red arrow. **(d)** Time course of calcium deposition by control MSC cultured with optimised osteogenic conditions. Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. Separate plates of MSC monolayers were cultured for each time point, 3 wells per time point. Black arrow indicates selected osteogenic conditions for subsequent osteoblast cultures. Mean ± SEM plotted.

### 5.3.4 MSC's as a model for Gaucher disease.

To determine whether the MSC-osteoblast cultures could be used as a model for Gaucher disease, control MSC were cultured in optimised osteogenic medium A10-OB in the presence of β-glucocerebrosidase inhibitors CBE (100µM) or isofagomine (100µM) for 35 days, assessed for degree of β-glucocerebrosidase inhibition after 4 days (figure 5.5a) and for viability at weekly time points for the duration of the culture (figure 5.5b). In addition, the effect of Gaucher specific therapies imiglucerase (1 unit/ml) and miglustat (50µM) on viability was also assessed at weekly time points for the duration of the culture (figure 5.5c). β-glucocerebrosidase activity was reduced to 8.3% of standard in the presence of CBE ($p = 0.005$) and 9.9% of standard in the presence of isofagomine ($p = 0.0006$). No statistically significant change to viability was observed in the presence of either inhibitor over the 3 week period.

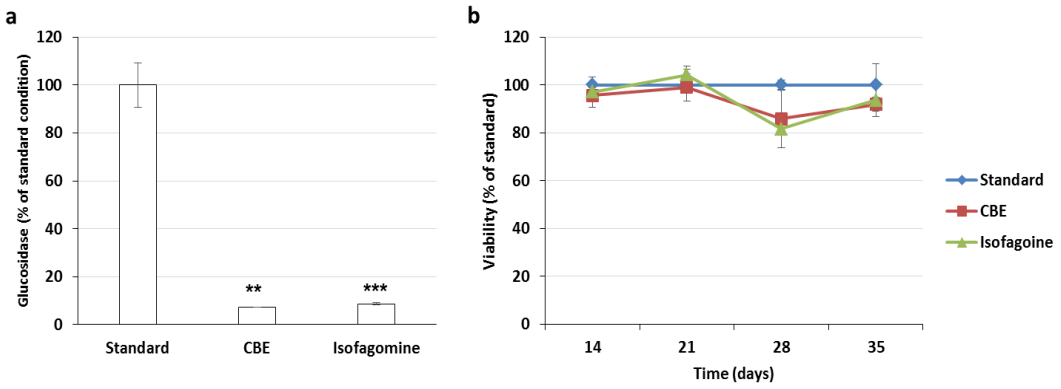



**Figure 5.5** Assessment of control MSC, sample DM001, differentiated into osteoblasts in A10-OB as a model of Gaucher disease. **(a)** Inhibition of β-glucocerebrosidase activity by addition of an irreversible inhibitor CBE (100µM) or reversible inhibitor isofagomine (100µM) cultured for 4 days in triplicate per condition. Activity in cell lysates measured by fluorometric assay. **(b)** Viability of control MSC when cultured for 35 days in optimised osteogenic conditions with CBE (100 µM) or isofagomine (100 µM) in triplicate per condition. Viability measured by MTS assay, 100% calculated from the averaged absorbance values obtained from the standard condition. Mean ± SEM plotted. Unpaired t-tests. ** $p \leq 0.01$, *** $p \leq 0.001$.

### 5.3.5 MSC viability was reduced by Gaucher specific therapies.

To assess the potential impact of inhibitors, therapy and sphingolipids on MSC viability when added exogenously to osteoblast cultures MTS assays were performed after 7 days of culture, the time point at which medium is normally replaced. In general, the addition of inhibitors CBE and isofagomine did not affect MSC viability. Statistically significant decreases were observed for control subject DM001 for both CBE ($p = 0.02$) and isofagomine ($p = 0.009$) and for control subject 2186 with isofagomine ($p = 0.04$). The addition of GD specific therapies to cultures led to a statistically significant decrease in viability for control and MM subject MSC's with the exception of MM subject 2167 for imiglucerase and miglustat, (table 5.1).

In comparison, the addition of exogenous sphingolipids to osteoblast cultures did not reduce MSC viability with the exception of glucosylsphingosine which significantly reduced viability in control subject DM001 ($p = 0.006$) and MM subjects 2036 ($p = 0.0001$), 2314 ($p = 0.023$). Culture with lactosylceramide or glucosylceramide resulted in a generally higher viability for all subjects and was statistically significantly higher for MM subject 2167 MSC's (lactosylceramide $p = 0.024$, glucosylceramide $p = 0.007$) as shown in table 5.2.

| | CBE | | Isofagomine | | Imiglucerase | | Miglustat | | Ambroxol | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample | % | p | % | p | % | p | % | p | % | p |
| DM001 | 96 | * | 96 | ** | 87 | *** | 87 | *** | 81 | *** |
| 2186 | 104 | NS | 93 | * | 88 | ** | 78 | *** | 82 | *** |
| 2036 | 95 | NS | 100 | NS | 94 | NS | 94 | NS | 90 | ** |
| 2167 | 100 | NS | 93 | NS | 87 | *** | 83 | *** | 85 | *** |
| 2314 | 104 | NS | 98 | NS | 92 | * | 88 | * | 94 | NS |

**Table 5.1** Viability of MSC's when cultured in A10-OB with β-glucocerebrosidase inhibitors CBE (100µM), isofagomine tartrate (100µM) or GD specific therapies imiglucerase (1 unit/ml), miglustat (50µM), ambroxol hydrochloride (20µM) for 7 days. Viability determined by MTS assay, percentage viability calculated as percentage of standard condition i.e. MSC's in osteoblast differentiation medium alone. 9 replicates per condition. Control samples highlighted in blue, MM samples in red. Unpaired t-tests. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. NS = No statistically significant difference. All p values are in comparison to standard condition i.e. A10-OB only.

| | Sphingosine | | Sphingosine-1-phosphate | | Glucosyl sphingosine | | Ceramide | | Lactosyl ceramide | | Glucosyl ceramide | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | % | p | % | p | % | p | % | p | % | p | % | p |
| DM001 | 98 | NS | 101 | NS | 90 | ** | 96 | NS | 97 | NS | 103 | NS |
| 2186 | 101 | NS | 100 | NS | 95 | NS | 93 | NS | 107 | NS | 108 | NS |
| 2036 | 94 | NS | 95 | NS | 79 | *** | 99 | NS | 100 | NS | 105 | NS |
| 2167 | 99 | NS | 101 | NS | 94 | NS | 109 | NS | 111 | * | 114 | ** |
| 2314 | 95 | NS | 98 | NS | 87 | * | 102 | NS | 103 | NS | 103 | NS |

**Table 5.2** Viability of MSCs when cultured in A10-OB with sphingolipids (1µM) for 7 days. Viability determined by MTS assay, percentage viability calculated as percentage of carrier i.e. MSCs in osteoblast differentiation medium with methanol (1:10,000 dilution). 9 replicates per condition. Control samples highlighted in blue, MM samples in red. Unpaired t-tests. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. NS = No statistically significant difference. All p values are in comparison to carrier condition i.e. A10-OB + methanol.

### 5.3.6 Calcium deposition by osteoblasts differentiated from a control subjects MSC's was reduced when cultured with imiglucerase, lactosylceramide or glucosylceramide.

MSC's isolated from the bone marrow of a control subject (2186) were cultured in optimised osteoblast medium A10-OB for 35 days in the presence of β-glucocerebrosidase inhibitors, Gaucher specific therapies or sphingolipids involved in the synthesis pathway of glucosylceramide for the duration of the culture, medium replaced once per week. Addition of irreversible inhibitor CBE (100μM) or reversible inhibitor isofagomine (100μM) to MSC osteoblast cultures did not significantly affect the amount of calcium deposition (CBE $p = 0.29$, isofagomine $p = 0.54$, figure 5.6a). Addition of enzyme replacement therapy (ERT) imiglucerase resulted in a 61.5% decrease ($p = 0.03$) while addition of another ERT velaglucerase did not significantly alter the degree of calcium deposition ($p = 0.98$). Addition of substrate reduction therapy (SRT) miglustat or potential chaperone therapy ambroxol hydrochloride also did not significantly affect calcium deposition ($p = 0.32$ and $p = 0.54$ respectively, figure 5.6b). In regards to MSC-osteoblast culture with exogenous sphingolipids, no significant changes in calcium deposition were observed between standard and carrier (methanol), $p = 0.12$, or between carrier and sphingosine based lipids (sphingosine $p = 0.23$, glucosylsphingosine $p = 0.46$, sphingosine-1-phosphate $p = 0.15$). However, a significant reduction in calcium deposition was observed for lactosylceramide (55.4% decrease, $p = 0.016$) and glucosylceramide (48.9% decrease, $p = 0.025$) when compared to carrier, figure 5.6c.

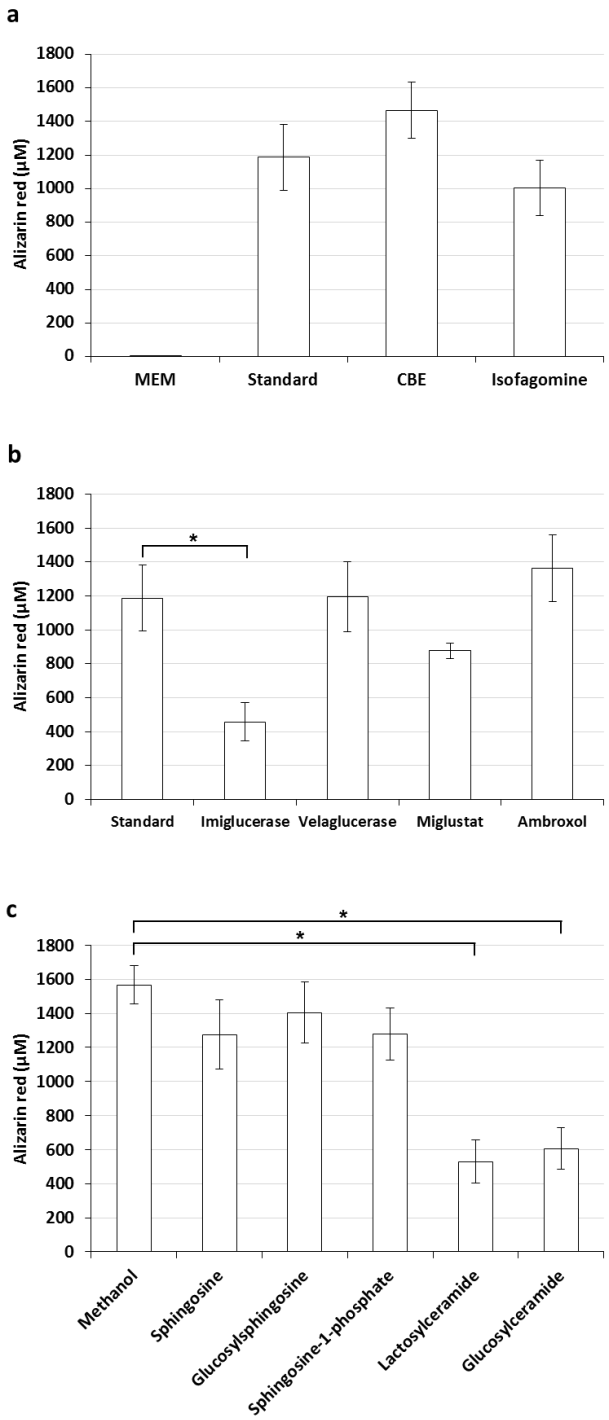



**Figure 5.6** Exogenous effects on calcium deposition of control osteoblasts, subject 2186. **(a, b, c)** Control MSC's cultured for 35 days in optimised osteogenic medium A10-OB with **(a)** irreversible inhibitor CBE (100µM) or reversible inhibitor isofagomine (100µM), **(b)** Gaucher disease specific therapies imiglucerase (1 unit/ml), velaglucerase (1 unit/ml), miglustat (50µM) or ambroxol hydrochloride (20µM), **(c)** sphingolipids (1µM) or carrier (methanol, 1:10,000 dilution). Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. Minimum of 6 replicates per condition. Mean ± SEM plotted. *$p \leq 0.05$, unpaired t-tests.

### 5.3.7 Calcium deposition by osteoblasts differentiated from a multiple myeloma patients MSC's was reduced when cultured with imiglucerase, miglustat, lactosylceramide or glucosylceramide.

The same experimental approach was used for MSC's isolated from the bone marrow of a multiple myeloma subject, sample 2036. MSC's were cultured in optimised osteoblast medium for 35 days in the presence of β-glucocerebrosidase inhibitors, Gaucher specific therapies or sphingolipids for the duration of the culture, medium replaced once per week. As for the control sample, addition of inhibitors CBE (100μM) or isofagomine (100μM) to MSC osteoblast cultures did not significantly affect the amount of calcium deposition (CBE $p = 0.84$, isofagomine $p = 0.25$, figure 5.7a). While addition of ERT imiglucerase significantly reduced the amount of calcium deposition as seen for the control. In this case deposition was reduced by 56.1% ($p = 0.002$). However, for this MM subject, the SRT miglustat decreased calcium deposition by 49% ($p = 0.021$), a decrease not observed in the control MSC cultures. There were no significant changes in calcium deposition for another ERT velaglucerase ($p = 0.51$) or for the potential chaperone therapy ambroxol hydrochloride ($p = 0.23$), figure 5.7b. A similar pattern of calcium deposition to the control MSC was observed for the MM MSC osteoblast culture when sphingolipids were added exogenously. There were no significant changes in calcium deposition between standard and carrier (methanol) or between carrier and sphingosine ($p = 0.68$), glucosylsphingosine ($p = 0.55$), sphingosine-1-phosphate ($p = 0.41$) or ceramide ($p = 0.37$). However, as in control cultures, calcium deposition was significantly reduced in the presence of exogenous lactosylceramide (32.7% decrease, $p = 0.03$) or glucosylceramide (32.7% decrease, $p = 0.016$), figure 5.7c.

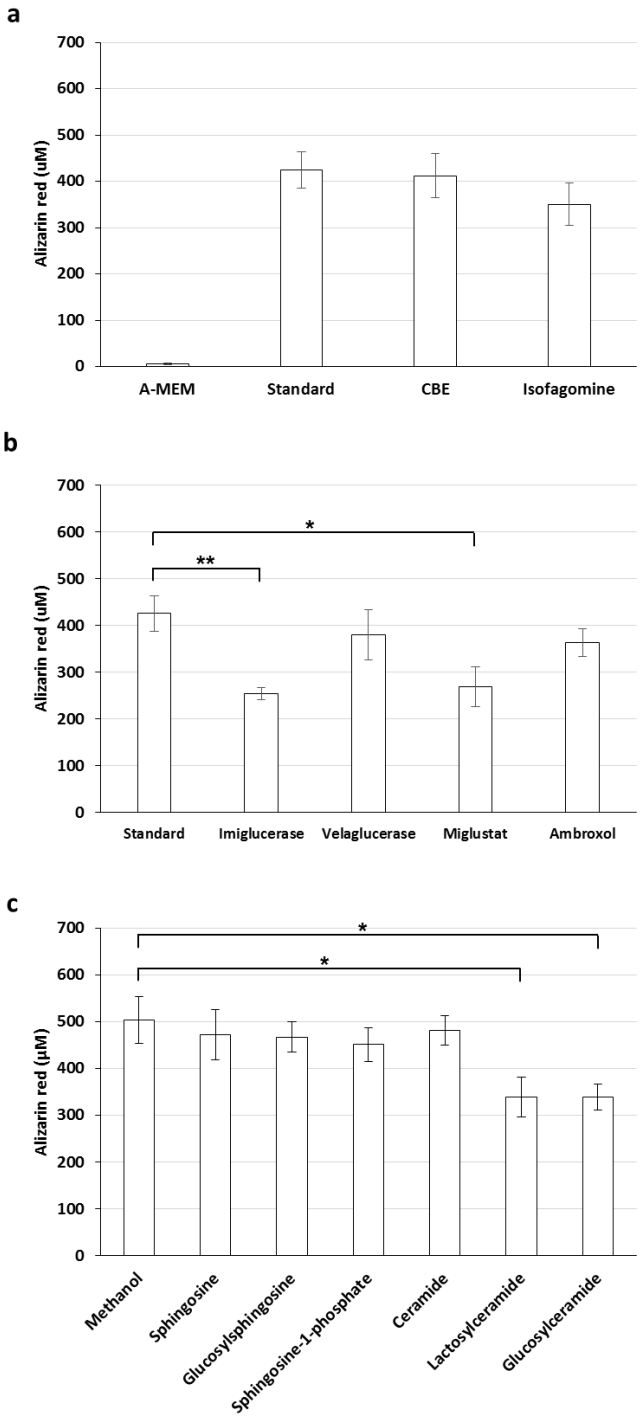



**Figure 5.7** Exogenous effects on calcium deposition of a multiple myeloma subjects osteoblasts, subject 2036. **(a, b, c)** MSC's cultured for 35 days in optimised osteogenic medium A10-OB with **(a)** irreversible inhibitor CBE (100µM) or reversible inhibitor isofagomine (100µM), **(b)** Gaucher disease specific therapies imiglucerase (1 unit/ml), velaglucerase (1 unit/ml), miglustat (50µM) or ambroxol hydrochloride (20µM), **(c)** sphingolipids (1µM) or carrier (methanol, 1:10,000 dilution). Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. 6 replicates per condition. Mean ± SEM plotted. *p ≤ 0.05, **p ≤ 0.01, unpaired t-tests.

### 5.3.8 Calcium deposition by osteoblasts differentiated from a second multiple myeloma patients MSC's was reduced when cultured with imiglucerase, velaglucerase or miglustat.

MSC's isolated from the bone marrow of a second multiple myeloma subject (2167) were cultured in optimised osteoblast medium for 35 days in the presence of β-glucocerebrosidase inhibitors, Gaucher specific therapies or sphingolipids for the duration of the culture, medium replaced once per week. Addition of inhibitors CBE (100µM) or isofagomine (100µM) to MSC osteoblast cultures did not significantly affect the amount of calcium deposition (CBE $p = 0.59$, isofagomine $p = 0.27$, figure 5.8a). However, addition of current Gaucher specific therapies significantly reduced the amount of calcium deposition over 35 days of culture for this subject. Both enzyme replacement therapies led to the greatest reductions, addition of imiglucerase resulted in a 69% decrease ($p < 0.0001$) and velaglucerase in a 53.7% decrease ($p = 0.001$). While addition of substrate reduction therapy miglustat reduced calcium deposition to a lesser extent (19.5% decrease, $p = 0.049$) addition of a potential chaperone therapy, ambroxol hydrochloride, did not significantly alter the degree of calcium deposition ($p = 0.843$, all therapy conditions shown in figure 5.8b). In contrast, addition of a range of sphingolipids (all at 1µM) including glucosylsphingosine and glucosylceramide did not significantly affect the amount of calcium deposition when compared to carrier only (methanol, 1:10,000 dilution ratio) as shown in figure 5.8c.

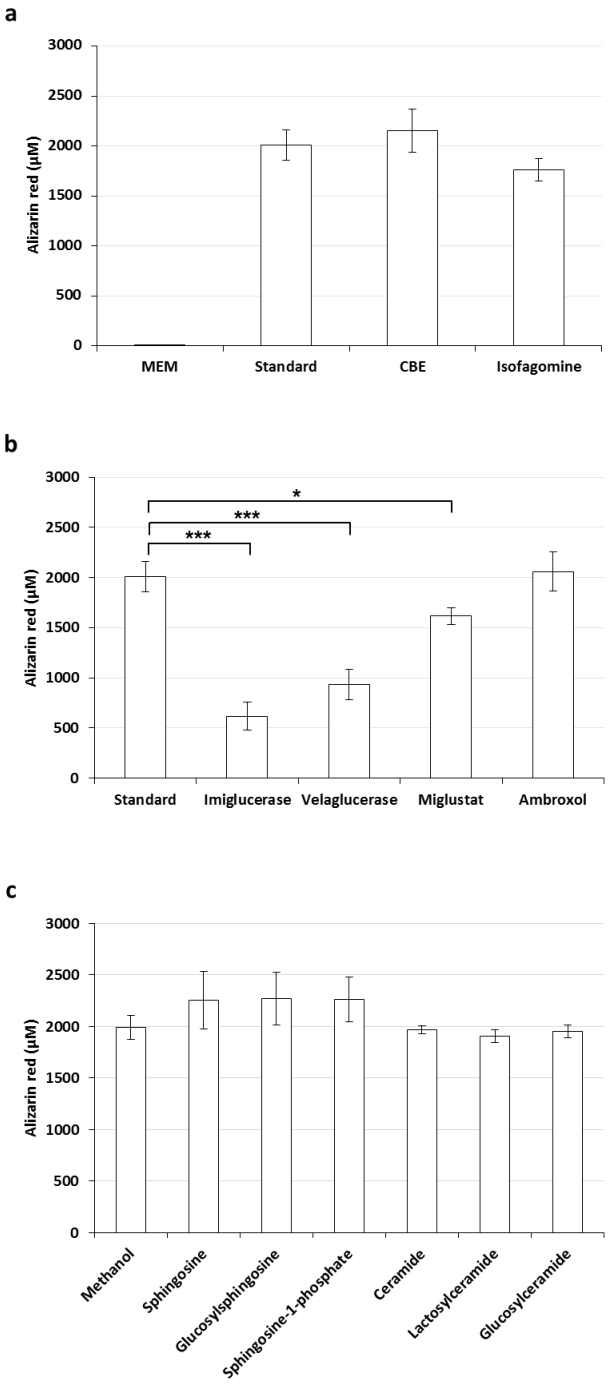



**Figure 5.8** Exogenous effects on calcium deposition of a multiple myeloma subjects osteoblasts, subject 2167. **(a, b, c)** MSC's cultured for 35 days in optimised osteogenic medium A10-OB with **(a)** irreversible inhibitor CBE (100µM) or reversible inhibitor isofagomine (100µM), **(b)** Gaucher disease specific therapies imiglucerase (1 unit/ml), velaglucerase (1 unit/ml), miglustat (50µM) or ambroxol hydrochloride (20µM), **(c)** sphingolipids (1µM) or carrier (methanol, 1:10,000 dilution). Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. 6 replicates per condition. Mean ± SEM plotted. *$p \leq 0.05$, ***$p \leq 0.001$, unpaired t-tests.

### 5.3.9 Calcium deposition by osteoblasts differentiated from a third multiple myeloma subjects MSC's was unaffected by addition of Gaucher specific therapies or sphingolipids.

MSC's isolated from the bone marrow of a third multiple myeloma subject (2314) were cultured in optimised osteoblast medium for 35 days in the presence of β-glucocerebrosidase inhibitors, Gaucher specific therapies or sphingolipids for the duration of the culture, medium replaced once per week. Unlike the previous control or MM subjects no statistically significant changes in calcium deposition were observed for any condition when compared to either standard (CBE $p = 0.27$, isofagomine $p = 0.91$, imiglucerase $p = 0.93$, velaglucerase $p = 0.85$, miglustat $p = 0.91$, ambroxol hydrochloride $p = 0.91$, carrier $p = 0.41$) or carrier (sphingosine $p = 0.73$, glucosylsphingosine $p = 0.85$, sphingosine-1-phosphate $p = 0.91$, lactosylceramide $p = 0.38$, glucosylceramide $p = 0.32$), figures 5.9a, b and c.

### 5.3.10 Calcium deposition by osteoblasts differentiated from GD subjects MSC's was suboptimal.

MSC's isolated from the bone marrow of two GD subjects (2158 and 2371) were cultured in optimised osteoblast medium for 35 days in the presence of β-glucocerebrosidase inhibitors, Gaucher specific therapies or sphingolipids for the duration of the culture, medium replaced once per week. However, due to limited expansion of the 2158 sample MSC's too few experiments could be carried out to obtain statistical analysis. In addition, osteoblast cultures of samples 2158 and 2371 produced suboptimal amounts of calcium preventing assessment of the effects of exogenous factors. The amount of calcium deposited in GD osteoblast cultures in the presence of GD-specific therapies are shown in figures 5.10a and 5.10b.

