Hydrogen sulfide (H$_2$S) based therapeutics for bone diseases: translating physiology to treatments.
A mio fratello Tommy,

Con te, per sempre
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<tbody>
<tr>
<td>Ahr</td>
<td>Aryl-hydrocarbon receptor</td>
<td>Cyr61</td>
<td>Axin2, cysteine rich protein 61</td>
</tr>
<tr>
<td>Ao</td>
<td>Aortic</td>
<td>DAO</td>
<td>D-aminoacid oxidase</td>
</tr>
<tr>