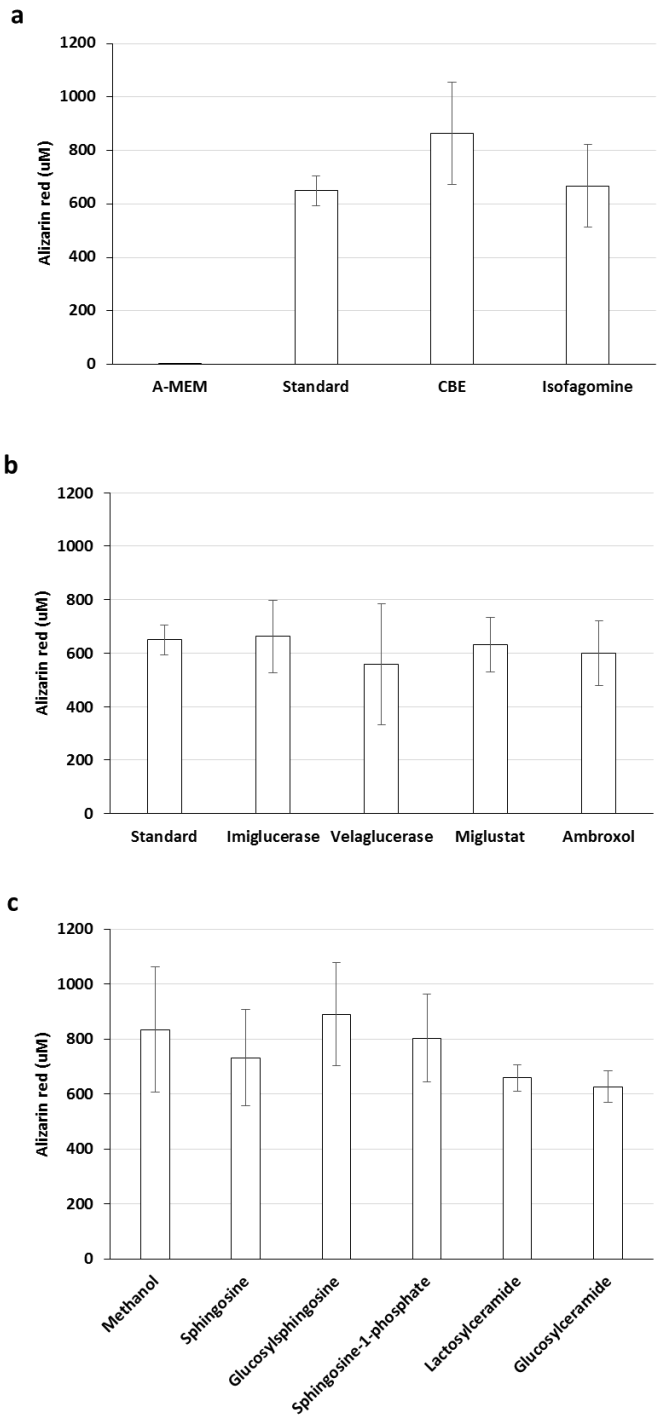



**Figure 5.9** Exogenous effects on calcium deposition of a multiple myeloma subjects osteoblasts, subject 2314. **(a, b, c)** MSC's cultured for 35 days in optimised osteogenic medium A10-OB with **(a)** irreversible inhibitor CBE (100µM) or reversible inhibitor isofagomine (100µM), **(b)** Gaucher disease specific therapies imiglucerase (1 unit/ml), velaglucerase (1 unit/ml), miglustat (50µM) or ambroxol hydrochloride (20µM), **(c)** sphingolipids (1µM) or carrier (methanol, 1:10,000 dilution). Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. 6 replicates per condition. Mean ± SEM plotted. ***$p \leq 0.001$, unpaired t-tests.

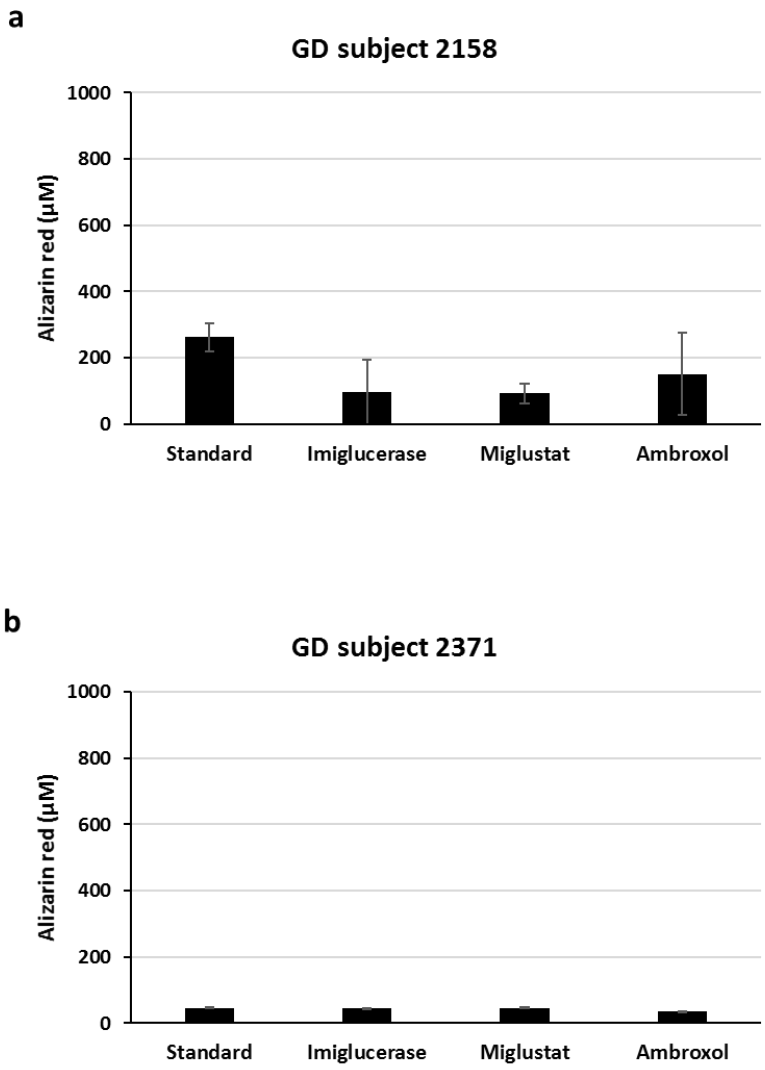



**Figure 5.10** Exogenous effects of GD specific therapies on calcium deposition of GD subjects' osteoblasts. **(a, b)** MSC's cultured for 35 days in optimised osteogenic medium A10-OB with imiglucerase (1 unit/ml), miglustat (50µM) or ambroxol hydrochloride (20µM). Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. **(a)** Minimum of 2 replicates per condition. **(b)** 6 replicates per condition. Mean ± SEM plotted. Unpaired t-tests, no significance.

### 5.3.11 Optimisation of culture conditions for the human osteoblast cell line SaOs-2.

To further assess osteoblast function in Gaucher disease an osteoblast cell culture model was optimised. The human osteoblast cell line SaOs-2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES, 100 units/ml penicillin, 0.1mg/ml streptomycin, 10nM dexamethasone and a range of concentrations of β-glycerophosphate and ascorbate-2-phosphate for up to 4 weeks to induce calcium deposition on the monolayer surface, medium replaced twice per week. In order to permit the detection of increased or decreased calcium deposition due to modifications in culture conditions an intermediate degree of calcium deposition was selected, as indicated by a red arrow in figure 5.11d and a representative image, figure 5.11b. SaOs-2 cells were cultured in these conditions i.e. supplemented DMEM + 10nM dexamethasone, 4mM β-glycerophosphate, 100μM ascorbate-2-phosphate, for 21 days for all subsequent calcium deposition experiments for this cell line, referred to as D10-OB for subsequent experiments.

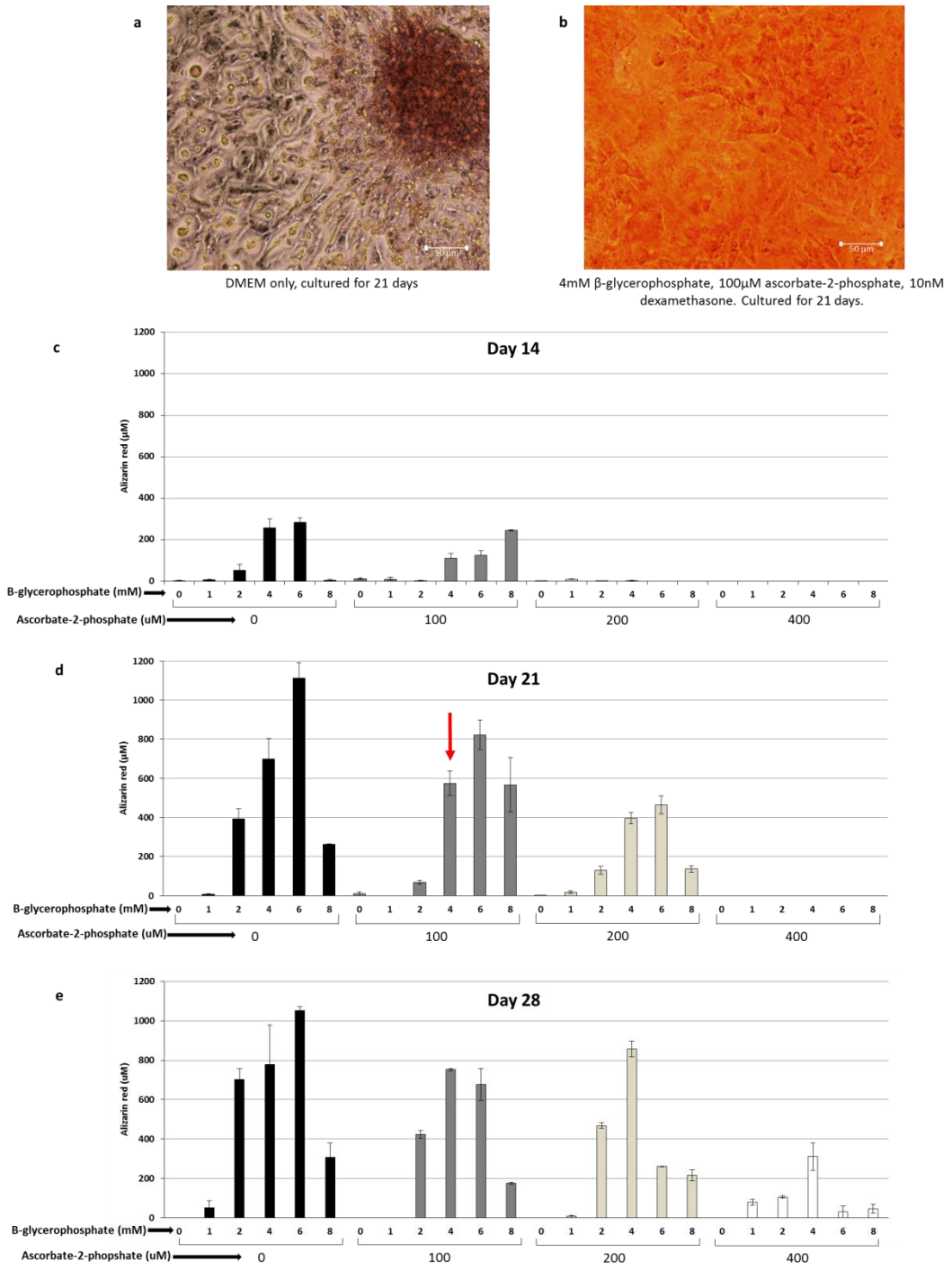



**Figure 5.11** Optimisation of conditions required for calcium deposition by osteoblast cell line SaOs-2. **(a, b)** Representative images of SaOs-2 cells cultured **(a)** without and **(b)** with optimised osteogenic conditions for 21 days, stained with alizarin red. **(c, d, e)** SaOs-2 cells cultured with 10nM dexamethasone and a range of concentrations of β-glycerophosphate and ascorbate-2-phosphate for **(c)** 14 days, **(d)** 21 days, **(e)** 28 days. Duplicates per condition, mean ± SEM plotted. Selected osteogenic conditions for subsequent cultures indicated by red arrow. Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. Mean ± SEM plotted.

### 5.3.12 SaOs-2 as a model for Gaucher disease subject osteoblasts.

To determine if the SaOs-2 cell line could be used as a model of Gaucher disease these cells were cultured in a range of CBE concentrations in optimised calcium deposition medium for 4 days. β-glucocerebrosidase activity was reduced by 92.8% when cultured with 100μM CBE. Maximal inhibition was achieved with 250μM CBE (95.9%). No significant reduction in viability, measured using MTS assay, was observed for any of the CBE concentrations however to minimise the potential of off-target effects SaOs-2 cells were cultured with 100μM CBE in subsequent experiments, figure 5.12a. To determine whether addition of therapies or sphingolipids in the absence or presence of CBE would affect viability and therefore potentially affect the amount of calcium deposition through cell death rather than osteoblast activity MTS assays were performed for all conditions after 4 days of culture in the presence of the condition in optimised osteoblast medium with a minimum of six cultures per condition. Cultures with GD specific therapies did not lead to significant changes in viability either in the absence or presence of CBE. However, SaOs-2 cells cultured with glucosylsphingosine resulted in highly significant decreases in viability in the absence or presence of CBE (no CBE $p < 0.0001$, with CBE $p < 0.0001$). Significant decreases in viability were also observed for glucosylceramide (no CBE $p < 0.008$, with CBE $p = 0.001$). To a lesser degree viability was also decreased in the presence of CBE for sphingosine-1-phosophate ($p = 0.025$), ceramide ($p = 0.033$) and lactosylceramide ($p = 0.006$), figures 5.12b, c and d.

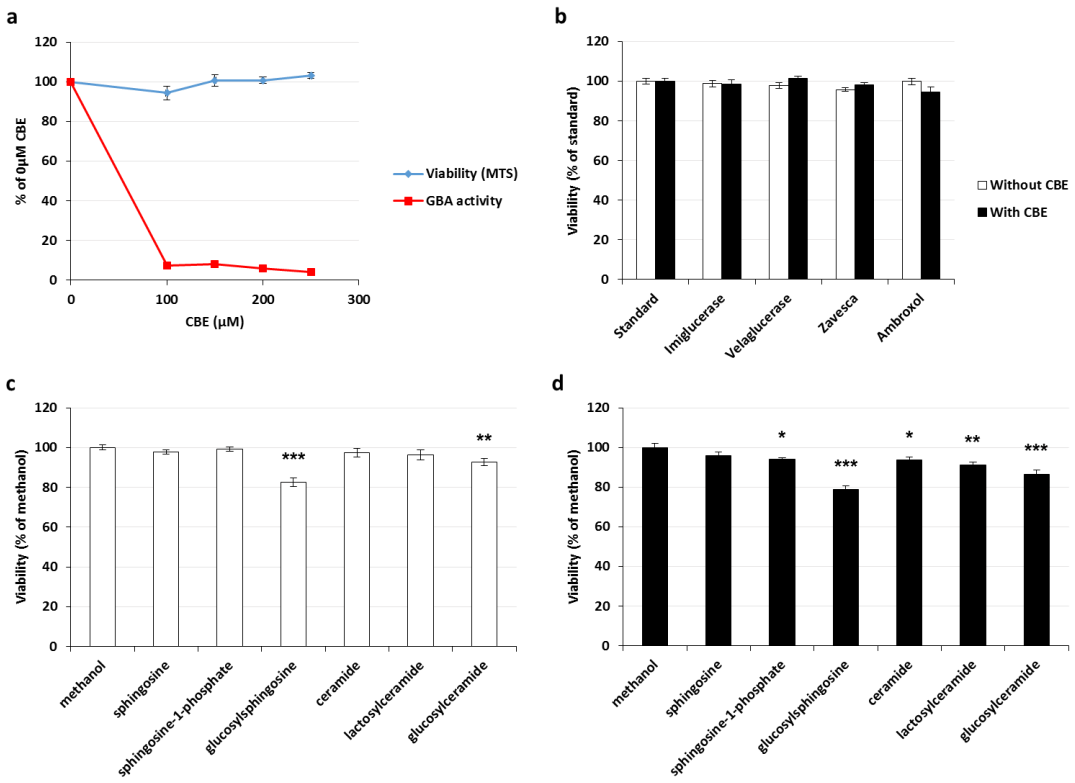



**Figure 5.12** Assessment of SaOs-2 osteoblast cell line as a model of Gaucher disease. **(a)** Viability of cell line (blue line) measured by MTS assay, inhibition of β-glucocerebrosidase activity (red line), by addition of an irreversible inhibitor CBE at a range of concentrations when cultured in D10-OB for 4 days in triplicate. Activity measured by fluorometric assay. **(b, c, d)** Viability of cell line when cultured in D10-OB for 4 days with **(b)** Gaucher disease therapies or **(c, d)** sphingolipids in the absence (white bars) or presence (black bars) of 100μM CBE, minimum of 6 cultures per condition. Viability measured by MTS assay, 100% calculated from the averaged absorbance values obtained from the standard condition. Mean ± SEM plotted. Unpaired t-tests. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

## 5.3.13 Calcium deposition was reduced in the presence of current Gaucher specific therapies in the absence or presence of CBE.

To further assess the potential effects of GD specific therapies on osteoblast activity a human osteoblast cell line, SaOs-2, was cultured in optimised osteogenic medium D10-OB for 3 weeks in the presence of ERT's imiglucerase or velaglucerase, SRT miglustat or potential pharmacological chaperone therapy ambroxol hydrochloride in the absence or presence of 100μM CBE for 3 weeks, medium changed twice per week. As seen in figures 5.6, 5.7 and 5.8 for primary MSC derived osteoblast

cultures, SaOs-2 cells cultured in the presence of imiglucerase resulted in the most substantial decrease in calcium deposition (27.3% decrease, $p < 0.0001$). However, unlike in the majority of the MSC osteoblast cultures, addition of the other GD specific therapies also resulted in statistically significant reduction in calcium deposition (velaglucerase: 14% decrease, miglustat: 7.7% decrease, ambroxol hydrochloride: 19.3% decrease, $p < 0.0001$ for each condition), figure 5.13a. Addition of GD specific therapies in the presence of CBE generated a very similar pattern in regards to calcium deposition as seen in cultures without CBE inhibition with the exception of ambroxol hydrochloride. Again, addition of imiglucerase resulted in the greatest reduction (26.8% decrease, $p < 0.0001$) while addition of velaglucerase or miglustat led to more modest reductions in calcium deposition (8.6%, 4.4% decrease, $p < 0.0001$, $p = 0.0002$ respectively). Conversely, addition of ambroxol hydrochloride in the presence of CBE led to an increase in calcium deposition (11%, $p < 0.0001$), figure 5.13b.

In addition, SaOs-2 cultured in the presence of CBE led to statistically significant decreases in calcium deposition for all equivalent conditions e.g. comparing imiglucerase to imiglucerase+CBE, again with the exception of ambroxol hydrochloride cultures which deposited significantly more calcium in the presence of CBE than those without CBE, percentage changes and statistical significances shown in table 5.3 below and figure 5.13c.

| | **+ CBE** | |
|---|---|---|
| | **% change in $Ca^{2+}$ deposition** | **p value** |
| **Standard** | **-14.5** | **< 0.0001** |
| **Imiglucerase** | **-13.9** | **0.018** |
| **Velaglucerase** | **-9.1** | **0.001** |
| **Miglustat** | **-14.5** | **< 0.0001** |
| **Ambroxol hydrochloride** | **+17.6** | **< 0.0001** |

**Table 5.3** Percentage changes in calcium deposition by SaOs-2 cell line when cultured for 3 weeks in optimised osteogenic medium D10-OB in the presence of GD specific therapies in the presence of CBE relative to conditions in the absence of CBE. p values refer to statistical significance of percentage changes in calcium deposition. Unparied t-tests.

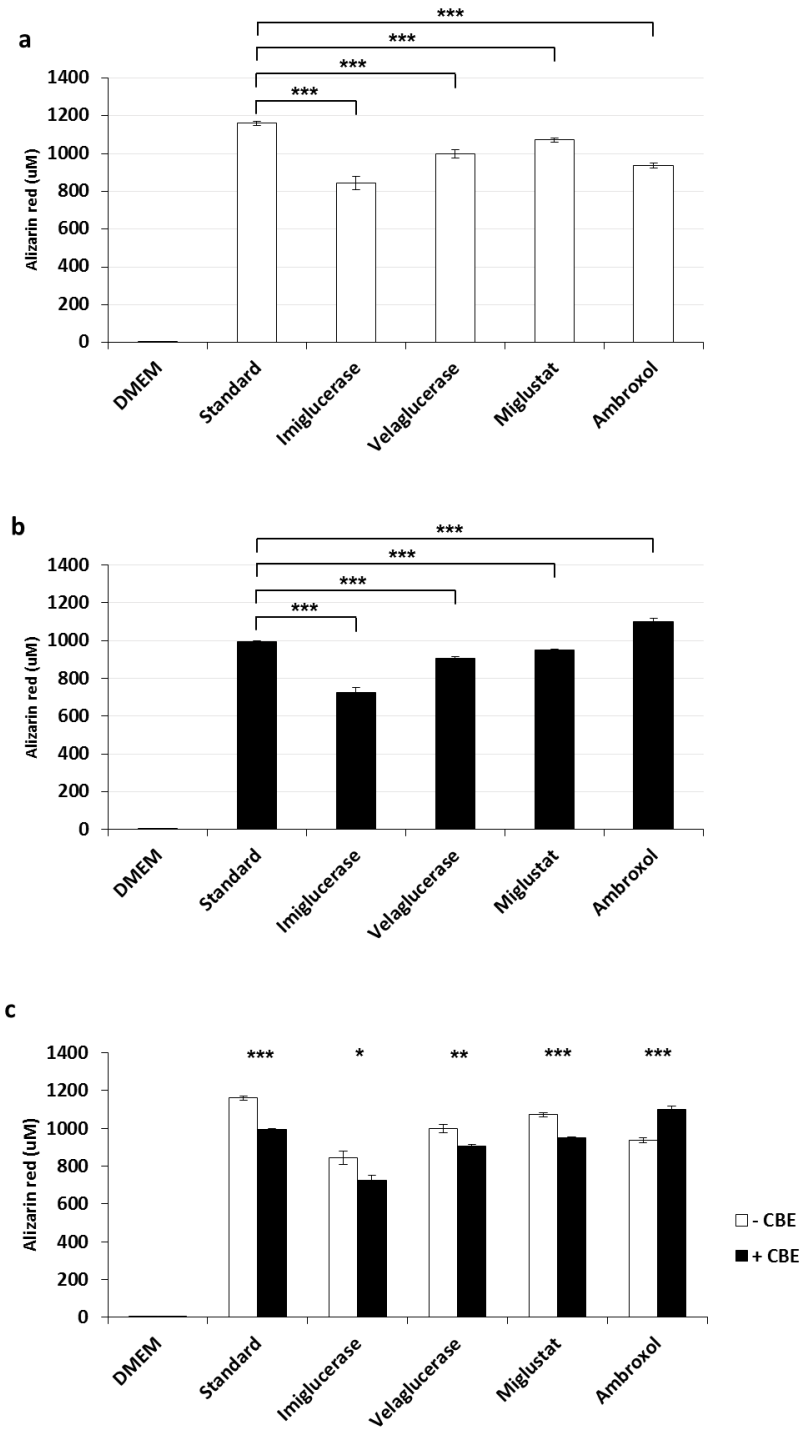



**Figure 5.13** Culture with therapy and CBE reduce calcium deposition by osteoblast cell line SaOs-2. **(a, b, c)** SaOs-2 cultured for 3 weeks in optimised osteogenic conditions in D10-OB in the absence (white bars) or presence (black bars) of CBE with either carrier (PBS) labelled as standard, imiglucerase (1 unit/ml), velaglucerase (1 unit/ml) or miglustat (50µM), ambroxol hydrochloride (20µM). Quantification of calcium deposition by human osteoblast cell line SaOs-2 by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted. 9 repeat experiments per condition. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Unpaired t-tests.

### 5.3.14 Calcium deposition by SaOs-2 is increased when cultured with sphingosine-1-phosphate or glucosylsphingosine in the presence of CBE.

To assess the effect of exogenous sphingolipids on calcium deposition by the GD osteoblast model, SaOs-2 cells were cultured in D10-OB medium for 3 weeks in the presence of carrier (methanol) or sphingolipid in the absence or presence of CBE for the duration of the culture, medium changed twice per week. No statistically significant differences in calcium deposition were observed when comparing carrier with any sphingolipid in the absence of CBE or when comparing carrier with CBE with any sphingolipid in the presence of CBE, figure 5.14a.

In contrast to the previous figure (figure 5.13), addition of CBE to SaOs-2 cultures in osteogenic medium D10-OB did not result in any statistically significant changes in calcium deposition with the exception of SaOs-2 cultured in the presence of exogenous sphingosine-1-phosphate or glucosylceramide in which addition of CBE resulted in statistically significant increases in calcium deposition (31% and 25.6%, $p = 0.0016$ and 0.005 respectively), figures 5.14b and c.

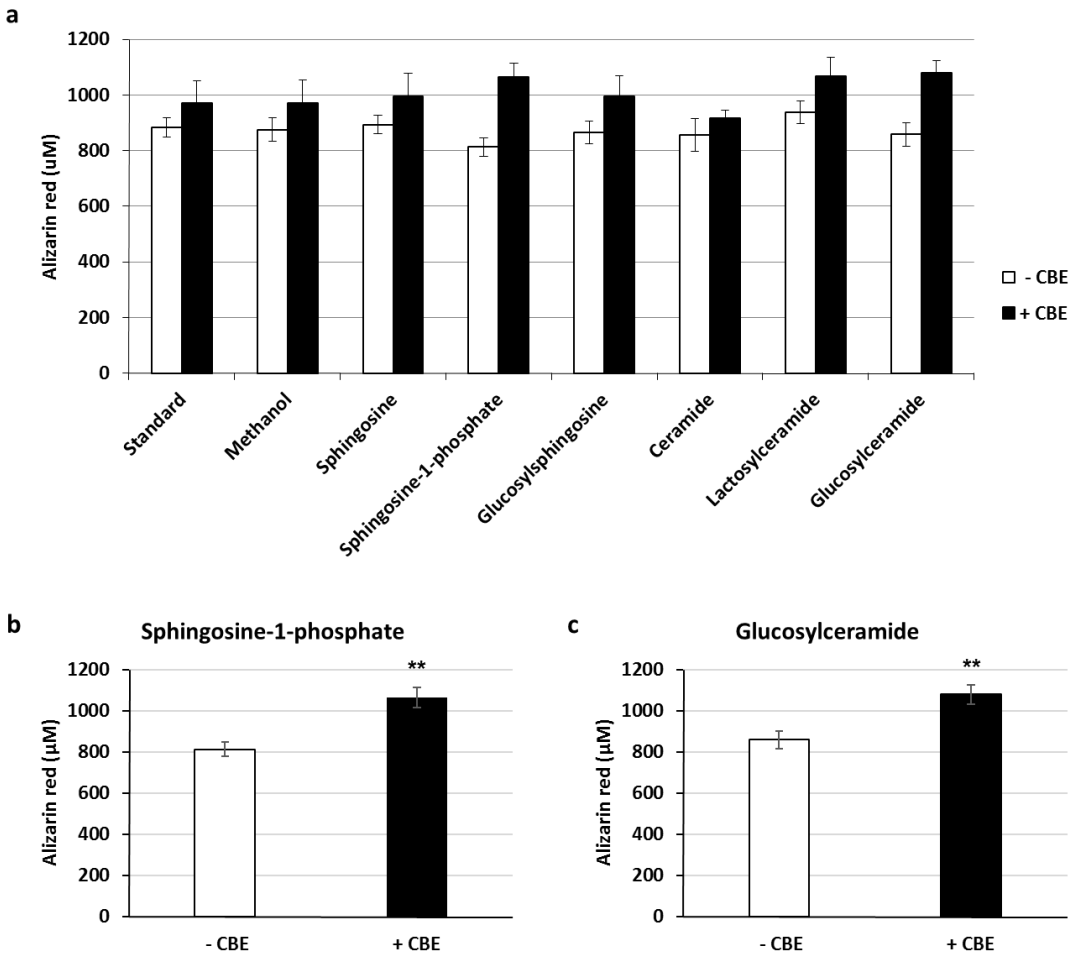



**Figure 5.14** Exogenous effects of sphingolipids on calcium deposition of human osteoblast cell line SaOs-2. **(a, b, c)** SaOs-2 cultured for 21 days in optimised osteogenic medium D10-OB with sphingolipids (1µM) or carrier (methanol, 1:10,000 dilution) in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100µM). **(b, c)** selected data from **(a)**. Quantification of calcium deposition by absorbance spectrometry of alizarin red solubilised post staining of calcium deposits on cell monolayer surface. 6 repeat experiments per condition. Mean ± SEM plotted. **p ≤ 0.01. Unpaired t-tests.

## 5.4 Discussion

The main paradigm for bone homeostasis is the interaction and signalling between osteoclasts and osteoblasts, commonly referred to as coupling (289,436). Such a close relationship would suggest that changes in osteoclast generation, size and activity may have a direct effect on osteoblast number and activity but it may also be the case that osteoblasts are abnormal in GD due to factors other than osteoclast abnormalities. Murine models of GD have suggested that the bone disease may be caused by reduced osteoblastic activity (272,541). Other publications have shown that type 1 GD patient MSC's have a decreased capacity to differentiate into osteoblasts and normal MSC's have impaired proliferation with altered secretion of soluble factors when inhibited with CBE (138,524). These findings suggest there may be an imbalance between osteoclasts and osteoblasts in GD both in terms of numbers and activity. To investigate the potential imbalance and dysfunction of GD patient osteoblasts MSC's were isolated from the bone marrow of control, Gaucher and multiple myeloma subjects and differentiated into functional osteoblasts to allow for characterisation and comparison of GD MSC's and osteoblasts with control and myeloma patient MSC's and osteoblasts. A model of GD osteoblasts was also created using the SaOs-2 human osteoblast cell line and the β-glucocerebrosidase inhibitor CBE.

The results discussed below indicate possible uncoupling between osteoclasts and osteoblast precursors in GD patients and investigate for the first time the effects of GD-specific therapies and sphingolipids on the function of a human osteoblast cell line and human primary osteoblasts.

**Evidence of uncoupling between GD osteoclasts and osteoblasts.**

No difference was found in the number of circulating osteoblast precursors between control and GD cohorts. This may indicate that initial commitment of MSC to osteoblast precursors is not impaired in GD patients. If the initial stages of differentiation in regards to either rate or proportion of MSC's was reduced fewer

would be in the peripheral blood assuming the migration rate is the same for control and GD subjects. However, this does not mean the anabolic aspect of bone metabolism is normal in GD as osteoblast precursor migration to the bone marrow niche from the peripheral blood could be impaired and maturation of precursors in the bone marrow could also be impaired as a number of publications have reported altered differentiation capacity of GD patient MSC's (138,141). In addition, migration and maturation could be affected by extracellular factors in the bone marrow and peripheral blood which may prevent precursors from either reaching a state in which they respond to the migration factors or reduce their ability to differentiate into functional osteoblasts once they reach the bone marrow.

Analysis of osteoclast generation and osteoblast precursors present in peripheral blood showed there may be uncoupling between GD osteoclasts and osteoblast precursors. While there was a correlation between the control cohort osteoclast generation and osteoclast numbers there was no correlation between these cells for GD patients which in combination with previous data showing increased osteoclast generation in vitro, correlating with clinical bone features, provides a strong case for bone microenvironment dysregulation in GD favouring an imbalance towards high resorption and low deposition leading to overall bone loss. Evidence of an osteoblast role is supported by findings of defects in osteoblast formation in a GD murine model which exhibited bone disease similar to that observed in GD patients including osteopenia and avascular necrosis (272). However, the lack of correlation of GD osteoclast generation with osteoblast precursors only demonstrates uncoupling between immature cells and may not lead to uncoupling between the mature forms of the osteoblasts and osteoclasts.

**CBE inhibition reduces control MSC viability.**

A reduction in viability of control MSC's in the presence of CBE and isofagomine was detected. However, the reduction in percentage viability was small and although statistically significant may not have a significant effect on the cell culture outcome. In addition, it should be noted that the assay used, MTS, can also be used to

measure proliferation and may indicate reduced proliferation instead of reduced viability as MSC's actively divide. In either case it indicates a lower number of mitochondria which, assuming the number of mitochondria per MSC is equal in all conditions, equates to a lower total cell number. This result may also indicate deficiency in GBA activity may reduce either proliferation or viability or both of MSC's resulting in a decreased pool of MSC's to differentiate into osteoblasts further skewing the balance between osteoblasts and osteoclasts towards catabolism of bone.

**Effects of GD-specific therapies on in vitro cultures of MSC's and primary osteoblasts.**

As mentioned in the chapter 3 discussion, treatment of GD patients with ERT has resulted in significant BMD increases in a number of long term studies however BMD can take several years to show improvement (84,507,571) with considerable residual skeletal disease after several years of ERT (84,571). To investigate the potential effects of GD-specific therapies on MSC's and osteoblasts, MSC's were differentiated into functional osteoblasts or a human osteoblast cell line, SaOs-2, were cultured with GD-specific therapies added to the medium for the duration of the culture. Viability of MSC's and SaOs-2 cells was assessed and calcium deposition by osteoblasts was measured by alizarin red staining.

GD-specific therapies imiglucerase, miglustat and ambroxol reduced the viability of non-GD MSC. The decreases in viability/proliferation in those samples affected were considerably greater than the reduction observed for the inhibitors. Again, whether these reductions are substantial enough to affect the outcome of the cell culture is unknown. While unexpected, the reduction in proliferation/viability of the MSC's in the presence of therapies may in part explain the slow response of bone improvement in GD to therapy reported (180) as while these therapies have been shown to decrease osteoclast generation this may be offset by a concomitant reduction in osteoblast numbers leading to a smaller than expected overall gain. This finding may also provide some rationale as to why some GD patients do not

appear to improve with therapy as if the osteoclast generation in these patients is particularly high, as suggested by previous data, the reduction in generation may not be sufficient to outweigh the reduction in osteoblast numbers resulting in continued bone loss. However, these findings were generated from an in vitro culture with several limitations including being a mono-culture and planar and may not recreate the effects of therapy in vivo. The potential mechanisms behind the reduction in MSC viability/proliferation remains unclear but may involve GD-specific therapy mediated alteration in intracellular sphingolipid ratios including glucosylceramide and sphingosine-1-phosphate which have been found to stimulate proliferation of cells (276,278), though these publications do not look specifically at their effects on MSC's. It should be noted that other lipids such as ceramide can have pro-apoptotic effects (285) and glucosylsphingosine has been reported to both enhance proliferation and to cause cell death depending on the cell type (272,572) therefore the ratios between these sphingolipids and their specific effects on MSC's need to be elucidated.

The activity of osteoblasts differentiated from subject MSC's was assessed by the amount of calcium deposited in culture, determined by staining of the calcium with alizarin red followed by solubilisation and quantification of the stain. Substantially reduced calcium deposition was measured in one control and two of the three MM samples when cultured with imiglucerase of up to 69% measured for MM sample 2167. Culture with miglustat also resulted in substantially reduced calcium deposition for two of the three MM samples, with the greatest reduction of 37% for MM sample 2036 and while the decrease in the control sample did not reach statistical significance for miglustat it was still a substantial decrease in calcium of 26%. A statistically significant decrease was also observed for velaglucerase for MM sample 2167 of 54%. As the differentiation of the MSC's into osteoblasts is not initiated until the MSC's are confluent in every well to minimise variation between wells and cultures the decrease in calcium deposition is unlikely to be related to reduction in proliferation. It is therefore likely that these effects are either due to increased apoptosis as indicated by the MTS assay or due to a reduction in capacity for the MSC's to differentiate into mature, functional osteoblasts or both. However,

the fact that for ERT this effect was observed to a greater degree for imiglucerase in comparison to velaglucerase raises questions as to the specificity and mechanism/s of this effect. It is possible that this effect is mediated by the carrier for imiglucerase rather than the enzyme itself, an effect not anticipated as the volume of enzyme added to culture equated to 1:400 of the total volume and that this carrier had been formulated by the manufacturer for administration to patients.

To address this possibility cultures with carrier alone need to be carried out which would require the provision of the specific formula from the manufacturer. However, as miglustat also reduced calcium deposition and for one MM sample addition of velaglucerase to culture did lead to a substantial and statistically significant decrease in calcium deposition this effect may be therapy specific and therefore warrants further investigation. In addition, as none of these cultures were carried out with GD patient MSC's it cannot be determined whether addition of GD-specific therapies would affect calcium deposition. It should also be noted that in these cultures the cells were continuously cultured in the presence of the therapies for the 35-day duration of the culture which may not equate to physiological conditions for GD patients, especially for imiglucerase, as enzyme replacement therapy is most commonly administered as an infusion once every two weeks. With a half-life reportedly ranging from 3.6 to 10.4 minutes (573) in patient plasma MSC's may not be exposed to imiglucerase continuously as the half-life of imiglucerase has been shown to be 14.1 hours (618). For control cells β-glucocerebrosidase is continuously present and active however in this case the majority of the enzyme is intracellular whereas in our cultures the enzyme is added exogenously and internalised via the mannose receptor.

**Effects of sphingolipids on in vitro cultures of MSC's and primary osteoblasts.**

Research into the potential roles of sphingolipids in relation to MSC's and osteoblasts has shown S1P produced by osteoclasts to be a chemoattractant for MSC's resulting in their migration to the bone marrow (431) and its receptor S1PR affects proliferation, migration and differentiation of MSC's (574). In addition,

catabolism of sphingomyelin by neutral sphingomyelinase 2 (nSMase2) has been shown to be essential for mineralisation in a *fro/fro* mouse model (432). To investigate the potential effects of sphingolipids present in the synthesis and degradation pathway of glucosylceramide (including glucosylsphingosine), these sphingolipids were added to osteoblast medium for the duration of the cultures and their effects on viability of MSC's and SaOS-2 and calcium deposition by primary osteoblasts and SaOS-2 was assessed.

The majority of sphingolipids do not reduce cell viability with the exception of glucosylsphingosine which resulted in a reduction in conversion of MTS substrate for all 5 samples, three of which reaching a statistically significant reduction of up to 21% in the case of MM sample 2036. However, this decrease could indicate decreased viability or proliferation, in either case the data suggests glucosylsphingosine reduced the number of MSC's present. As this sphingolipid has been found to be elevated over 10 fold in GD plasma compared to an average of 3 fold for glucosylceramide (155) with one study reporting <4pmol/ml for normal population plasma elevated up to 1860pmol/ml in GD plasma (173), its potential effect on circulating MSC's/pre-osteoblasts and MSC's/pre-osteoblasts resident in the bone marrow may be considerable. Treatment with ERT has been shown to reduce plasma glucosylsphingosine which may in part reverse the detrimental effects of glucosylsphingosine and thus aid in the gradual increase in bone mineral density observed in a number of therapy related GD patient studies (506–510). However, the potential effect of ERT reducing MSC viability may limit this effect.

Addition of glucosylceramide and lactosylceramide consistently increased viability/proliferation however in the majority of samples the increase was not statistically significant only reaching significance for multiple myeloma sample 2167. This data would suggest elevated concentrations of glucosylceramide may be beneficial for MSC's in contrast to the effect of glucosylsphingosine. This at least in part may explain the detrimental effect of GD-specific therapies on MSC's as none of the samples were from GD patients all would have a normal level of glucosylsphingosine and glucosylceramide. Therefore, the beneficial effect of GD-specific therapies on lowering the concentration of glucosylsphingosine would not

be observed while the potentially detrimental effect of lowering glucosylceramide would be. To investigate this further, GD and control subject derived MSC's could be cultured in the presence of ERT, glucosylsphingosine and glucosylceramide both individually and in combination.

Interestingly, addition of lactosylceramide or glucosylceramide to the control and MM sample 2036 resulted in substantial and statistically significant decreases in calcium deposition. Previous data from the MTS assay suggested MSC proliferation may be enhanced in the presence of glucosylceramide. However, as previously stated the MSC's in these cultures were already confluent and therefore this effect may have been limited. These results suggest lactosylceramide and glucosylceramide may inhibit MSC to osteoblast differentiation. While this effect was only observed in two of the four samples tested it may indicate that the high concentrations of glucosylceramide in bone marrow of GD patients may reduce osteoblast activity again to investigate this further it would be necessary to increase the size of the control cohort and to culture GD patient samples. However, if this is the case it would be expected that addition of GD-specific therapies designed to reduce the amount of glucosylceramide present would result in an increase in MSC differentiation into osteoblasts which suggests lactosylceramide concentration is also a factor. A histochemical study of GD bone marrow and spleen Gaucher cells found decreased concentrations of lactosylceramide compared to control macrophages (553) and a separate study found significantly lower concentrations in GD versus control plasma (154). Addition of GD-specific therapy to MSC cultures may result in an increase in lactosylceramide as the sphingolipid pathway may be closer to functioning normally. If this is the case the effect of reducing the amount of glucosylceramide may be negated by a concomitant increase in lactosylceramide.

GD patient derived MSC's did not produce sufficient calcium to allow assessment of exogenous factors such as inhibitors, therapies and sphingolipids. In the case of sample 2158 the MSC's were very slow to expand which made it very difficult to set up sufficient culture replicates for statistical analysis. While MSC's isolated from subject 2371 expanded normally they still did not produce sufficient calcium to assess. Whether this was due to experimental failure or due to a reduction in

differentiation capacity, reported by Lecourt et al (138) would require repeating this experiment in these samples and in a larger cohort of GD subject MSC's.

**Effects of GD-specific therapies and sphingolipids on in vitro cultures of the SaOS-2 cell line.**

In comparison to primary cells the SaOs-2 cell line responded differently to exogenous factors showing no reduction in viability to either inhibitors or GD specific therapies. This may be due to a number of factors. Firstly, the SaOs-2 cell line is not comparable to the primary MSC's as they are described as osteoblast-like cells and therefore already committed to the osteoblast lineage which may alter their response to external factors. Secondly, the SaOs-2 cells proliferate much more quickly than primary MSC's.

In contrast, SaOS-2 cells did exhibit reduced viability/ proliferation in the presence of certain sphingolipids, namely glucosylsphingosine (both in the absence and presence of CBE), sphingosine-1-phosphate, ceramide, lactosylceramide and glucosylceramide but only in the presence of CBE. While the reduction in viability in the presence of glucosylsphingosine was present for both primary MSC's and the SaOS-2 cell line the effect of sphingosine-1-phosphate and ceramide on the SaOS-2 cell line was not observed in the MSC cultures. Moreover, the presence of lactosylceramide and glucosylceramide in cultures led to opposite results from appearing to increase proliferation in MSC cultures to reducing viability/proliferation in SaOs-2 cultures. These differences again could relate to the different rates of proliferation between the SaOs-2 and MSC's but may also be due to the SaOs-2 being osteoblast-like. If the latter is the case it may suggest the viability effects of inhibitors, therapies and lipids affect MSC's differently to osteoblasts and therefore may have stepwise effects in terms of MSC numbers differentiating into osteoblasts and the number of osteoblasts maturing into functional cells. However, glucosylsphingosine appears to have a more consistent effect of reducing viability/proliferation further supporting the hypothesis that high

concentrations of glucosylsphingosine in the bone marrow may lead to a reduction in MSC and osteoblast numbers resulting in reduced calcium deposition.

Although different results were obtained in regards to viability between primary MSC's and the SaOs-2 cell line the effects of GD-specific therapies on calcium deposition were similar. Calcium deposition by this cell line was reduced in the presence of each therapy with the exception of ambroxol hydrochloride which showed a slight but statistically significant increase in calcium deposition. As seen with the MSC's the greatest decrease was seen when SaOs-2 cells were cultured with imiglucerase. In this case the decrease of 27.3% without CBE and 26.8% with CBE although substantial was not as marked as for some of the MSC's with decreases of up to 69% for multiple myeloma sample 2167. This may indicate that imiglucerase is affecting differentiation with its effect being limited in regards to the SaOs-2 due to already being differentiated into the osteoblast lineage unlike the MSC's. As previously mentioned, whether this effect is specific to the enzyme or due to the carrier is unknown and requires investigation. Again, as with MSC's, cultures with velaglucerase and miglustat resulted in less substantial decreases in calcium and although these are statistically significant whether this is clinically relevant is unknown. The increase in calcium deposition in the presence of ambroxol hydrochloride was similar to that observed for several MSC cultures although in all cases the increase is very small and few reached statistical significance suggesting either at worst ambroxol has no detrimental effect and at best has a small beneficial effect on bone mineralisation.

Inhibition by CBE resulted in consistently reduced calcium deposition in the cultures set up with GD specific therapies however this result is not replicated in a separate experiment in which sphingolipids were added to culture. In this experiment calcium levels were not affected by addition of CBE under standard or carrier conditions. These findings suggest results may vary between cultures. To determine if CBE does reduce calcium deposition several separate repeat experiments will be required to account for this apparent variation.

In contrast to effects observed on calcium deposition for MSC samples 2186 (control) and 2036 (MM) culture of SaOs-2 with lactosylceramide or

glucosylceramide did not reduce calcium deposition. In the presence of CBE, the SaOs-2 culture with sphingosine-1-phosphate or glucosylceramide conversely resulted in substantial increases in calcium deposition suggesting these sphingolipids, both found to be elevated in GD plasma and serum, may act as a counterbalance to the detrimental effects on cell number to glucosylsphingosine at least for the osteoblast-like cell line. MSC osteoblast cultures with CBE and sphingolipids will need to be carried out to determine if this effect is also present for primary cells. Again, the effect of glucosylsphingosine on cell viability/proliferation may have been negated by the necessity to only initiate differentiation and mineralisation once the cells had reached confluence.

**Limitations**

In addition to the limitations stated within the above discussion it should be noted that all of the above findings were generated from in vitro culture which has several limitations compared to in vivo studies. Firstly, MSC's and osteoblasts were cultured and differentiated in isolation which does not take into account the potential effects of other cells in the bone marrow such as osteoclasts, osteocytes, plasma cells, and T-cells. Secondly, these cells were cultured as a monolayer which does not replicate the three-dimensional environment present in vivo. Thirdly, cell cultures were performed in static medium. This method can lead to a depletion of nutrients and osteogenic factors and a build-up of waste products between medium changes.

**Summary**

In summary, the results in this chapter based on data from the human osteoblast cell line SaOs-2 and the majority of MSC derived osteoblasts suggested that GD-specific therapies reduced calcium deposition and MSC viability, as did glucosylsphingosine. In comparison, glucosylceramide increased MSC viability but decreased their degree of calcium deposition. However, the effects of GD-specific

therapies and sphingolipids on GD MSC's was not investigated due to difficulties encountered in expanding and differentiating the MSC's isolated from GD patient bone marrow. Inhibition of β-glucocerebrosidase using CBE did not appear to affect calcium deposition by osteoblasts differentiated from MSC's and was inconclusive in regards to the human osteoblast cell line.

# 6. In vitro plasma cell culture

## 6.1 Introduction

Several studies have reported an increased risk of cancer in GD, in particular for multiple myeloma (MM) with reported risk ratios of 25 in an N370S/N370S GD cohort of 367 (242) up to a standardised rate ratio of 51.1 in a mixed GD population of 131 from western Europe (157). In addition a conditional knock out murine model of GD was found to have increased incidences of B-cell lymphoma and monoclonal gammopathy (575). Pre-treatment of this model with a substrate reduction therapy, eliglustat tartrate, appeared to reduce the incidence of B-cell lymphoma and myeloma (576) suggesting the cause of the lymphoma and myeloma was directly linked to GD in this murine model.

A major feature of MM is extensive and recurrent bone disease with 70-80% of patients suffering osteolytic lesions at diagnosis (577). The development of cell culture techniques for the isolation and differentiation of cells present in the bone marrow microenvironment, especially osteoclasts and osteoblasts, has facilitated research into the interaction between plasma cells and other components of the bone marrow niche. A major component for survival and proliferation of the plasma cells has been found to be the monocyte/macrophages in the bone marrow shown to secrete factors such as IL-1b, IL-10, TNF-α and IL-6 (578). Studies have also shown that physical contact between macrophages and plasma cells protect the MM plasma cells from apoptosis by chemotherapy (579). Similarly, in vitro osteoclast-plasma cell cultures were found to greatly increase the survival of plasma cells when in direct contact (580) while plasma cells were also found to increase osteoclast generation by physical contact (581). Secretion of osteoclastogenic chemokines such as macrophage inflammatory protein 1α (MIP1α) and MIP1β by MM plasma cells have also been shown to affect adhesion of the MM plasma cells to the bone marrow stroma leading to increased RANKL expression and thus osteoclastogenesis (388).

In addition, recent research has provided evidence that many sphingolipids are bioactive and are involved in migration, proliferation and survival of several cell types (582,583) including plasma cells (584) osteoclasts and osteoblasts (380,585). Several sphingolipids including sphingosine-1-phosphate and ceramide-1-phosphate have been linked to cancers both solid tumour such as breast cancer for which Maczis et al demonstrate that S1P acts as an anti-apoptotic and growth-promoting factor (586) and pancreatic cancers in which Rivera et al suggest an increase in ceramide-1-phosphate enhanced migration of pancreatic cancer cells (587) and haematological such as multiple myeloma for which S1P has been suggested to increase plasma cell migration and inhibit dexamethasone-induced apoptosis (584).

The findings that GD macrophages differentiated either from monocytes isolated from peripheral blood (131) or from induced pluripotent stem cells (iPSC) (132) showed inflammasome activation with increased secretion of IL-1β and IL-6, in conjunction with evidence of elevated concentrations of several sphingolipids including sphingosine, sphingosine-1-phosphate and glucosylsphingosine in the serum and plasma of GD patients (152,153), with our findings of increased osteoclast generation in vitro suggests that the GD bone marrow may contain a combination of factors which provide both a pro-proliferation and pro-survival environment for plasma cells which may in part explain the increased incidence of multiple myeloma in GD.

## 6.2 Aims and Hypotheses

**Aim:** To understand the relationship between plasma cells and in vitro GD osteoclastogenesis.

**Objectives:**

(1) To determine whether plasma cells affect GD patient osteoclastogenesis in vitro.

(2) To determine whether sphingolipids found to be increased in GD plasma affect plasma cell survival and proliferation.

**Hypothesis 1:** GD osteoclastogenesis is further enhanced when cultured with myeloma plasma cells e.g. the NCI-H929 myeloma plasma cell line.

**Rationale:** Myeloma patient plasma cells and myeloma plasma cell lines have been reported to express cytokines such as IL-6, RANKL, TNF-α, MIP-1α known to stimulate osteoclastogenesis, contributing to the osteopenia and bone lesions found in over 80% of myeloma patients.

**Methods:** Co-culture of control or GD patient osteoclast cultures with a myeloma plasma cell line. Osteoclasts will be identified by being histochemically positive for TRAP.

**Hypothesis 2:** The altered sphingolipid profile in GD patients creates a pro-survival environment for plasma cells which in turn stimulate osteoclast generation, creating a positive effector loop between plasma cells and osteoclasts in GD.

**Rationale:** Several sphingolipids including glucosylsphingosine and glucosylceramide have been suggested to have roles as bioactive molecules in cancer. Both sphingolipids have been linked with increasing cellular proliferation and reducing apoptosis and glucosylceramide has been suggested to be involved in drug resistance.

**Methods:** Culture of a myeloma plasma cell line with a range of exogenous sphingolipids. Cell number determined by haemocytometer.

# 6.3 Results

## 6.3.1 Myeloma plasma cell line NCI-H929 Gaucher disease model.

Gaucher patients have been shown to have an increased relative risk of for developing multiple myeloma (157,241,260).

To address the role of plasma cells in Gaucher disease and the bone microenvironment a human plasma cell line NCI-H929 was used to model reduced β-glucocerebrosidase activity in plasma cells. To validate this model $2x10^4$ per ml NCI-H929 cells were cultured in RPMI-1640 supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES, 100 units/ml penicillin, 0.1mg/ml streptomycin (R10) with a range of concentrations of irreversible inhibitor CBE. Substantial inhibition of β-glucocerebrosidase was achieved with 100µM CBE (91.5% reduction in activity, figure 6.1a). While further reduction in activity was achieved with higher concentrations of CBE (250µM CBE resulted in a 95.7% decrease, figure 6.1a) 100µM was chosen for future experiments to minimise possible off target affects associated with this inhibitor (593). However, no significant toxicity was observed either through MTS assay (figure 6.1b) or through annexin V/ PI assay (figure 6.1c).

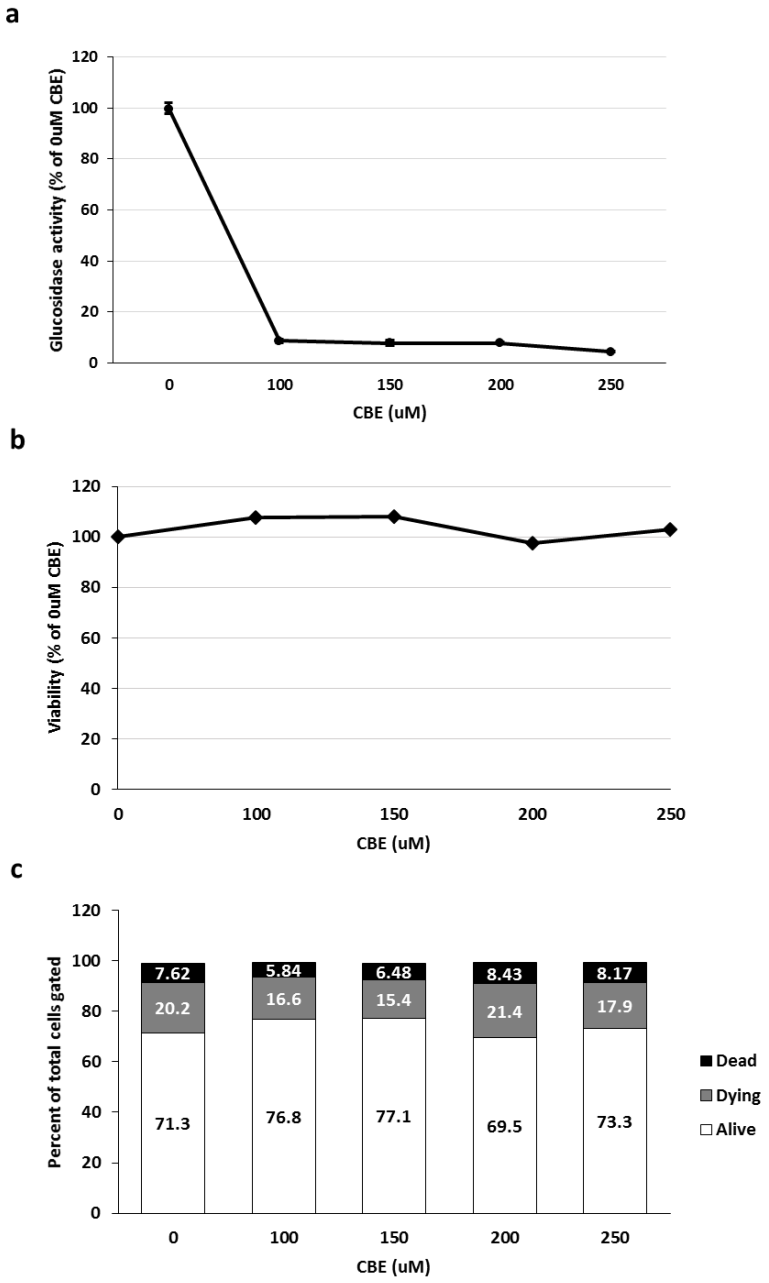



**Figure 6.1** Validation of NCI-H929 human plasma cell line as a model of Gaucher disease. **(a)** Inhibition of glucocerebrosidase activity by addition of an irreversible inhibitor CBE at a range of concentrations, cultured for 4 days in triplicate. Activity measured by fluorometric assay, mean ± SEM plotted. **(b)** Viability of cell line when cultured for 4 days with CBE at a range of concentrations in triplicate. Viability measured by MTS assay, 100% calculated from the averaged absorbance values obtained from the standard condition. Mean ± SEM plotted. **(c)** Viability of cell line when cultured for 4 days with CBE at a range of concentrations. Viability measured by annexin V/ PI flow cytometric assay, minimum of 50,000 gated events per condition.

### 6.3.2 NCI-H929 increased osteoclast generation when cultured with control subject mononuclear cells.

To assess the effect of plasma cells on osteoclast generation mononuclear cells were isolated from control and Gaucher subjects and cultured for 14 days in osteoclastogenic medium at a density of $1.25x10^5/cm^2$ on glass coverslips in wells of a 24 well plate. At day 14 NCI-H929 plasma cells were added to the culture for the remaining 7 days of culture at a density of $2x10^4$ cells/well either in direct contact or physically separated by being pipetted into transwells comprising a membrane with a pore size of 0.4μm which permitted exchange of chemicals and proteins while preventing migration of plasma cells and thus contact between osteoclasts and plasma cells. To investigate the Gaucher model in this system osteoclastogenic medium was supplemented with CBE (100μM), medium replaced twice per week for the 21 days of culture. NCI-H929 plasma cells were also pre-treated with 100μM CBE for 14 days in $25cm^2$ tissue culture flasks prior to addition to the osteoclast cultures. As previously observed control subject osteoclast generation was significantly increased when the mononuclear cells were cultured in the presence of CBE ($p = 0.035$, figure 6.2a). Similarly, control subject osteoclast generation was significantly increased in the presence of plasma cells ($p = 0.006$) when in direct contact. However, culture of control subject mononuclear cells with osteoclastogenic medium, CBE and plasma cells in direct contact, while significantly increasing osteoclast generation compared to standard conditions ($p = 0.037$), did not increase osteoclast numbers beyond those obtained for the individual conditions i.e. CBE only, plasma cells only. Similarly, cultures with pre-treated plasma cells either in the absence or presence of CBE during the 21 days of culture resulted in significant increases in osteoclast numbers (NCI pre-treated $p = 0.018$, NCI pre-treated + CBE $p = 0.021$) but not above those attained for individual conditions suggesting inhibition of β-glucocerebrosidase in this plasma cell line does not lead to further enhancement of osteoclast generation. Cultures in which the NCI-H929 plasma cells were physically separated from the osteoclasts by transwell showed no change in osteoclast generation either in the absence ($p = 0.252$) or presence ($p = 0.362$) of CBE suggesting direct contact is necessary for increased

osteoclastogenesis. Interestingly, while marked increases in osteoclast numbers were observed for control subjects no significant increases in osteoclast numbers were obtained in Gaucher subject cultures for any of the mentioned conditions (figure 6.2b).

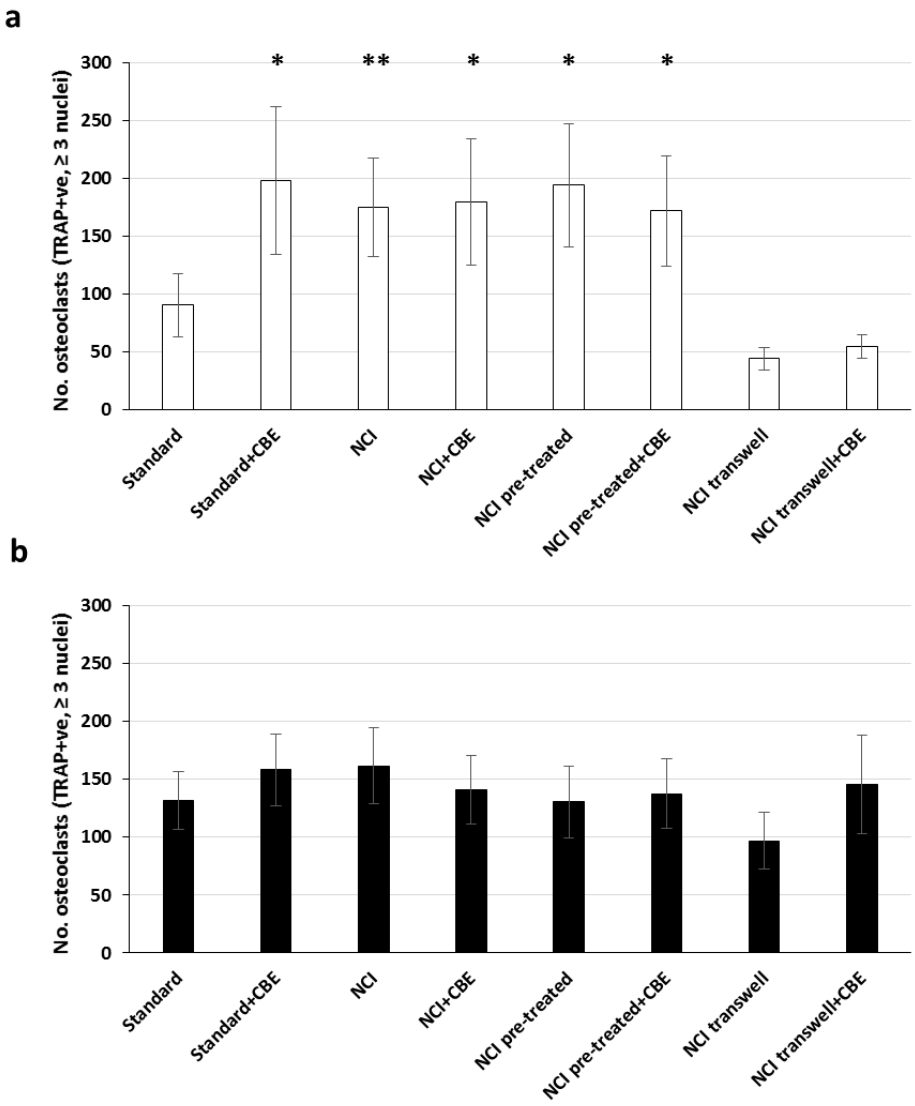



**Figure 6.2** Increased generation of control subject derived osteoclasts when co-cultured in contact with plasma cell line NCI H929. Number of osteoclasts generated from **(a)** control subjects, **(b)** Gaucher subjects, adherent mononuclear cells after 21 days of culture on glass with irreversible β-glucocerebrosidase inhibitor CBE (100µM), NCI H929 plasma cells, NCI H929 plasma cells pre-treated with CBE (100µM) for 14 days or a combination there of. Plasma cells were cultured either in contact or in transwells at a cell density of $2x10^4$ cells/well for the final 7 days of culture. For **(a)** n = 7 per condition except for transwell where n = 4 per condition, **(b)** n = minimum of 12 per condition except for transwell where n = 5 per condition. Mean ± SEM plotted, *p ≤ 0.05, **p ≤ 0.01, paired t-tests.

### 6.3.3 Exogenous sphingolipids affected NCI-H929 cell numbers over a 14-day culture.

To investigate the effects of exogenous sphingolipids on plasma cell proliferation and survival an initial experiment was carried out in which the NCI-H929 plasma cell line was cultured at a density of $4x10^4$ cells/ml with carrier (methanol 1:10,000 dilution) or sphingolipid (1μM) in the absence or presence of CBE (100 μM). Samples were collected for up to 14 days and cell density was determined by phase contrast microscopy using a haemocytometer. After 3 days, plasma cell density was significantly decreased when cultured in the presence of sphingosine ($p = 0.0038$) sphingosine-1-phosphate ($p < 0.0001$) or glucosylsphingosine ($p < 0.0001$) when compared to carrier alone (figure 6.3a). Cultures with CBE and sphingolipids resulted in similar reductions in cell density (sphingosine $p = 0.0001$, sphingosine-1-phosphate $p = 0.0041$, glucosylsphingosine $p < 0.0001$), figure 6.3b. Conversely, plasma cells cultured for 3 days in the presence of ceramide showed a significant increase in cell density ($p = 0.0006$) when compared to carrier alone. Again, a similar effect was observed for plasma cells cultured with CBE and ceramide ($p = 0.0004$). Plasma cell density was also higher in day 3 cultures with glucosylceramide however these did not reach significance (glucosylceramide $p = 0.17$, glucosylceramide + CBE $p = 0.205$), figures 6.3c and 6.3d. After 7 days of culture both glucosylceramide and ceramide cultures resulted in significantly increased cell density (glucosylceramide $p = 0.001$, ceramide $p = 0.0008$, figures 6.4a and 6.4b). Although these conditions in the presence of CBE also showed an increase in cell density the increases were not statistically significant (glucosylceramide + CBE $p = 0.064$, ceramide + CBE $p = 0.121$). Cultures with glucosylsphingosine continued to show decreased cell density after 7 days both in the absence or presence of CBE ($p < 0.0001$ for both, figure 6.4c). However, in the presence of CBE the decrease of cell density was significantly larger from days 7 to 14 compared to plasma cell cultures with glucosylsphingosine alone (day 7 $p = 0.00019$, day 10 $p = 0.015$, day 14 $p = 0.003$, figure 6.4d). Interestingly, the effects observed for sphingosine and sphingosine-1-phosphate were not present from days 7 to 14 (data not shown) suggesting their effects may be short lived.

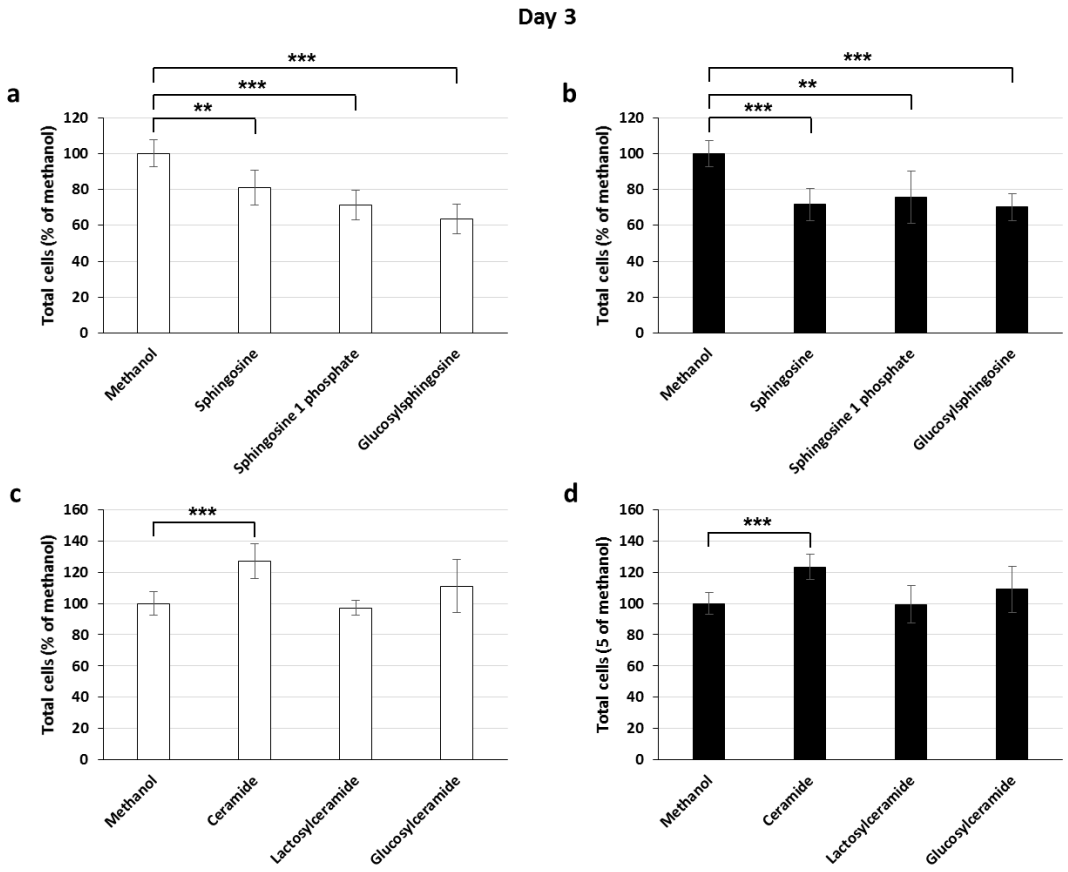



**Figure 6.3** Addition of exogenous sphingolipids in culture affect the total cell number of NCI-H929 plasma cells after 3 days. **(a, c)** Plasma cell line cultured with carrier (methanol 1:10,000 dilution) or sphingolipids (1µM) for 3 days. **(b, d)** Plasma cell line cultured with carrier (methanol 1:10,000 dilution) or sphingolipids (1µM), all with irreversible β-glucocerebrosidase inhibitor CBE (100µM) for 3 days. Total cell number determined by haemocytometer. Initial cell density $4x10^4$ cells/ml. 6 cultures per condition. 100% calculated as the averaged cell number of carrier or carrier + CBE. Mean ± SEM plotted. **p ≤ 0.01, ***p ≤ 0.001, unpaired t-tests.

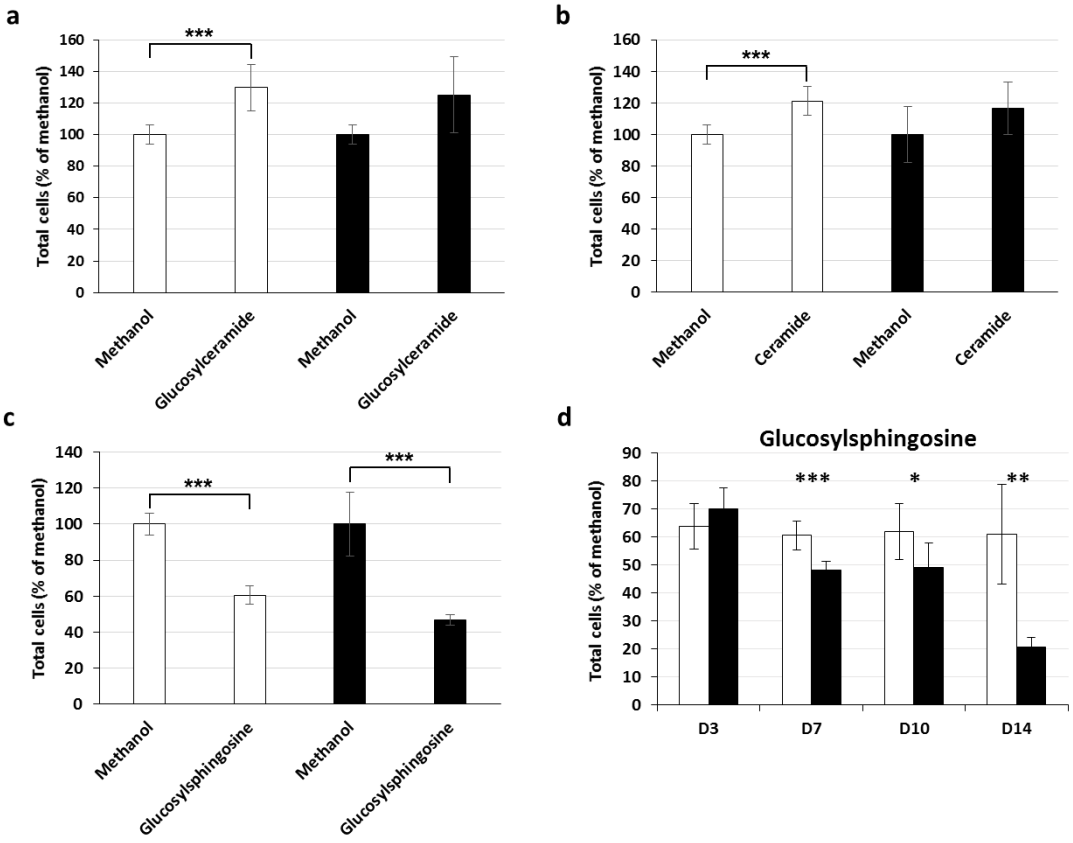



**Figure 6.4** Addition of exogenous sphingolipids in culture affect the total cell number of NCI-H929 plasma cells over time. **(a, b, c)** Plasma cell line cultured with carrier (methanol 1:10,000 dilution) or sphingolipids (1µM) in the absence (white bars) or presence (black bars) of irreversible β-glucocerebrosidase inhibitor CBE (100µM) for 7 days. **(d)** Plasma cell line cultured with glucosylsphingosine (1µM), cell number counted at days 3, 7, 10 and 14 of culture. Total cell number determined by haemocytometer. Initial cell density $4x10^4$ cells/ml. 6 cultures per condition. 100% calculated as the averaged cell number of carrier or carrier + CBE. Mean ± SEM plotted. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, unpaired t-tests.

## 6.4 Discussion

Several studies have reported an increased risk of cancer in GD, in particular for multiple myeloma (MM) up to a standardised rate ratio of 51.1 (157). In addition a conditional knock out murine model of GD was found to have increased incidences of B-cell lymphoma and monoclonal gammopathy (575). In vitro osteoclast-plasma cell cultures were found to greatly increase the survival of plasma cells when in direct contact (580) and plasma cells were found to increase osteoclast generation by physical contact (581). Recent research has provided evidence that many sphingolipids are bioactive and are involved in migration, proliferation and survival of several cell types (582,583,589) including plasma cells (584). Several sphingolipids including sphingosine-1-phosphate have been linked to haematological cancers such as multiple myeloma for which S1P has been suggested to increase plasma cell migration and inhibit dexamethasone-induced apoptosis (584). These findings in conjunction with our own finding of increased in vitro osteoclast generation in GD cultures suggest that GD patient bone marrow might provide an environment which supports plasma cell proliferation and survival. Therefore, to investigate this a human myeloma plasma cell line NCI-H929 was cultured with control or GD osteoclasts or with sphingolipids added to plasma cell culture medium.

The results discussed below demonstrate for the first time how osteoclast generation from GD patient samples is affected by the presence of a myeloma plasma cell line and how plasma cell number may be affected by sphingolipids found to be elevated in GD patient plasma, namely glucosylsphingosine and glucosylceramide.

**The NCI-H929 plasma cell line increase osteoclastogenesis when cultured in contact.**

As published by previous groups co-culture of plasma cells with control monocytes leads to an increase in osteoclast generation when in contact (590,591). While a

similar increase of osteoclast generation was seen with addition of CBE to osteoclast cultures there appeared to be no additive effect as cultures with both CBE and plasma cells added to the osteoclast culture did not result in a further increase in osteoclast number. Similarly, no significant increase in osteoclast number was observed for GD subject monocytes co-cultured with plasma cells suggesting the effect of reduced β-glucocerebrosidase activity and plasma cell co-culture stimulate osteoclastogenesis through the same signalling pathway. The lack of further increase when combined suggests that individually these factors maximally stimulate through this pathway, at least over the duration of the three weeks of culture, and therefore cannot further enhance the stimulation when combined. Pre-treatment of the plasma cell line with CBE prior to addition to osteoclast cultures again did not further enhance the osteoclast generation suggesting either that maximal stimulation had already been achieved by the plasma cell line alone or that reducing β-glucocerebrosidase activity in plasma cells does not affect their capability for stimulating osteoclast generation.

**Sphingolipids affect NCI-H929 plasma cell number.**

Certain sphingolipids such as ceramide (285,592), ceramide-1-phosphate (593), sphingosine-1-phosphate (278), glucosylsphingosine (272) and glucosylceramide (277,594), can affect cell proliferation, differentiation and apoptosis. In regards to the NCI-H929 myeloma plasma cell line a time course revealed that culture with exogenous sphingosine based lipids including sphingosine, sphingosine 1-phosphate and glucosylsphingosine resulted in lower total cell numbers after 3 days suggesting these sphingolipids either inhibited cell proliferation, induced apoptosis or a combination thereof. However, this effect was no longer present after 7 days of culture for sphingosine and sphingoine-1-phosphate suggesting this effect is short lived. In addition, no significant difference was observed between cell densities for any sphingolipids in the absence or presence of CBE over the 14-day time course with the exception of glucosylsphingosine. Previous MTS assays of MSC and SaOs-2 cultures with glucosylsphingosine suggested it may reduce proliferation or cell viability. This finding is further supported with this plasma cell line as the plasma

cell density was consistently lower across the 14-day time course in the absence of CBE (~65% of standard conditions). In the presence of CBE this effect appears to have been exacerbated as cell density continued to decrease over time decreasing from 70% of standard condition at day 3 to 20% by day 14. Whether this is a direct consequence of reduced β-glucocerebrosidase activity or related to altered sphingolipid processing is unclear.

Conversely, addition of ceramide resulted in a consistent increase in cell density over time of roughly 20% while culture with exogenous glucosylceramide induced a gradual increase of cell density, reaching statistical significance after 7 days of around 29% above standard conditions both in the absence or presence of CBE.

Assessment of viability using annexin V/Pi staining showed no significant difference in the percentage of viable cells for any sphingolipids suggesting their effects are related to proliferation rather than viability. The contrasting effects of glucosylsphingosine, ceramide and glucosylceramide suggests the relative concentrations of these sphingolipids in the bone marrow would dictate whether their overall effect would be to inhibit or stimulate proliferation of plasma cells and possibly MSC's.

**Limitations.**

The above findings have several limitations, firstly all of the data is generated using a cell line which may not be representative of the interaction of primary plasma cells with monocytes and osteoclasts or with sphingolipids. Secondly, these cells were cultured in vitro either in isolation or with osteoclasts which does not take into account the potential effects of other cells in the bone marrow such as mesenchymal stem cells, osteoblasts, osteocytes and T-cells. Thirdly, an assay to measure proliferation was not used therefore increased proliferation can only be implied, not confirmed. Fourthly, this culture system, as for all the monocultures performed in this project, is planar (2D) i.e. all the cells are cultured on a flat surface. In vivo, these cells would exist in a three-dimensional (3D) lattice. Fifthly, cell cultures were performed in static medium. This method can lead to a depletion

of nutrients and osteogenic factors and a build-up of waste products between medium changes. Finally, the plasma cells were cultured in the continuous presence of sphingolipids which may not be the case within the bone marrow. In addition, several sphingolipids would be present at varying concentrations within the bone marrow and not in isolation.

**Summary**

In summary, osteoclast cultures with the human plasma cell line NCI-H929 increased osteoclast numbers in control cultures but not GD cultures. Culture of the plasma cell line with glucosylsphingosine reduced plasma cell number consistently over a 14-day period, further reduced when also cultured with CBE. Conversely, culture with glucosylceramide increased plasma cell numbers. These findings demonstrate firstly that sphingolipids may play a significant role in the survival and proliferation of plasma cells and the development of multiple myeloma in GD patients. Secondly, that quantitation of the sphingolipid composition of GD patient bone marrow may provide insight as to whether the microenvironment is stimulating plasma cell proliferation potentially increasing the likelihood of developing multiple myeloma as different sphingolipids have been shown both in this chapter and other publications to affect proliferation and apoptosis.

# 7. In vitro co-culture of osteoblasts, osteoclasts and plasma cells

## 7.1 Introduction

In the previous chapters, some of the cells present in the bone marrow, namely osteoclasts, osteoblasts and plasma cells have been investigated in relation to reduced β-glucocerebrosidase activity, GD specific therapies and exogenous sphingolipids. In order to characterise these cells, it was necessary to culture these cells in isolation for the majority of experiments. Historically, this has also been the case for the majority of research into the differentiation and function of human cells in the bone microenvironment primarily due to the fact that establishment of techniques enabling isolation and culture of these cell types, in particular osteoclast precursors from peripheral blood, are relatively recent. This in turn has required substantial research focusing on determining what defines a cell as being for example an osteoclast or osteoblast and from which cell lineage these cells differentiated. As a result, there is now a wealth of data in relation to what constitutes for example an osteoclast, that they originate from the monocyte/macrophage lineage and their pre-cursors are present in the peripheral blood prior to homing to the bone marrow where they mature to become active, resorbing osteoclasts (595).

However, in the bone microenvironment these cells along with several other cell types co-exist in close proximity affecting each-others differentiation and function through both direct contact and secretion of several signalling molecules. One of the most common methods of research into these interactions is through use of murine models. Creation of murine gene knock-in or knock-out models both global and conditional have identified several genes and their respective proteins such as OPG (596) and MCSF (597) as being critical in the development and function of osteoblasts and osteoclasts. Murine models are also being used to investigate several cancers including breast (598) and prostate (599) in relation to bone metastasis and osteolytic disease in multiple myeloma (600). Similarly, several

murine models have been created to investigate cancers, Parkinson's disease and bone disease in GD (272,575,601,602). However, due to the fact that many of these murine models including GBA null (603), N370S homozygous (555), RecNcil homozygous and L444P homozygous (604) die shortly after birth or in the case of models homozygous for V394L, D409H, D409V (555) do not mimic the human phenotype, the creation of conditional knock-out models in specific cell types has been required to recapitulate the phenotypes seen in Gaucher patients, especially in regards to bone disease. The conditional knock-out model which most closely mimics the bone disease observed in GD is the Mistry murine model in which exons 8 to 11 are conditionally deleted, 2 days postnatal, in the cells of the hematopoietic and mesenchymal stem cell lineages (272). While this model provides essential data in regards to possible causes of bone disease in GD the fact that the exon deletions do not mimic mutations of Gaucher patients and that GBA1 activity is not reduced in all cell types means that the observed causes of bone disease in the murine model may not reflect the causes in the Gaucher patient. Therefore, it is necessary to develop in vitro models of the bone microenvironment using both human cells isolated from Gaucher patients and human cell lines to assess the data acquired from GD murine models. As a first step towards developing this model system osteoblasts, either the SaOs-2 cell line or MSC-derived, will be cultured with osteoclasts differentiated from a control subject and the human myeloma plasma cell line NCI-H929 in this chapter.

## 7.2 Aims and Hypotheses

**Aims:** To understand the effects of interaction between osteoblasts, osteoclasts and plasma cells in Gaucher disease.

**Objectives:** (1) To create an in vitro culture model which allows for the assessment of the interactions between osteoclasts, osteoblasts and plasma cells.

**Hypothesis 1:** GD osteoblast-osteoclast interaction with plasma cells enhances overall bone loss.

**Rationale:** Plasma cells have been shown to increase osteoclastogenesis and to secrete cytokines which induce osteoclast generation and function and to decrease differentiation of MSC's into osteoblasts. In addition, MGUS patients have been reported to have lower BMD.

**Methods:** Co-culture of primary and cell line osteoblasts with primary osteoclasts and myeloma plasma cell line. Mineralisation quantified by alizarin red staining and solubilisation.

**Hypothesis 2:** GD osteoclasts can protect plasma cells from apoptosis.

**Rationale:** Osteoclasts have been shown to secrete cytokines including IL-6 which stimulate plasma cell proliferation and survival.

**Methods:** Co-culture of primary and cell line osteoblasts with primary osteoclasts and myeloma plasma cell line. Viability assessed by flow cytometry.

## 7.3 Results

### 7.3.1 Co-culture of osteoblasts, osteoclasts and plasma cells.

To partially recreate the bone microenvironment a model culture system was developed in which osteoblast, osteoclast and plasma cells were co-cultured in a number of combinations and conditions to assess their interactions and the possible effects of having reduced β-glucocerebrosidase activity on these interactions.

In the first model culture system, the human osteoblast cell line SaOs-2 was cultured in previously optimised osteoblast medium D10-OB for 21 days to induce calcium deposition. Subsequently, for cultures which included osteoclasts, control subject mononuclear cells were isolated from peripheral blood and seeded at a density of $2.5x10^5$ cells per well either on their own or on top of the SaOs-2 monolayer in direct contact or physically separated by being seeded into transwells. All cells were cultured in osteoblast medium with osteoclastogenic cytokines MCSF and RANKL for the remainder of the culture. After a further 14 days, for cultures which included plasma cells, NCI-H929 cells were added at a density of $2x10^4$ cells per well either on their own, on top of SaOs-2 only, osteoclasts only or SaOs-2 with osteoclasts. Again these were seeded either in direct contact or physically separated using transwells which were either devoid of other cell types or already contained maturing osteoclasts. All conditions were cultured in the absence or presence of 100µM CBE. All cultures were continued for a further 7 days (42 days in total) prior to assessment.

All the above-mentioned combinations of cells involving the SaOs-2 cell line are shown in figure 7.1 showing the degree of calcium deposition as determined by alizarin red staining, solubilisation and quantitation. In order to aid visualisation of significant effects on calcium deposition the data in figure 7.1 has been sub-divided into graphs highlighting differences observed between certain conditions.

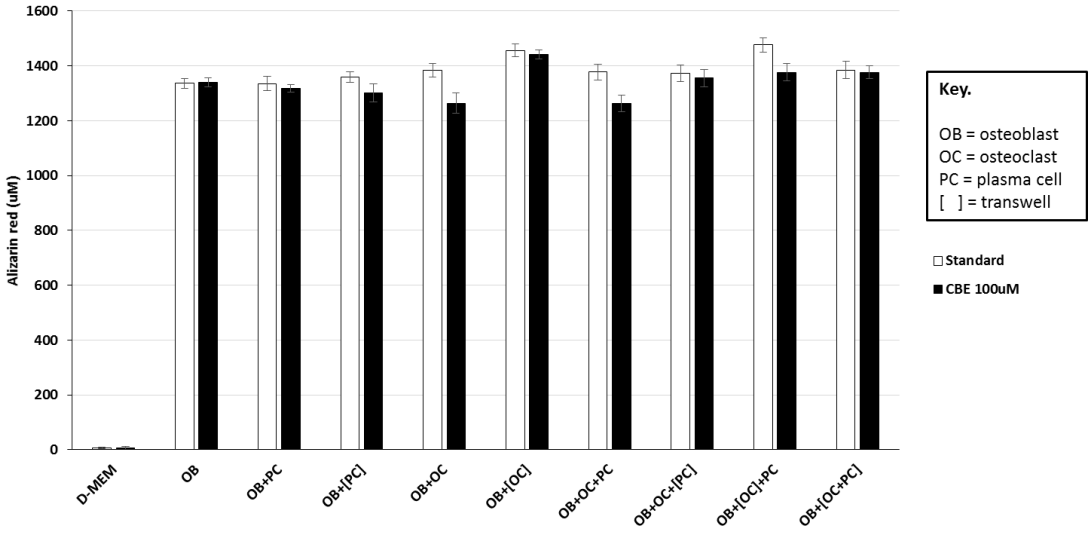



**Figure 7.1** An in vitro model of some of the cellular interactions of the bone microenvironment. A human osteoblast cell line SaOs-2 was cultured in osteogenic medium for 21 days in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100μM) prior to addition of control subject adherent mononuclear cells at a density of $2.5x10^5$ cells/well either in direct contact or physically separated by being seeded into transwells. Both cell types were cultured for a further 14 days in the presence of osteogenic medium comprised of both osteoblast and osteoclast differentiation and maturation factors (MCSF 25ng/ml, RANKL 30ng/ml). After these 14 days NCI H929 plasma cells were added for the final 7 days of culture at a cell density of $2x10^4$ cells/well. Total cell culture time for each combination was 42 days. 9 cultures per condition. Quantification of calcium deposition by human osteoblast cell line SaOs-2 by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted. Unpaired t-tests, significant differences shown in subsequent figures.

## 7.3.2 Inhibition with CBE resulted in reduced calcium deposition.

Comparison of conditions in the absence or presence of CBE showed that osteoblast cultures which included osteoclasts with or without plasma cells had significantly reduced calcium deposition in the presence of CBE when in direct contact with the osteoblasts (osteoblasts + osteoclasts vs osteoblasts + osteoclasts + CBE p = 0.016, osteoblasts + osteoclasts + plasma cells vs osteoblasts + osteoclasts + plasma cells + CBE p = 0.0136, figures 7.2a and 7.2b). Interestingly, the presence of osteoclasts when physically separated from the osteoblasts led to a statistically significant increase in calcium deposition (osteoblasts + osteoclasts in transwell + plasma cells vs osteoblasts + osteoclasts in transwell + plasma cells + CBE p =

0.0289, figure 7.2c) however when cultured with CBE this effect was abrogated suggesting CBE may affect calcium deposition in addition to increasing osteoclast resorption in the presence of plasma cells.

### 7.3.3 Osteoclasts in contact with osteoblasts resulted in lower calcium deposition.

When assessing the effect of osteoclasts on calcium deposition, cultures in which osteoclasts were in direct contact with the osteoblast monolayer and thus the calcium matrix had significantly reduced amounts of calcium compared to cultures with osteoclasts physically separated by transwell (osteoblasts + osteoclasts vs osteoblasts + osteoclasts in transwell $p = 0.0498$, osteoblasts + osteoclasts + plasma cells vs osteoblasts + osteoclasts in transwell + plasma cells $p = 0.0214$, figure 7.3).

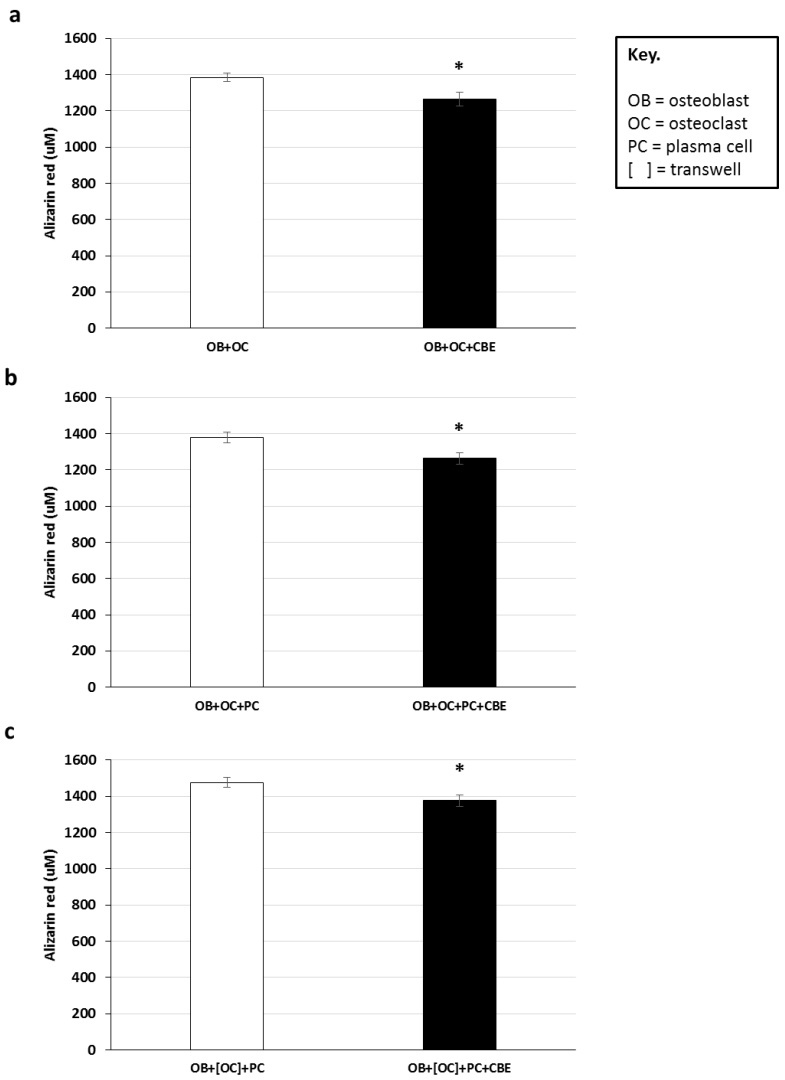



**Figure 7.2** Addition of CBE to osteoblast cultures reduced calcium deposition. Human osteoblast cell line SaOs-2 was cultured in osteogenic medium for 21 days in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100μM) prior to addition of **(a)** control subject adherent mononuclear cells at a density of $2.5x10^5$ cells/well. Both cell types were cultured for a further 21 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors in the absence (white bars) or presence (black bars) of irreversible β-glucocerebrosidase inhibitor CBE (100μM). **(b)** Cultured as in (a) but with NCI-H929 plasma cells ($2x10^4$ cells/well) added after 14 days of culture with the adherent mononuclear cells, 35 days into the overall culture. All three cell types were co-cultured for a further 7 days in the same medium in the absence or presence of CBE. **(c)** Cultured as in (b) except the control subject adherent mononuclear cells were physically separated from both osteoblasts and plasma cells by being seeded into transwells. 9 cultures per condition. Quantification of calcium deposition by human osteoblast cell line SaOs-2 by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted. *$p \leq 0.05$, unpaired t-tests.

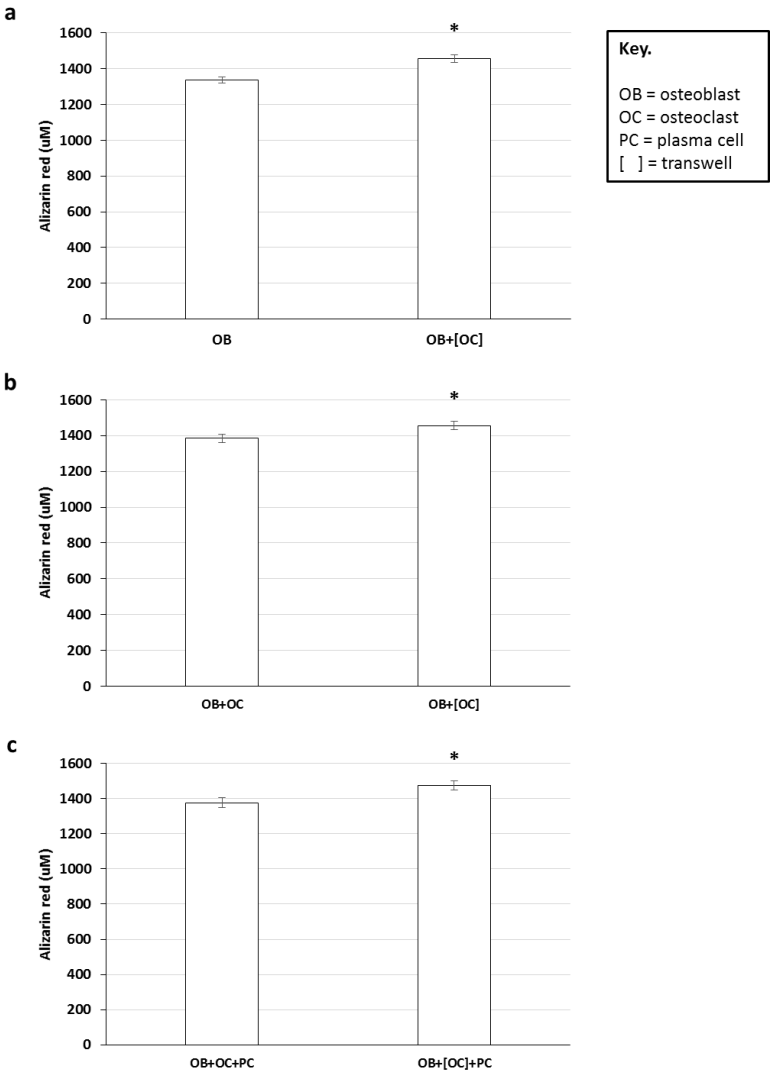



**Figure 7.3** Osteoclasts require contact to reduce calcium deposition. Human osteoblast cell line SaOs-2 was cultured in osteogenic medium for 21 days in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100µM) prior to addition of **(a)** control subject adherent mononuclear cells at a density of $2.5x10^5$ cells/well physically separated from osteoblasts by being seeded into transwells. Both cell types were cultured for a further 21 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors. Osteoblasts cultured on their own, labelled OB, were cultured for a total of 42 days. **(b)** Cultured as in (a) except control subject adherent mononuclear cells were added either in direct contact or physically separated by transwell. **(c)** Cultured as in (b) but with NCI-H929 plasma cells ($2x10^4$ cells/well) added after 14 days of culture with the adherent mononuclear cells, 35 days into the overall culture. All three cell types were co-cultured for a further 7 days in the same medium. 9 cultures per condition. Quantification of calcium deposition by human osteoblast cell line SaOs-2 by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted. *$p \leq 0.05$, unpaired t-tests.

### 7.3.4 Myeloma plasma cells increase calcium deposition when in contact with osteoblast cells.

Osteoblast-osteoclast co-cultures in which plasma cells were in direct contact with the osteoblast monolayer resulted in significantly higher calcium deposition compared to cultures in which the plasma cells were physically separated (osteoblasts + osteoclasts in transwell + plasma cells vs osteoblasts + osteoclasts + plasma cells both in transwell $p = 0.04$, osteoblasts + osteoclasts in transwell + plasma cells vs osteoblasts + osteoclasts + plasma cells in transwell $p = 0.02$, figures 7.4a and 7.4b). However, the difference in deposition in figure 7.4b may also be due to direct contact between osteoblasts and osteoclasts. The presence of osteoclasts in the culture when separated from osteoblasts led to a significantly higher amount of calcium deposition compared to osteoblasts cultured in isolation, $p = 0.0004$, which was further increased in the presence of plasma cells, $p = 0.0001$ (figure 7.4c). Suggesting there may be signalling factors from both plasma cells and osteoclasts which may increase calcium deposition.

### 7.3.5 Addition of CBE increases the effect observed by different cell types on calcium deposition.

Similar effects were observed in the presence of CBE. Osteoblast cultures in which osteoclasts were in direct contact again showed reduced calcium deposition however statistical significance was only achieved when plasma cells were also in direct contact (osteoblasts + CBE vs osteoblasts + osteoclasts + plasma cells + CBE $p = 0.0398$, figure 7.5a). Cultures in which osteoclasts were separated from osteoblasts by transwell led to significantly increased calcium deposition (osteoblasts + CBE vs osteoblasts + osteoclasts in transwell + CBE $p = 0.0005$, osteoblasts + osteoclasts + CBE vs osteoblasts + osteoclasts in transwell + CBE $p = 0.0005$, figures 7.5b and 7.5c). When all three cell types were in direct contact calcium deposition was significantly lower than when either plasma cells or osteoclasts were physically separated from the osteoblast monolayer as shown in

figure 7.5d. As it has been previously shown that direct contact between plasma cells and mononuclear cells is required for increased osteoclast generation it would be logical to conclude that direct contact between these cells and the calcium matrix would result in lower amounts of calcium overall.

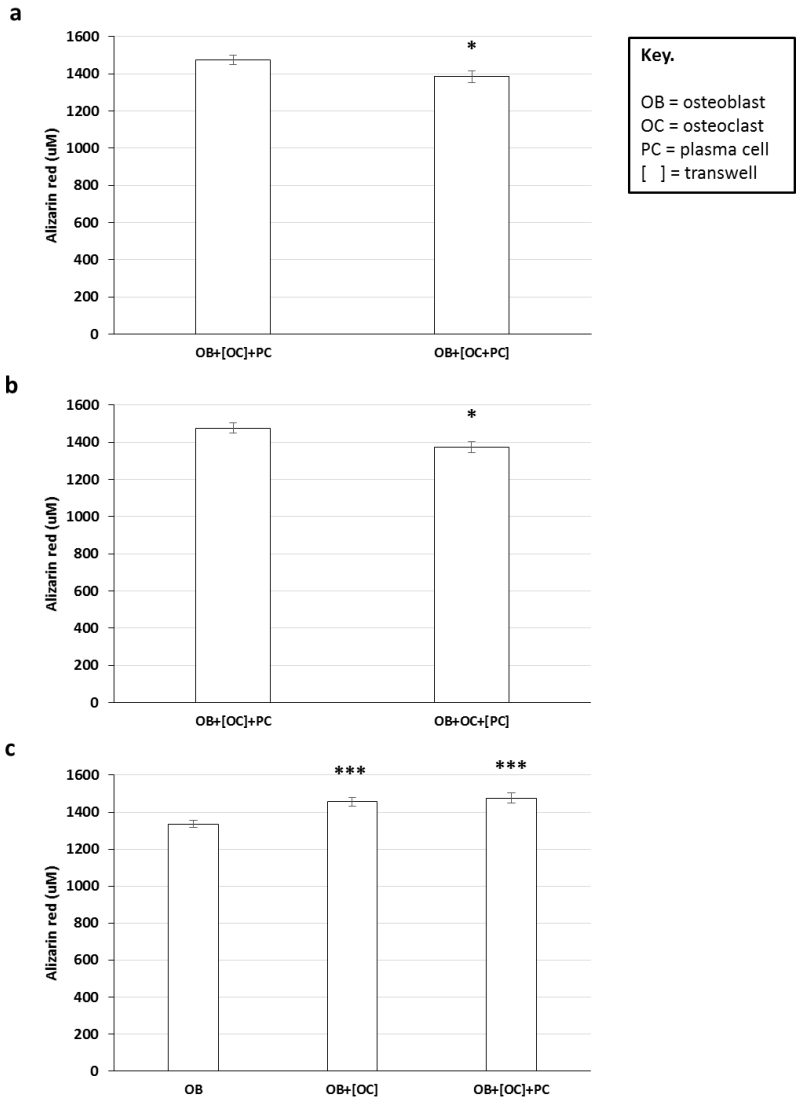



**Figure 7.4** Myeloma plasma cells increase calcium deposition when in contact with osteoblast cells. Human osteoblast cell line SaOs-2 was cultured in osteogenic medium for 21 days in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100µM) prior to addition of **(a)** control subject adherent mononuclear cells at a density of $2.5x10^5$ cells/well physically separated from osteoblasts by being seeded into transwells. Both cell types were then cultured for a further 14 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors. After these 14 days NCI H929 plasma cells were added for the final 7 days of culture at a cell

density of $2x10^4$ cells/well either in direct contact or physically separated by addition to transwells already containing the adherent mononuclear cells. **(b)** Cultured as in (a) except the transwells contained only plasma cells. **(c)** Cultured as in (a) except plasma cells were cultured in direct contact with osteoblasts. 9 cultures per condition. Quantification of calcium deposition by human osteoblast cell line SaOs-2 by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted. *p ≤ 0.05, ***p ≤ 0.001, unpaired t-tests.

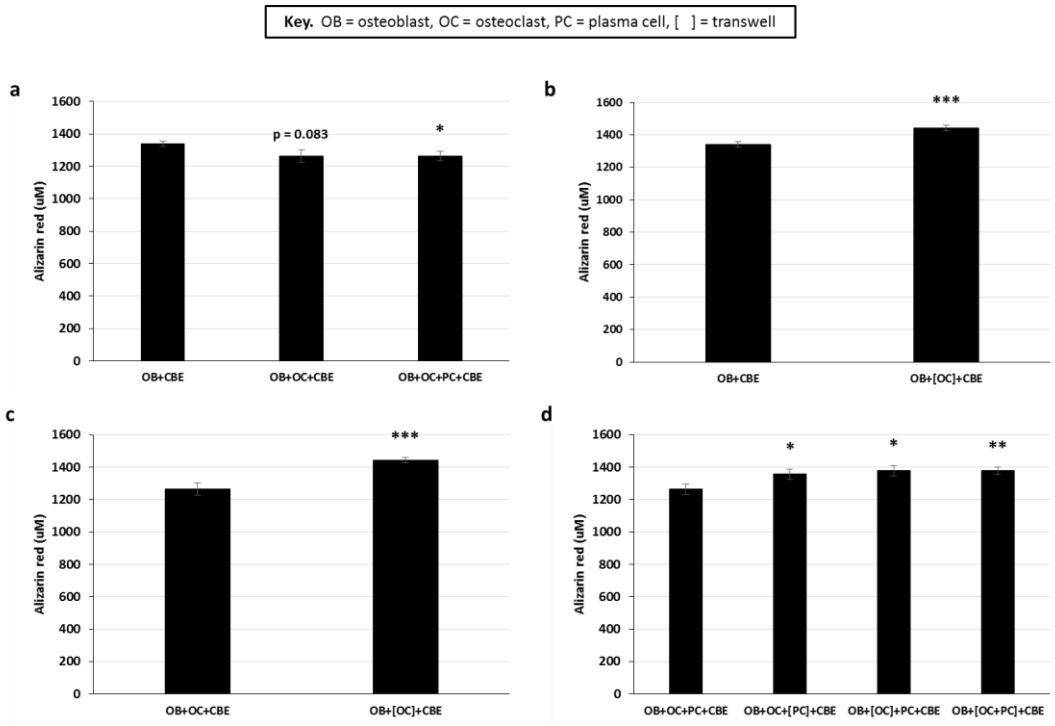



**Figure 7.5** Addition of CBE increases the effect observed by different cell types on calcium deposition. Human osteoblast cell line SaOs-2 was cultured in osteogenic medium D10-OB for 21 days in the presence of irreversible β-glucocerebrosidase inhibitor CBE (100µM) prior to addition of **(a)** control subject adherent mononuclear cells at a density of $2.5x10^5$cells/well. Both cell types were cultured for a further 14 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors and irreversible β-glucocerebrosidase inhibitor CBE (100µM). NCI-H929 plasma cells ($2x10^4$ cells/well) were added after a further 14 days of culture with the adherent mononuclear cells, 35 days into the overall culture. All three cell types were co-cultured for a further 7 days in the same medium. **(b)** Cultured as in (a) except control subject adherent mononuclear cells were physically separated by being seeded into transwells and without plasma cell addition. **(c)** Cultured as in (b) except adherent mononuclear cells were cultured either in direct contact or physically separated by being seeded into transwells. **(d)** Cultured as in (c) except control subject adherent mononuclear cells and plasma cells were either added directly or physically separated by culture

in transwell. 9 cultures per condition. Quantification of calcium deposition by human osteoblast cell line SaOs-2 by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, unpaired t-tests.

### 7.3.6 Plasma cell viability is greatly increased when cultured in contact with osteoclasts.

At the end of the 42 day cultures the NCI-H929 plasma cells, added to the cultures for the final seven days, were harvested and assessed for viability by flow cytometry using annexin V/Pi staining with a minimum of 50,000 gated events per condition. Comparison of all conditions is shown in figure 7.6a in order of increasing percentage viability. Unexpectedly, direct contact between plasma cells and the osteoblast cell line SaOs-2 resulted in a substantial decrease in plasma cell viability decreasing from 64.3% when cultured in isolation to 5.1% when in contact with the osteoblasts. This effect was largely reversed when the plasma cells were physically separated by transwell, figure 7.6b. The presence of osteoclasts in the cultures also appeared to affect plasma cell viability, again mediated through direct contact. In cultures where osteoclasts were separated from osteoblasts and plasma cells by transwell viability was again markedly lower at 3.9% without CBE and 5.6% with CBE. Culture of plasma cells in contact with both osteoblasts and osteoclasts partially rescued plasma cell viability increasing to 23.5% without CBE and 26.9% with CBE. Separation of plasma cells from the osteoblasts and osteoclasts by transwell returned viability to a similar percentage observed for plasma cells cultured in isolation (64.6% without CBE, 62.3% with CBE) while the most favourable conditions appeared to be when plasma cells were cultured in direct contact with osteoclasts but separated from the osteoblasts, viability reaching 85.5% without CBE and 85.9% with CBE, figure 7.6c. Suggesting osteoclasts may play a key role in plasma cell viability in the bone marrow microenvironment. Inclusion of the irreversible inhibitor CBE did not noticeably affect plasma cell viability in any of the co-culture combinations.

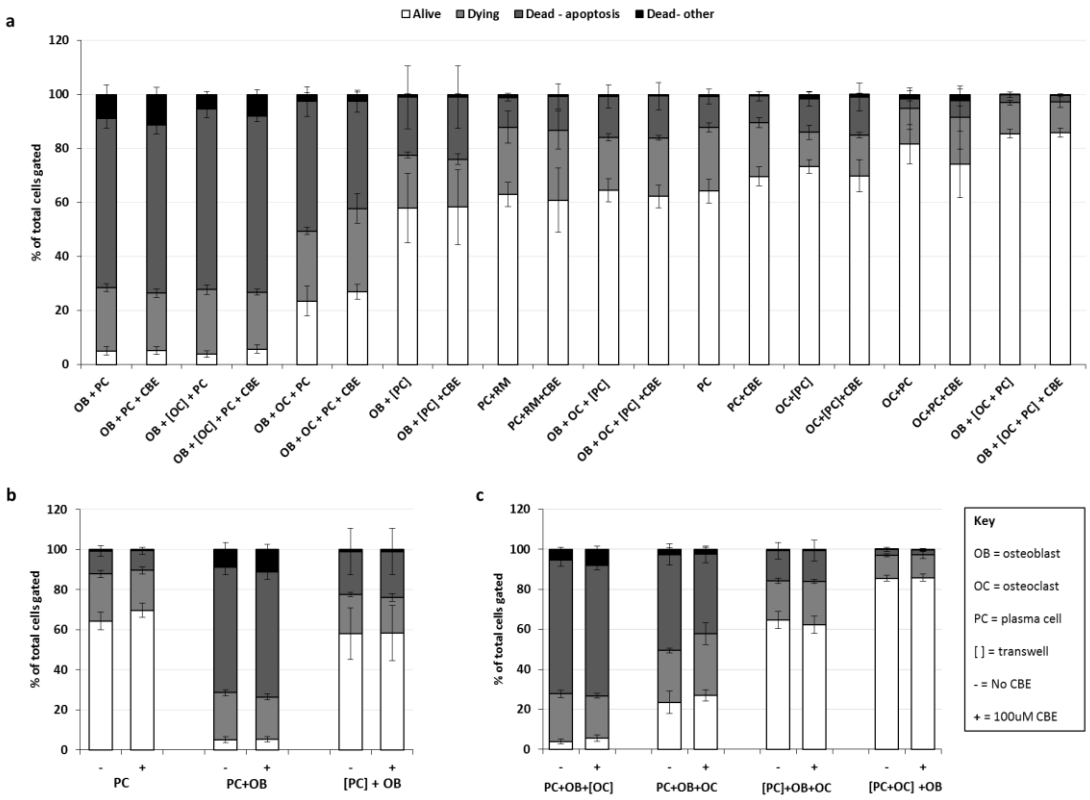



**Figure 7.6** Plasma cell viability is greatly increased when cultured in contact with osteoclasts. **(a, b, c)** Human osteoblast cell line SaOs-2 was cultured in osteogenic medium D10-OB for 21 days in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100µM) prior to addition of control subject adherent mononuclear cells at a density of $2.5x10^5$cells/well either in direct contact or physically separated by being seeded into transwells. Both cell types were cultured for a further 14 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors. After these 14 days NCI H929 plasma cells were added for the final 7 days of culture at a cell density of $2x10^4$ cells/well. Total cell culture time for each combination was 42 days. Plasma cells were also cultured either in isolation or for the final 7 days of a 21 day culture with control subject adherent mononuclear cells in osteogenic medium comprised of both osteoblast and osteoclast differentiation and maturation factors. Plasma cells were removed from culture at day 42 and percentage viability determined by annexin V/ Pi assay. **(b, c)** Selected data from **(a)**. Minimum of 50,000 gated events per condition. All conditions in triplicate. Mean ± SEM plotted.

### 7.3.7 Plasma cells reduce calcium deposition by control subject osteoblasts.

Further development of the culture system to partially recreate the bone microenvironment led to cultures with primary human mesenchymal stem cells (MSC's) from a control subject differentiated into osteoblasts in place of the human osteoblast cell line SaOs-2. This system required culturing the MSC's in optimised osteoblast medium A10-OB for 5 weeks prior to the addition of control subject mononuclear cells and plasma cell line NCI-H929. The remaining culture protocol remained the same as with the SaOs-2 cell line with an overall culture duration of 56 days. The degree of calcium deposition, determined by alizarin red staining, solubilisation and quantitation for all combinations, again the same as those used for the SaOs-2 co-cultures, is shown in figure 7.7. In comparison to cultures with SaOs-2, physical contact between osteoblasts and osteoclasts led to a reduction in calcium deposition in the absence of CBE (20.7% decrease) however this decrease was not statistically significant. Cultures in which osteoclasts were in physical contact also had lower calcium deposition when compared to cultures with osteoclasts in transwell but again these differences were not statistically significant.

Similarly, osteoblasts cultured with NCI-H929 plasma cells either in contact or transwell resulted in decreased calcium deposition in the absence of CBE (24.5% and 23.8% respectively, figure 7.7a) but again this decrease was not statistically significant in either case. However, this reduction in calcium deposition appears to be rescued in the presence of osteoclasts when the osteoclasts are physically separated by culture in transwell. Cultures in which plasma cells were in contact with osteoblasts with osteoclasts in transwell had significantly higher calcium deposition than cultures with osteoblast and plasma cells only both in the absence (48.7% increase, $p = 0.02$) or presence of CBE (40.4% increase, $p = 0.01$), figure 7.7b. Calcium deposition was also higher when plasma cells and osteoclasts were both physically separated from the osteoblasts by co-culture in transwell compared to osteoblasts cultured with plasma cells only either when the plasma cells were cultured in contact (44.9% increase, $p = 0.007$) or transwell (46.2%, $p = 0.004$) with osteoblasts, figure 7.7c.

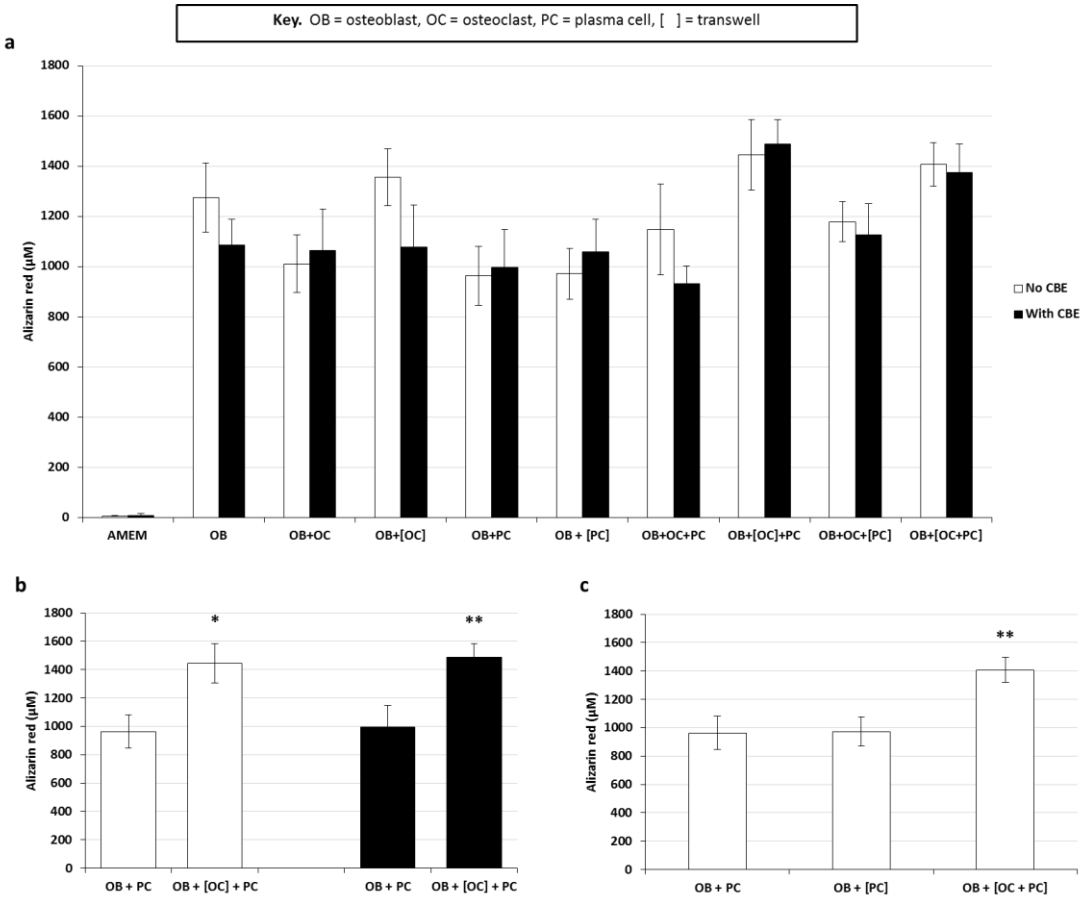



**Figure 7.7** Plasma cells reduce calcium deposition by control subject osteoblasts, rescued by osteoclasts in transwell. Control subject 2186 MSC were differentiated into active osteoblasts by culture in osteogenic medium A10-OB for 35 days in the absence (white columns) or presence (black columns) of irreversible β-glucocerebrosidase inhibitor CBE (100µM) prior to addition of **(a, b, c)** control subject adherent mononuclear cells at a density of $2.5x10^5$ cells/well either in direct contact or physically separated by being seeded into transwells. Both cell types were cultured for a further 14 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors. After these 14 days NCI H929 plasma cells were added either in direct contact or physically separated by being seeded into transwells for the final 7 days of culture at a cell density of $2x10^4$cells/well. Total cell culture time for each combination was 56 days. **(b, c)** selected data from (**a**). A minimum of 6 cultures per condition. Quantification of calcium deposition by MSC-derived osteoblasts determined by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted.

### 7.3.8 Addition of CBE increases the effect of osteoclast related reduction of calcium deposition by osteoblasts.

The same experimental approach was used for MSC's isolated from the bone marrow of a multiple myeloma subject, sample 2167. While no significant differences were observed for any combination of cells without CBE, the addition of CBE to the culture system did result in some notable differences. Cultures in which osteoblasts and osteoclasts were in direct contact showed no significant differences in calcium deposition without CBE ($p = 0.45$) however in the presence of CBE calcium deposition was deceased by over 30% compared to osteoblasts cultured in isolation ($p = 0.022$). While significance was not obtained when comparing osteoblasts cultured in isolation with CBE and osteoblasts cultured in direct contact with osteoclasts with CBE ($p = 0.12$). Similarly, osteoblasts cultured in direct contact with osteoclasts in the presence of CBE resulted in significantly lower calcium deposition compared to osteoblast-osteoclast cultures without CBE ($p = 0.044$), figure 7.8b. The inclusion of plasma cell line NCI-H929, physically separated by transwell, in the osteoblast-osteoclast cultures also resulted in significantly lower calcium deposition ($p = 0.035$) compared to osteoblasts cultured in isolation, figure 7.8c. For all conditions the degree of calcium deposition in the presence of CBE was lower than that obtained without CBE. Although statistical significance was not attained for many of these combinations it may be worth increasing the number of cultures with this subjects MSC's and with other subjects to determine whether this general decrease is consistent and a potential consequence of reduced β-glucocerebrosidase activity.

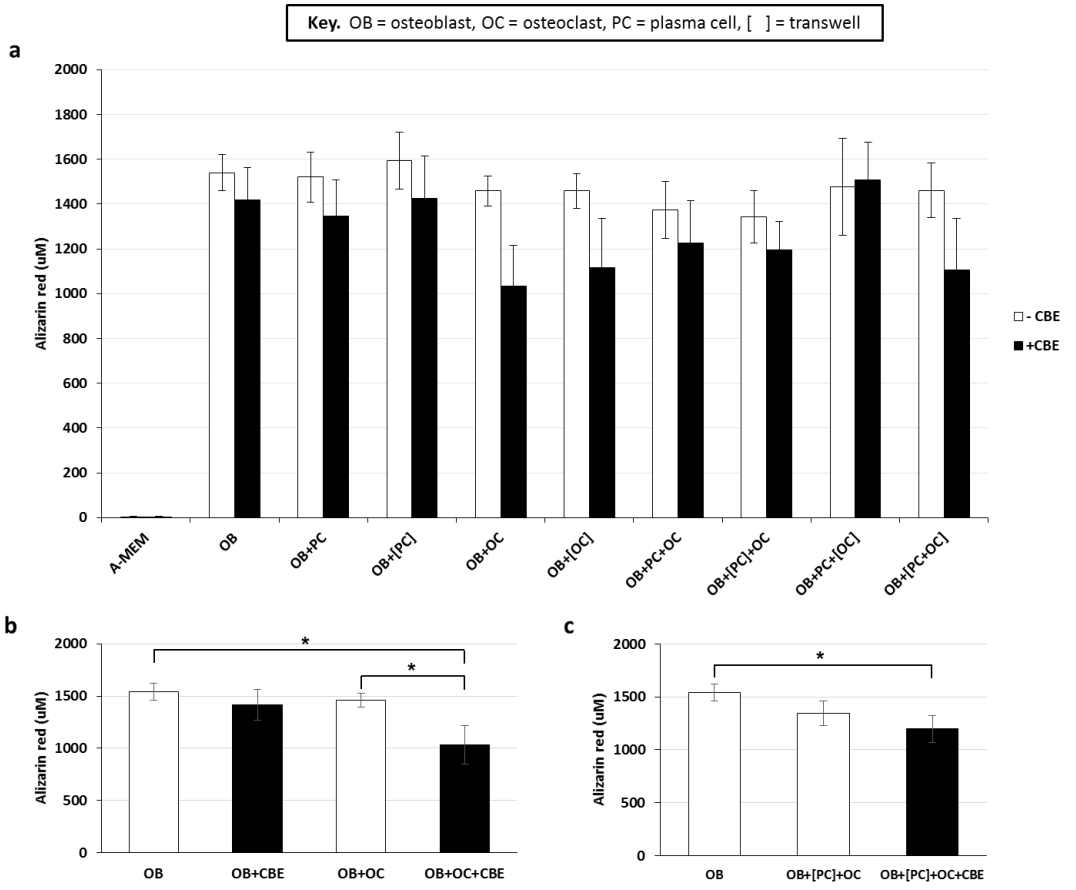



**Figure 7.8** Addition of CBE increases the effect of osteoclast related reduction of calcium deposition by osteoblasts. Multiple myeloma subject 2167 MSC were differentiated into active osteoblasts by culture in osteogenic medium A10-OB for 35 days in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100µM) prior to addition of **(a, b, c)** control subject adherent mononuclear cells at a density of $2.5x10^5$cells/well either in direct contact or physically separated by being seeded into transwells. Both cell types were cultured for a further 14 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors. After these 14 days NCI H929 plasma cells were added either in direct contact or physically separated by being seeded into transwells for the final 7 days of culture at a cell density of $2x10^4$cells/well. Total cell culture time for each combination was 56 days. **(b, c)** selected data from (a). 9 cultures per condition. Quantification of calcium deposition by MSC-derived osteoblasts determined by absorbance spectrometry of alizarin red solubilised post staining of calcium on cell monolayer surface. Mean ± SEM plotted. *$p \leq 0.05$, unpaired t-tests.

### 7.3.9 NCI-H929 viability was not affected by culture with MSC derived osteoblasts, osteoclasts or combinations thereof.

At the end of the 56 day cultures the NCI-H929 plasma cells, added to the cultures for the final seven days, were harvested and assessed for viability by flow cytometry using annexin V/Pi staining with a minimum of 50,000 gated events per condition. Comparison of all conditions is shown in figures 7.9a and b in order of increasing percentage viability for control subject 2186 and MM subject 2167 respectively. No statistically significant differences in viability were observed between any co-culture cell combination either in the absence or presence of CBE or when comparing cultures with or without CBE with the same cell components and arrangements.

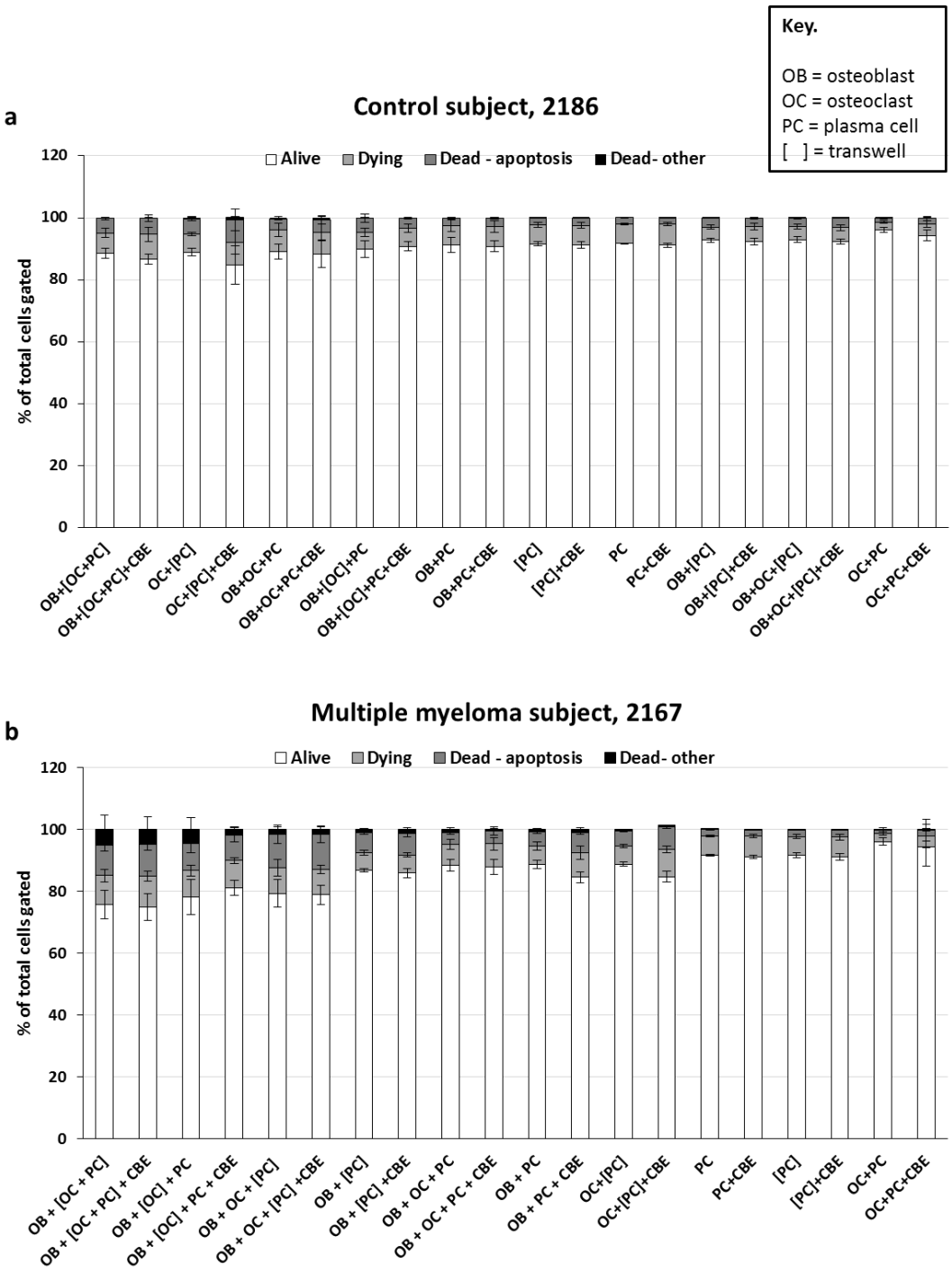



**Figure 7.9** Plasma cell viability is unaffected when cultured in contact with primary osteoblasts. **(a, b)** Human MSC from **(a)** control subject 2186 and **(b)** MM subject 2167 were cultured in osteogenic medium A10-OB for 35 days in the absence or presence of irreversible β-glucocerebrosidase inhibitor CBE (100µM) prior to addition of control subject adherent mononuclear cells at a density of $2.5x10^5$ cells/well either in direct contact or physically separated by being seeded into transwells. Both cell types were cultured for a further 14 days in the presence of osteogenic medium comprising of both osteoblast and osteoclast differentiation and maturation factors. After these 14 days NCI H929 plasma cells were added for the final 7 days of culture at a cell density of $2x10^4$ cells/well. Total cell culture time

for each combination was 56 days. Plasma cells were also cultured either in isolation or for the final 7 days of a 21-day culture with control subject adherent mononuclear cells in osteogenic medium comprised of both osteoblast and osteoclast differentiation and maturation factors. Plasma cells were removed from culture at day 56 and percentage viability determined by annexin V/ Pi assay. Minimum of 50,000 gated events per condition. All conditions in triplicate. Mean ± SEM plotted.

## 7.4 Discussion

In the bone microenvironment osteoblasts, osteoclasts and plasma cells, along with several other cell types, co-exist in close proximity affecting each-others differentiation and function through both direct contact and secretion of several signalling molecules. One of the most common methods of research into these interactions is through use of murine models. The murine model which most closely mimics the bone disease observed in GD is the Mistry model in which exons 8 to 11 are conditionally deleted, 2 days postnatal, in the cells of the hematopoietic and mesenchymal stem cell lineages (272). While this model provides essential data in regards to possible causes of bone disease in GD the fact that the exon deletions do not mimic mutations of Gaucher patients and that GBA1 activity is not reduced in all cell types means that the observed causes of bone disease in the murine model may not reflect the causes in the Gaucher patient. Therefore, it is necessary to develop in vitro models of the bone microenvironment using both human cells isolated from Gaucher patients and human cell lines to further assess the data acquired from GD murine models. As a first step towards developing this model system osteoblasts, either the SaOs-2 cell line or MSC-derived, were cultured with osteoclasts differentiated from a control subject and the human myeloma plasma cell line NCI-H929 in vitro.

For the first time a human cell based model of the GD bone microenvironment has been developed to investigate the interactions between osteoclasts, osteoblasts and plasma cells in the GD bone marrow microenvironment.

**SaOS-2 co-cultures.**

Culture with CBE led to a significantly lower amount of calcium deposition when SaOs-2 cells were co-cultured with osteoclasts with or without plasma cells. This supports previous findings that CBE increases osteoclast generation which in turn would result in increased bone matrix degradation and resorption resulting in overall lower calcium deposition. However, the same could be expected of cultures in which all three cell types were in contact in the absence of CBE as previous data showed that plasma cells in contact with monocytes also increase osteoclast generation. This was not the case as the triple culture with all cells in direct contact produced a small but not significant increase in calcium deposition in comparison to osteoblasts alone. However, this effect may have been negated as subsequent data showed that culture of the plasma cell line in contact with the SaOS-2 cells resulted in substantial plasma cell apoptosis of ~95%.

A potentially interesting finding is that cultures in which osteoclasts were physically separated from the SaOs-2 resulted in higher amounts of calcium deposition in comparison to SaOs-2 on their own or co-cultured with osteoclasts in contact both in the absence or presence of plasma cells. This finding may suggest that osteoclasts and their precursors produce factors which stimulate osteoblast maturation and activity when not in physical contact, acting as coupling factors mediating bone metabolism.

Plasma cells appear to increase calcium deposition when in physical contact with SaOs-2 cells though in most cases the presence of plasma cells increases calcium deposition relative to SaOs-2 alone whether they are in contact or not. These findings suggest that while plasma cells are known to stimulate osteoclastogenesis resulting in the severe bone disease suffered by the majority of multiple myeloma patients they may also increase the activity of osteoblast mediated mineralisation though probably to a much lesser extent as the observed increases are quite small reaching a maximum of a 10.5% increase for SaOs-2 cultured in contact with plasma cells with osteoclasts physically separated. Again, this result may have been affected by the apoptosis of the plasma cells when cultured in contact with the SaOs-2 cells.

It should also be noted that in the majority of conditions the increase or decrease in calcium deposition was relatively small. This may be due to the fact that these cultures are with the SaOs-2 cell line which may be less sensitive to external factors due to its high rates of proliferation and calcium deposition in comparison to MSC derived osteoblast cultures.

**Plasma cell viability is markedly reduced when in contact with SaOs-2 cells.**

Unexpectedly, co-culture of NCI-H929 myeloma plasma cells with the SaOs-2 osteoblast-like cell line induced substantial apoptosis in the plasma cells of around 95%. An effect not observed in MSC derived osteoblast co-cultures. This effect appears to be contact dependent with the percentage of viable plasma cells equivalent to monocultures when separated from the SaOs-2 by transwell. The cause of this apoptosis was not investigated but it demonstrates potentially important differences between this cell line and primary cells suggesting that while cell lines are useful in regards to availability, ease of generating large cell numbers for culture and eliminates sample variation they may not faithfully replicate the function of primary cells. Therefore, if possible primary cells should be used to confirm any findings based on cell lines especially when they are involved in systems which require such interdependent interaction between several cell types.

Although this outcome appears to be an anomaly of this cell line it did provide an opportunity to investigate the interaction between osteoclasts and plasma cells in regards to plasma cell survival. Co-cultures in which osteoclasts were present increased the percentage of viable plasma cells with partial rescue of viability when all three cell types were in contact but a much more substantial effect when osteoclasts were in contact with the plasma cells in a transwell. As there was little difference in viability between cultures in the presence or absence of osteoclasts in contact with the SaOs-2 when plasma cells were cultured in transwell it would suggest this pro-survival effect is also contact dependent as the plasma cell cultures with the highest viability were those in which osteoclasts were in direct contact with plasma cells but not cultured with or not in contact with the SaOs-2 cells. This

finding suggests there may be a positive feedback loop between osteoclast generation and plasma cell survival in which plasma cells increase osteoclast numbers which in turn increase plasma cell survival. Increased plasma cell survival increases plasma cell numbers which in turn increases the pro-osteoclastogenic stimuli leading to a further increase in osteoclast generation etc.

Addition of CBE to cultures did not lead to any significant differences suggesting deficiency in β-glucocerebrosidase activity does not affect the SaOs-2 pro-apoptotic or osteoclast pro-survival effect on the plasma cell line or affect plasma cell viability directly.

**MSC-derived osteoblast co-cultures.**

Similarities were observed in MSC derived osteoblast co-cultures to SaOs-2 co-cultures. Osteoclasts in contact with osteoblasts again resulted in a decrease in calcium deposition though this did not reach significance for control or MM derived osteoblast co-cultures despite the decreases in many cases being greater than in SaOs-2 co-culture, a decrease of up to 20.7% in the control culture without inhibitor. Addition of CBE enhanced this osteoclast effect in MM co-culture resulting in a statistically significant difference but not in control culture.

In contrast to SaOs-2 co-cultures control MSC-derived osteoblasts cultured with plasma cells resulted in lower calcium deposition instead of higher calcium deposition, suggesting osteoblast activity may be inhibited by plasma cells. However, as seen in the SaOs-2 co-cultures, inclusion of osteoclasts when physically separated in MSC-osteoblast cultures reversed the effect of the plasma cells. This finding may add further support to the suggestion that osteoclasts and their precursors produce factors which stimulate osteoblast maturation and activity when not in physical contact, acting as coupling factors mediating bone metabolism.

However, while culture with primary cells appears preferable to cell lines certain limitations have been noted. Firstly, there was greater variation encountered with

primary cell MSC-osteoblast cultures compared to SaOs-2 cultures for both control and MM samples in regards to calcium deposition. Consequently, to confirm these results these cultures would need to be repeated with several subject samples with a number of replicates per sample. Generation of sufficient cells and the duration of these cultures may make such requirements very time consuming and possibly impractical suggesting alternative systems may need to be developed including immortalisation of primary MSC's, ensuring cellular characteristics are not altered in the process. The clear differences in cellular interactions of SaOs-2 and MSC with other cell types would suggest that use of the SaOs-2 cell line in co-cultures may not provide relevant data for modelling the bone microenvironment.

Unlike in SaOs-2 cultures, plasma cell viability was not affected by co-culture with MSC derived osteoblasts. In this system, the very high percentage of viable plasma cells may mask the pro-survival effects of osteoclasts in the bone microenvironment, preventing assessment of the potential pro-survival aspect of culture with osteoclasts. A potential solution may be to use primary plasma cells instead of a cell line as they are likely to be more susceptible to apoptosis than a cell line.

**Limitations.**

This model culture system has several limitations. Firstly, mature osteoblasts and osteoclasts do not co-exist in direct contact with each other in the bone microenvironment (294). Secondly, this culture system, as for all the monocultures performed in this project, is planar (2D) i.e. all the cells are cultured on a flat surface. In vivo, these cells would exist in a three-dimensional (3D) lattice. Thirdly, cell cultures were performed in static medium. This method can lead to a depletion of nutrients and osteogenic factors and a build-up of waste products between medium changes. In addition, all findings from primary co-cultures were based on only one control sample and one myeloma sample.

**Summary.**

In summary, data from cultures involving the human osteoblast cell line SaOs-2 indicated osteoclasts and plasma cells may act as coupling factors as calcium deposition by the SaOS-2 cells was increased when in the presence of the myeloma plasma cell line and when cultured with osteoclasts when not in physical contact. In addition, culture of the plasma cell line NCI-H929 in contact with the SaOs-2 cells resulted in substantial apoptosis of the plasma cells. This effect was partially rescued in the presence of osteoclasts with maximal effect attained when osteoclasts were in contact with plasma cells but separated from the SaOs-2 cells. When combined with data from the previous chapter which showed plasma cells can increase osteoclast generation it may indicate a positive feedback loop of plasma cell survival and osteoclast generation. While osteoclast generation was not further enhanced by plasma cells in GD osteoclast cultures the high number of osteoclasts present may still have a significant effect on plasma cell numbers. However, MSC osteoblast cultures did not induce a measurable increase in apoptosis in the human plasma cell line demonstrating that the use of the SaOs-2 cell line may not be representative of in vivo primary cell interactions. Due to the lack of availability of alternative mature human osteoblast cell lines co-culture systems with human primary MSC's or immortalised MSC's appears to be the most accurate model of the in vivo environment.

# 8. Main discussion

GD is an inherited disorder in which mutations in the GBA1 gene lead to deficient β-glucocerebrosidase activity and the consequent accumulation of its substrate glucosylceramide (46). GD is typically divided into three types distinguished by the presence of neurological features in types 2 and 3 and an absence of such features in type 1 (91), the most common form of GD comprising ~94% of the GD patient population (63). Common features for type 1 GD include hepatosplenomegaly, cytopenias and bone disease (120). Present in around 84% of GD patients (84,85) bone disease can manifest in a number of forms ranging from bone loss including osteopenia and osteonecrosis to abnormal bone remodelling in the form of Erlenmeyer flask formation of the femoral head (101). Historically the cause of bone disease in GD has been attributed to bone marrow infiltration of foamy macrophages, known as 'Gaucher cells' (605). In GD macrophages are the primary storage cell due to their function as phagocytic cells, degrading effete red blood cells, cell debris and microbes, providing an additional source of substrate (47). GD bone disease can be partially explained by bone marrow infiltration of Gaucher cells, hypothesised to cause displacement of marrow cells to the periphery (123) which may lead to marrow infarcts including osteonecrosis of joints (122) and to elicit an inflammatory response which may affect bone metabolism (122). However, the range of severity and the variety of types of bone disease found in GD patients indicate the involvement of several mechanisms which cannot be explained by Gaucher cell infiltration alone.

In this project we set out to investigate the unanswered question of the role of osteoclasts in GD and the interaction of osteoclasts with other cells in the bone marrow given the recent publications of GD animal models which have stated that osteoblasts may be the affected cells in the GD bone marrow.

**Osteoclasts in GD.**

Current knowledge of the cellular contributions to bone disease in GD were very limited. Since this projects instigation research by Mucci et al published in 2012 (525) demonstrated that soluble factors produced by CBE-treated PBMC's induced differentiation of osteoclasts precursors into mature and active osteoclasts. In support of this, results produced in this research project have shown that monocytes isolated from the peripheral blood of Gaucher patients generate more osteoclasts which are larger, have more nuclei and are more active in comparison to control subject osteoclasts. These finding being subsequently confirmed in a publication by Mucci et al in 2015 (134), following our publication in 2013 (526), which also reported that PBMC's isolated from type 1 GD patients generated more osteoclasts than controls. In addition, this effect was partially replicated by inhibiting control monocyte β-glucocerebrosidase with CBE which again led to increased osteoclast generation, size and activity indicating the enhanced osteoclastogenesis and activity is directly related to a deficiency in β-glucocerebrosidase activity.

Cultures which included exogenous glucosylceramide further enhanced osteoclastogenesis and indicate that elevated levels of this sphingolipid both intra and extra-cellularly due to this enzyme deficiency may contribute to the enhanced osteoclast generation in vitro. This conclusion is further supported by evidence in this thesis that GD-specific therapies intended to reduce substrate burden either by substrate degradation or synthesis inhibition reduced osteoclast generation in GD osteoclast cultures demonstrating that not only can the increased osteoclastogenesis observed in GD cultures be induced but also reversed.

**In vitro effects of GD-specific therapy on osteoclastogenesis.**

The finding that osteoclast generation in our in vitro culture system is responsive to GD-specific therapies suggests that this assay may be useful for initial studies of potential therapies for GD and to determine their possible effectiveness in treating bone disease. Indeed, our cultures which included ambroxol hydrochloride

demonstrated that it may reduce osteoclast generation in vivo but also demonstrated that unlike the majority of the GD-specific therapies ambroxol affected control osteoclast generation to a very similar degree suggesting its affect may be via a different pathway to the GD-specific therapies.

Further investigation into the function of ambroxol and other chaperone therapies on osteoclastogenesis via this system may prove useful to define their mode of action and whether this is of particular benefit in regards to GD bone disease. Such investigations may include western blot of lysed osteoclast cultures targeting pathways known to stimulate osteoclast precursor fusion and osteoclast maturation e.g. OC-STAMP (332,606), DC-STAMP (331), RANKL/RANK/OPG (342), c-fms (334,337) pathways and mass spectrometry of the sphingolipid content of osteoclasts pre and post exposure to GD-specific therapies to assess which sphingolipids are predominantly affected and whether these correlate with our findings of the potential effects of sphingolipids on osteoclast generation.

The potential utility of the osteoclast culture system is further supported by correlations made in chapter 4 which found that the increased osteoclast number and average number of nuclei per osteoclast correlated with GD patients with active bone disease and that osteoclast number and activity correlated with individual patient osseous manifestations in the majority of cases. In addition, higher osteoclast generation also correlated with bone pain and anaemia, identified as a risk factor for avascular necrosis in Gaucher patients (529). These findings suggest accumulation of glucosylceramide is relevant to the pathology of GD, that osteoclasts may play a major role in the pathology of bone disease in GD and that therefore in vitro culture of patient osteoclasts may be a useful tool for identifying patients with high osteoclast numbers and activity in vitro which may indicate a greater likelihood of developing bone complications later in life.

However, these findings, especially in regards to individual patient correlation, are based on a relatively small cohort. To confirm these findings a much larger cohort would be required which would include both patients with and without active bone disease to determine a potential cut off for osteoclast number and activity. A second study would also be required which would include younger patients yet to

develop bone complications which would be firstly grouped according to the previously defined osteoclast number cut off and then followed over a period of time to determine if this assay is predictive for patients developing bone complications.

**Correlation of GD patient in vitro osteoclastogenesis with in vivo response to GD-specific therapies.**

Treatment of Gaucher patients with GD-specific therapies such as enzyme replacement and substrate reduction have proven successful in reducing disease burden especially in regards to hepatosplenomegaly (202,205,209,216,221). However, response of bone disease in Gaucher patients has been found to take much longer although in the majority of patients has been regarded as successful due to a gradual increase of bone mineral density and a reduction in fracture events (506–510). Our findings in chapter 3 in regards to in vitro effects of GD-specific therapy on osteoclast generation support the effectiveness of these therapies for GD bone disease as all the therapies tested significantly reduced osteoclast generation in vitro and therefore would be expected to do so in vivo. This assumption is further supported by correlations made in chapter 4 between osteoclast generation and in vivo therapy. Firstly, patients receiving GD-specific therapy generated significantly fewer osteoclasts in vitro than untreated patients and secondly the majority of time courses of GD patients show either a decrease or maintenance of osteoclast generation while receiving therapy with one patient, followed over several years, achieving a substantial decrease over time when treated with miglustat. In addition to providing supportive evidence of the effectiveness of GD-specific therapies in treating bone disease in Gaucher patients, at least in regards to osteoclasts, these findings also demonstrate that the in vitro osteoclast system is responsive to the in vivo changes achieved by therapy. This system may therefore be useful for monitoring patient response to therapy in relation to bone resorption and could be used in conjunction with established methods such as MRI to follow patient progress.

Several publications have reported patients developing new bone events after initiation of GD-specific therapy (84,85) or with bone complications which do not improve after a number of years of therapy even though dosages were increased (511) and a publication by Baris et al in 2015 found small bone crises increased after the initiation of ERT (505). From these studies it could be concluded that there may be a sub-group of patients which have bone disease that does not respond to current GD-specific therapies. However, evidence for this although compelling is limited as the patient cohorts in these studies are relatively small. In support of this a small number of patient in vitro osteoclast cultures did not decrease over time. These findings and the stated publications have led to the initiation of a project by a colleague in our group investigating this potential sub-group.

To confirm the existence of this sub-group and their frequency in the GD population it would be necessary to assess and follow a much larger cohort of patients and divide them according to which therapy they are receiving. Inclusion of the in vitro osteoclast system may be informative in a prospective version of this study as we have shown that GD patients with active bone disease correlate with higher osteoclast generation in vitro. Therefore, if the bone disease of a Gaucher patient does not respond and remains active the assay may confirm this. In addition, if osteoclast generation strongly correlates with active bone disease in the potential non-responders in the prospective study it may prove to be a useful tool in identifying a potential non-responder indicating closer monitoring of the patient's bone health may be required. It may also be used to add further support to radiological and MRI evidence that a patient's bone manifestations are not responding to GD-specific therapies and may require adjunctive bone specific therapies such as bisphosphonates (607), parathyroid hormone (608) or therapies under development such as PTHrP analogues (609) and sclerostin antibodies (610).

**Osteoblasts in GD.**

Evidence from chapters 3 and 4 clearly indicate that osteoclasts may contribute to the various bone complications suffered by Gaucher patients. However, as described in the introduction, the maintenance of bone requires the co-ordination of a number of cell types but in particular between osteoclasts and osteoblasts. It is therefore likely that if osteoclast generation and activity is altered in GD osteoblast function either in regards to migration, differentiation or activity is also affected. Previous publications investigating Gaucher patient MSC's have provided some conflicting data with one showing no alteration in differentiation capacity (139) while two other studies reported a decrease in the capacity of GD-MSC's to differentiate into osteoblasts (138,141).

Animal studies have suggested osteoblast dysfunction may be a cause of bone disease in GD. A murine model of GD which exhibited bone involvement showed reduced proliferation of bone marrow stromal cells and reduced osteoblast differentiation (272) while a Zebra fish model exhibited impaired osteoblast differentiation and reduced bone mineralisation (523). However, in the murine model no alteration in osteoclast number or activity were found with the authors concluding that osteoclasts were unlikely to be involved in the pathogenesis of bone disease in GD.

Given the data generated in vitro and the clinical correlations attained in chapters 3 and 4 of this thesis the conclusion that osteoclasts are not involved in the pathogenesis of bone disease in GD from data generated by the murine model suggests the data in regards to osteoclast number and activity may be spurious in this murine model and highlights the necessity for human based cellular studies in addition to murine models (272). However, this is not to say that animal models are not of value as the findings in both the murine and zebra fish models of impaired MSC proliferation and osteoblast differentiation may provide vital mechanistic information with further investigation.

In regards to our investigations, correlation of osteoblast precursors in peripheral blood with osteoclasts generated from peripheral blood showed a strong

correlation for controls but a very poor correlation for Gaucher patients indicating uncoupling between osteoclasts and osteoblasts in Gaucher patients. In addition, the difficulties encountered in expansion and differentiation of the two GD MSC samples may support the reports of impaired MSC proliferation and differentiation capacity from both GD patient and animal model studies however this finding is based on only two Gaucher patient samples and requires further investigation. The fact that difficulty in expanding MSC's was only encountered for one of the two GD samples, not encountered for any of the control or multiple myeloma samples, suggests that the degree of proliferation impairment may vary from patient to patient. However, to assess this a much larger cohort of GD patient MSC's will be required.

The inability to obtain sufficient calcium deposition for accurate measurement with both GD MSC samples over the 5 weeks of osteoblast culture supports the theory that osteoblast differentiation may be impaired in GD leading to the reduction in bone mineralisation noted in the zebra fish model. However, to confirm this observation, additional repeats of the osteoblast culture with these MSC samples and osteoblast cultures of a much larger cohort of GD MSC samples will be required.

If the capacity of GD-MSC's to differentiate into osteoblasts is impaired, it may explain why GD patient bone disease is slow to respond to therapy as there may be fewer mature osteoblasts available to replace the resorbed bone and therefore a slower increase in bone mineral density than expected. In addition, if osteoblast differentiation is impaired it may have an impact on the effectiveness of both anti-catabolic therapies such as bisphosphonates and anabolic therapies such as the sclerostin antibody currently under development.

In regards to bisphosphonates, their mode of action of binding to calcium crystals, causing toxic effects on osteoclasts or affecting their signalling pathways (607) has the overall effect of reducing bone matrix resorption which in turn means a reduction in osteoblast signalling factors released from the bone matrix (289,436). Therefore, due to the coupling effect a reduction in osteoclast activity may lead to a reduction in osteoblast recruitment and activity. In this case, bisphosphonates

would be beneficial in terms of reducing osteoclast numbers, although our in vitro evidence suggests GD-specific therapies may be sufficient to reduce osteoclast numbers for GD patients in vivo, but the negative effect on osteoblast recruitment and activity may exacerbate the potentially already reduced number of osteoblasts due to the impaired differentiation capacity of the GD-MSC's and therefore may not have an overall beneficial effect.

The action of sclerostin antibody is to bind sclerostin, preventing it from binding to the low-density lipoprotein receptor-related proteins 5 and 6 thus preventing it from inhibiting osteoblast function (611). However, while it may increase the overall activity of osteoblasts in GD patients the fact that there may be fewer functional osteoblasts would limit its effectiveness and so the expected increase in bone mineral density may take considerably longer for GD patients than for non-GD patients.

**In vitro effects of GD-specific therapies on MSC's.**

Our novel research into the potential effects of GD-specific therapies on MSC viability and differentiation and osteoblast activity indicates some of these therapies may be detrimental, at least in the in vitro system. Inclusion of imiglucerase, miglustat or ambroxol in the MSC-osteoblast cultures led to a reduction in viability for all control and multiple myeloma samples for all therapies but did not reach statistical significance in some cases. Calcium deposition was also substantially reduced in the presence of imiglucerase for one control, two of the three multiple myeloma MSC samples and in the SaOs-2 cell line cultures. With reductions also observed for miglustat and velaglucerase in some cases.

However, as none of these samples are from GD patients it is possible that these effects are due to these cells having normal β-glucocerebrosidase expression and activity, thus addition of imiglucerase would lead to excessive amounts and activity of functional β-glucocerebrosidase altering the balance and potential signalling effects of a number of sphingolipids. An effect which would not be present in GD patient MSC's and osteoblasts due to their deficiency in β-glucocerebrosidase. As

glucosylceramide has been linked with cell proliferation a substantial decrease due to excessive β-glucocerebrosidase may lead to reduced viability. This may be particularly relevant for MSC's as they have been shown to have very high β-glucocerebrosidase activity, similar to monocytes (138). Culture with miglustat may have a similar effect as by inhibiting glucosylceramide synthase it would reduce the amount of glucosylceramide to below normal levels within the cell and therefore alter the balance of sphingolipids within the cell.

In contrast, calcium deposition in the presence of ambroxol was not significantly reduced for any of the samples. As ambroxol is a pharmacological chaperone it may not affect cells with normal lysosomal β-glucocerebrosidase activity as trafficking to the lysosome is already optimal and therefore further enhancement by ambroxol may be minimal. As these experiments were not carried out on GD patient MSC's due to difficulties faced in expansion and inducing measurable calcium deposition no data is available to determine if these therapies would have the same effect on viability or calcium deposition. To assess these effects, it may be necessary to further optimise culture conditions for GD MSC's to firstly enhance expansion and secondly to enhance differentiation and calcium deposition. Once achieved, additional repeats of the osteoblast culture with these MSC samples and osteoblast cultures of a much larger cohort of GD MSC samples will be required.

**Potential effects of sphingolipids on osteoblasts, osteoclasts and plasma cells.**

Research into the potential of sphingolipids as bioactive molecules has revealed several important roles in regards to the bone microenvironment, many of which have been found to be elevated in GD patient plasma and serum. Sphingosine-1-phosphate, found to be elevated in GD patient serum (153), has been shown to be an important regulator for migration of both osteoclast precursors and MSC's from the peripheral blood to the bone marrow, to increase proliferation and reduce apoptosis. Ceramides, of which certain species have been found to be elevated in GD plasma but which decrease in response to ERT (150), have several actions depending on the chain length of the species including decreasing osteoclast

activity (375), pro-apoptotic and anti-inflammatory effects (285). Glucosylceramide, the main cellular storage sphingolipid in GD, has been linked with cell proliferation and suggested to be involved in drug resistance (276,612). Glucosylsphingosine, found to be highly elevated in GD plasma and serum (151,152) has been suggested to mediate cellular dysfunction and lead to increased levels of sphingosine-1-phosphate (272).

To investigate the potential roles of sphingolipids in the bone microenvironment MSC's, osteoblasts, osteoclasts and plasma cells were cultured in isolation with a number of exogenous sphingolipids. Addition of glucosylsphingosine appears to have a consistent effect of reduced viability/proliferation as this effect was observed for MSC's, SaOs-2 and the plasma cell line NCI-H929. An effect found to be further enhanced in the case of the plasma cell line in the presence of CBE. Sphingosine, shown to decrease the viability of a mouse pre-osteoblast cell line MC3T3.E1 by Mistry et al (153), had no significant effect on viability/proliferation of any of the MSC samples or the SaOs-2 cell line nor on osteoblast activity which may suggest sphingosine has different roles in mouse and human bone microenvironments.

In contrast, addition of glucosylceramide led to a number of effects including increasing osteoclast generation in both control and GD cultures, increasing MSC differentiation/proliferation but decreasing MSC-derived osteoblast calcium deposition in 3 of the 5 samples cultured. Seemingly contradictory results in regards to the MSC's was obtained for SaOs-2 culture with glucosylceramide decreasing viability/proliferation but increasing calcium deposition. While again, culture of the plasma cell line with glucosylceramide led to an increase in total cell number.

The apparent contradiction of results between MSC's and SaOS-2 cell line may be explained by lineage commitment as glucosylceramide may have different effects depending on how committed the cells are to becoming osteoblasts. In the case of the MSC's they are at a very early stage of commitment therefore exposure to glucosylceramide may either reduce their differentiation capacity to forming osteoblasts or retard the differentiation process possibly redirecting cellular processes towards increasing proliferation of MSC's and away from differentiation.

As the SaOs-2 cells are further committed to becoming osteoblasts, described as osteoblast-like cells in literature (569), this effect on differentiation may be very limited for these cells. The increase in calcium deposition in the SaOS-2 cultures may indicate that glucosylceramide has an additional effect on mature osteoblasts, post-differentiation, increasing their activity. These findings suggest glucosylceramide has a dual role of inhibiting MSC differentiation to osteoblasts while stimulating mature osteoblast activity.

Together these findings suggest high concentrations of exogenous glucosylceramide in the GD patient bone marrow may create an environment in which osteoclast and plasma cell numbers are increased and osteoblast numbers are decreased which would create the uncoupling effect observed in figure 5.1 and may in part explain the bone complications present in up to 96% of GD patients, in particular the osteopenia present in 75% of GD patients (101).

**Potential roles of plasma cells in the GD bone microenvironment.**

Evidence that there may be a positive feedback loop between osteoclasts and plasma cells in which osteoclasts increase plasma cell survival, as shown both in the literature (475) and the findings from the SaOs-2 triple co-culture in figure 7.6, which in turn increase osteoclast generation, shown in figure 6.2, may be further enhanced by a high concentration of exogenous glucosylceramide in the bone marrow as our findings show that glucosylceramide increases osteoclast generation and total plasma cell number. In addition, myeloma plasma cells have also been found to decrease osteoblast differentiation and inhibit their maturation and activity by secretion of several factors including DKK-1, sFRP-2 and sclerostin (489–492). Our finding that GD monocytes generate more osteoclasts in vitro than control subjects suggests GD bone marrow may be a supportive environment for plasma cell proliferation which in turn may lead to a reduction in osteoblast differentiation, maturation and activity. However, the interaction between normal plasma cells and osteoblast differentiation and activity is currently unclear therefore the consequence of an increase in normal plasma cell numbers on

osteoblast number and activity is unknown at present. The potential of an environment supportive of plasma cell proliferation in GD bone marrow with the combination of high osteoclast number and high concentrations of glucosylceramide may also lead to the generation of clonal populations leading to MGUS which in time may lead to multiple myeloma, in part explaining the increased incidence of multiple myeloma in the GD population.

**Development of a co-culture system.**

While many of the findings discussed may expand our understanding of the GD bone microenvironment and provide some explanation for the bone complications present in the majority of GD patients, all the data was obtained from cells cultured in isolation, a state not found in the bone microenvironment and therefore does not account for the effects of the interactions between cells present in the bone microenvironment. To investigate these interactions novel co-culture models were created which included either MSC derived osteoblasts or SaOs-2 cells cultured with monocytes subsequently differentiated into osteoclasts and finally, once the osteoclasts had matured, the plasma cell line NCI-H929.

Co-culture of osteoblasts in contact with osteoclasts resulted in lower total calcium deposition in both the SaOS-2 and MSC model systems. Culture with CBE further reducing the amount of calcium present in the SaOs-2 and the MM MSC cultures. These results are expected as osteoclasts should resorb some of the calcium matrix deposited by osteoblasts resulting in a reduction in total calcium and based on results obtained in chapter 3, CBE was found to increase osteoclast generation in control cultures therefore an increase in osteoclast number would lead to further reduction in overall calcium deposition.

Osteoclasts cultured with but physically separated from osteoblasts resulted in an increase in calcium deposition both in the SaOS-2 system and in the MSC-derived system though this effect was inconsistent for the MM sample 2167 and in the presence of CBE for both MSC cultures. These findings support the concept of coupling between osteoclasts and osteoblasts suggested by publications which

have shown that osteoclasts secrete pro-osteoblast factors such as sphingosine-1-phosphate, BMP 6 and Wnt 10b (379).

The myeloma plasma cell line NCI-H929 appears to reduce the degree of calcium deposition when cultured with control MSC derived osteoblasts. Again, this outcome is in line with research by other groups which has found myeloma plasma cells decrease osteoblast differentiation and inhibit their maturation and activity (489–492). However, this data is from a single control sample and requires confirmation with additional control MSC-osteoblast cultures. In contrast, co-culture of the plasma cell line with the SaOs-2 osteoblast cell line resulted in a small increase in calcium deposition. This may again be due to the SaOs-2 cell line being osteoblast-like cells and thus more committed to differentiating to osteoblasts than the MSC's abrogating the effect of plasma cell secreted proteins such as IL-3 and IL-7 (493,494). In addition, this effect may be erroneous as the SaOs-2 cell line induced substantial apoptosis of the plasma cells with nearly 95% dead or dying when cultured in contact thus abrogating any inhibitory effect on osteoblast function the plasma cells may have.

The substantial apoptosis of the plasma cell line induced by the SaOS-2 cells was not observed in MSC derived osteoblast cultures and therefore may be a particular feature of this cell line. If this is the case this cell line may not be suitable for potential future co-culture experiments as cellular interactions comparable to those of primary cells is essential for the creation of an accurate model of the human bone microenvironment. However, this apoptotic effect did enable assessment of the reported pro-survival effect of osteoclasts on plasma cells (475,486,487,577,613). Using the SaOs-2 cell line we were able to demonstrate that osteoclasts promote plasma cell survival and that this effect is predominantly contact mediated as reported by Abe et al in 2004 (613).

**Limitations of this co-culture system.**

From these finding it can be concluded that this co-culture system has generated several results similar to those found by other groups and therefore may be

informative for further investigation into cellular interactions and the potential effects of GD-specific therapies on the bone microenvironment. To enable investigation of the GD osteoblast-osteoclast interaction it will be necessary to enhance the differentiation of GD MSC's into osteoblasts and possibly to adjust culture conditions to increase calcium deposition to allow accurate measurement.

However, this model culture system has several limitations. Firstly, mature osteoblasts and osteoclasts do not co-exist in direct contact with each other in the bone microenvironment (294). In this culture system, to create a mineralised matrix which incorporated the proteins secreted by osteoblasts during mineralisation to assess osteoclast interaction with this matrix and to investigate how this affects their activity, it was necessary to culture the MSC derived osteoblasts or SaOs-2 cells in osteogenic medium until substantial calcium deposition could be observed followed by direct co-culture with osteoclasts. As this mineralised matrix is deposited on the exposed surface of the osteoblast cells it is not possible to remove the osteoblasts and retain the bone matrix therefore it is necessary to culture osteoblasts with osteoclasts. This limitation was partially mitigated by the use of transwells to physically separate the cells and demonstrated that factors may be produced by osteoclasts which enhance calcium deposition by osteoblasts but it prevents osteoclast interaction with the mineralised surface and still creates the artificial scenario of osteoblasts and osteoclasts existing simultaneously in the local environment.

To address the potential interactions between osteoclasts and osteoblasts by in vitro culture one solution in regards to effects on osteoclast function may be to culture the monocytes on bone discs, as in chapter 3, with osteoblast conditioned medium with or without the pro-osteoclastogenic cytokines MCSF and RANKL. Similarly, MSC's could be cultured with osteoclast conditioned medium with or without the pro-osteoblastic factors dexamethasone, ascorbate-2-phosphate and β-glycerophosphate. However, this approach also has potential limitations as the conditioned medium may be at least partially depleted of nutrients and may contain cell debris and waste products from the initial culture. Additionally, the medium may still contain pro-osteoclastic or pro-osteoblastic factors depending on

the initial culture. While this can be partially mitigated by diluting the conditioned medium with unused medium specific for the culture and control conditions can be included these potential confounding factors cannot be completely accounted for.

Secondly, this culture system, as for all the monocultures performed in this project, is planar (2D) i.e. all the cells are cultured on a flat surface. In vivo, these cells would exist in a three-dimensional (3D) lattice, adhering to the trabecular or intracortical surface. Several studies have demonstrated that cell morphology, differentiation and gene expression differs in a 3D environment compared to 2D and suggest the 3D model system may be more physiologically relevant (614,615). Of particular interest is the study by Marino et al 2014 (616), which uses X-ray μCT images of trabecular bone to print 3D scaffolds to which are adhered and cultured SaOS-2 cells. Through this approach this group demonstrates that SaOs-2 morphology, viability, differentiation and gene expression are altered in comparison to 2D cultures.

Thirdly, cell cultures were performed in static medium, replaced every 3 to 7 days depending on the cell type and stage of culture. This method can lead to a depletion of nutrients and osteogenic factors and a build-up of waste products between medium changes.

**Future development of a 3D cell culture system.**

To address these limitations a different approach may be required. The creation of 3D scaffolds similar to those used by Marino et al with cells cultured in bioreactors with continuous perfusion of culture medium, as shown in figure 8.1, images published by Bouet et al (617). In addition to providing a closer physiological approximation with the 3D scaffold and continuous perfusion providing a constant supply of nutrients and removal of waste products the use of a cubic scaffold may permit quantitation of resorption by osteoclasts.

This protocol may involve seeding the scaffold with MSC's, culture in osteogenic medium until calcium deposition has led to a measurable mass increase, with a

control scaffold with undifferentiated MSC's to account for cell division, followed by seeding with monocytes, re-weighing post cell adhesion, culture in osteoclastogenic medium for 3 weeks followed by re-weighing. If resorption has occurred the final mass should be less than the post-cell adhesion mass. The mass differential would therefore quantify the amount of calcium resorption.

While this approach does not solve the first limitation of having osteoblasts and osteoclasts present at the same time it may provide a more physiological measurement of the difference in activity between control and GD osteoclasts. However, several factors will need to be optimised including the chemistry and architecture of the scaffold, the density of both MSC's and monocytes seeded, the duration of each phase of the culture and the method for accurately weighing the scaffold at each stage.

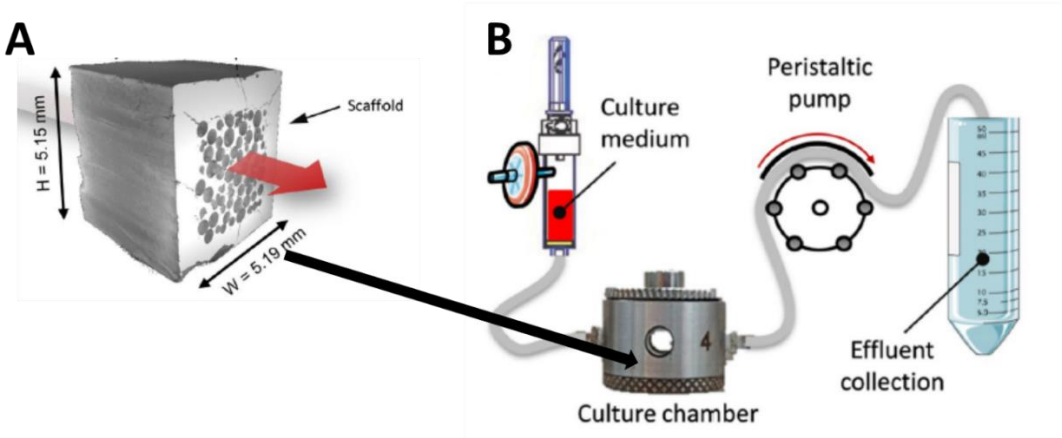



**Figure 8.1** Example of 3-dimensional scaffold based culture system with continuous perfusion. **(a).** Scaffold image, direction of medium perfusion indicated with red arrow. **(b).** Bioreactor system with culture chamber, medium reservoir and effluent collector. Adapted from Bouet et al, European cells and materials, 2015 (617).

**Future work.**

**1.** Given that GD osteoclast generation appears to be increased in vitro I would suggest investigating osteoclast signalling pathways in regards to differentiation and fusion such as c-fms, RANK/RANKL/OPG and DC-STAMP, measuring both RNA

and protein expression, comparing GD and controls to see firstly if there is a higher proportion of monocytes pre-disposed to forming osteoclasts and why this formation appears to be accelerated in GD.

**2.** Data in this thesis showed a decrease in osteoclast numbers in the presence of GD-specific therapies in vitro. I suggest investigating the cellular mechanisms for the reduction in osteoclast generation in the presence of GD-specific therapies. Initially assessing the same pathways stated in point 1.

**3.** Findings in this thesis suggest glucosylceramide may increase osteoclastogenesis and decrease osteoblast function while glucosylsphingosine may reduce cell viability. I would therefore suggest investigating the protein pathways involved in sphingolipid signalling in osteoclasts and osteoblasts and MSC's especially in regards to glucosylsphingosine and glucosylceramide, focusing on differentiation/fusion signalling pathways in regards to glucosylceramide (e.g. DC-STAMP, OC-STAMP, c-fms for osteoclasts and RunX-2, Dlx-5, Osx for osteoblasts) and apoptotic/proliferation pathways in regards to glucosylceramide (e.g. pro-apoptotic proteins BIM, Bcl-2, pro-survival proteins Bcl-XL, survivin and proliferation proteins b-FGF, VEGF).

**4.** Given the difficulty encountered in both expanding and differentiating GD MSCs' into osteoblasts, I would suggest further assessment of the differentiation capacity of GD vs control MSC into osteoblasts in vitro by assessing the rate and proportion which differentiate into adipocytes, chondrocytes and osteoblasts using adipogenic, chondrogenic or osteogenic medium or a combination thereof.

**5.** As an extension to the previous point, I would suggest the assessment of components of the mineralised matrix produced by control and GD MSC's including collagenous and non-collagenous proteins such as TGF-β, BMP-2, PDGF, and IGF's.

**6.** Our findings in this thesis that GD-specific therapies reduced the viability of control and MM MSCs'. I would suggest investigating whether the effects of GD-specific therapies on MSC viability and osteoblast calcium deposition observed in some of our samples are valid and the same effect is seen for GD MSCs'. If so it would be necessary to investigate the cellular mechanisms involved by increasing

cohorts and quantification of RNA and protein of apoptotic and proliferation pathways stated in point 3.

**7.** Data in this thesis indicates that several sphingolipids including glucosylceramide and glucosylsphingosine can affect plasma cell viability and proliferation. Therefore, I would suggest investigating the effects of glucosylceramide and glucosylsphingosine on plasma cells in regards to proliferation and apoptotic pathways respectively by quantification of RNA and protein of apoptotic and proliferation pathways stated in point 3.

**8.** Given the limitations discussed in regards to the culture system used I would suggest the development of 3D scaffolds and culture systems to enable further investigation of cellular interaction in the Gaucher bone microenvironment possibly as depicted in figure 8.1.

**Summary.**

These studies have demonstrated that in vitro osteoclast culture of Gaucher patient monocytes results in increased osteoclast generation, size and activity in comparison to control cultures and that this finding was partially replicated by inhibition of β-glucocerebrosidase with CBE in control cultures. Evidence from other research groups and this study indicate that Gaucher patient MSC's may have a reduced potential for differentiating into osteoblasts and that these osteoblasts have reduced calcium deposition. In addition, plasma cells were found to increase osteoclastogenesis in control osteoclast cultures which have been shown by other researchers to support plasma cell survival and proliferation which may result in a positive feedback loop resulting in increasing osteoclast and plasma cell numbers. Our finding that exogenous glucosylceramide increases both osteoclast and plasma cell number may further exacerbate this feedback loop in the Gaucher bone marrow. This combination of excessive osteoclast number and activity and reduced osteoblast number and activity may have the overall effect of an uncoupling between osteoclasts and osteoblasts in the GD bone microenvironment leading to

excessive bone resorption resulting in the bone pathology observed in GD patients, a simplified representation of which is shown below in figure 8.2.

The findings discussed in this thesis may provide further insight into potential therapies for bone disease in GD possibly tailored to reduce osteoclastogenesis and increase MSC differentiation into osteoblasts, redressing the balance between these cells. In addition, the findings in relation to sphingolipids, especially glucosylceramide and glucosylsphingosine, may increase our understanding not only of their roles in the GD bone microenvironment but also their roles in non-GD bone homeostasis. These findings may also help to further expand current understanding of the roles of these sphingolipids in multiple myeloma and possibly other cancers.

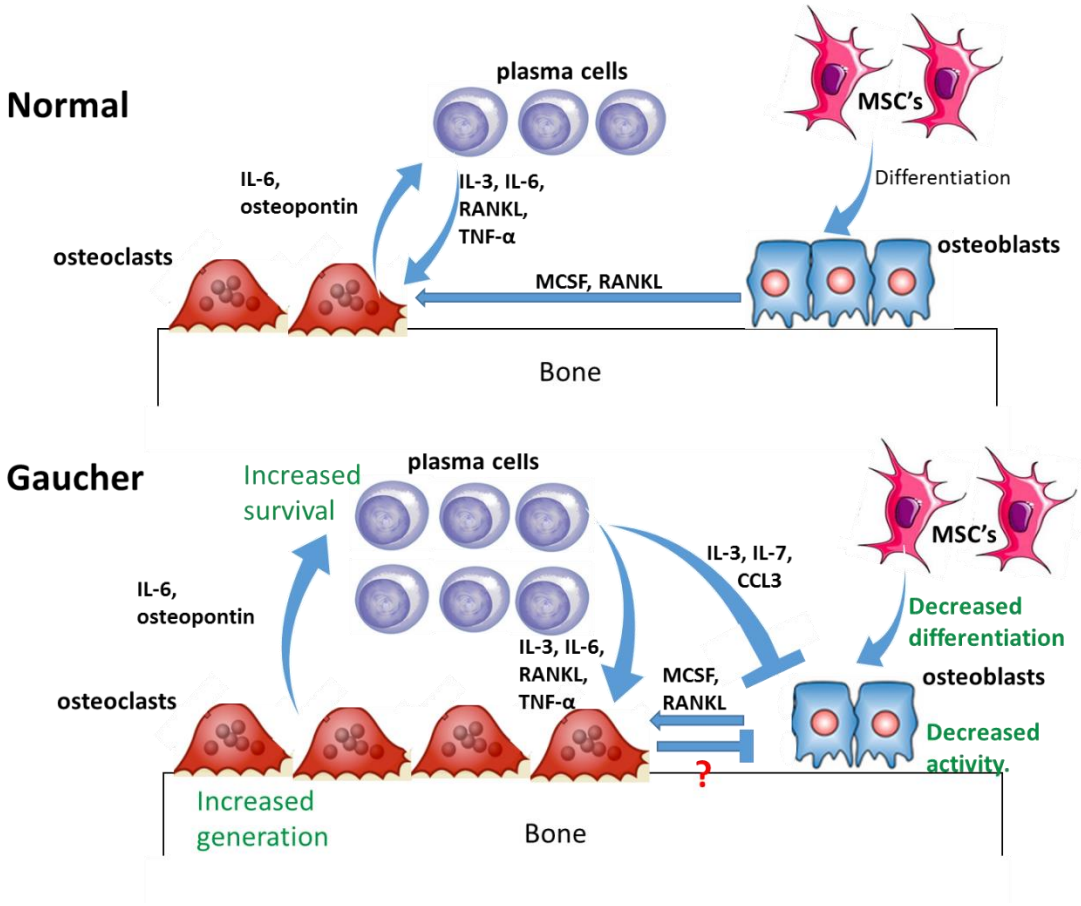



**Figure 8.2** Summary of potential cellular interaction in GD which may be the cause of the bone pathology found in the majority of GD patients. Own figure.

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# 10. Publications

### Papers

Reed M, Baker RJ, Mehta AB, Hughes DA. Enhanced differentiation of osteoclasts from mononuclear precursors in patients with Gaucher disease. Blood Cells Mol Dis. 2013 Oct;51(3):185-94.

### Posters

Reed, M., Hughes, D.A. Potential roles of glucosylceramide and glucosylsphingosine in bone metabolism and multiple myeloma. WORLD symposium. Feb 2016. San Diego, California. USA.

Reed, M., Cunningham, N., Bauernfreund, Y., Mehta, A.B., Hughes, D.A. Changes in peripheral blood osteoclast cultures in relation to therapeutic effects in Gaucher disease. WORLD symposium. Feb 2015. Orlando, Florida. USA.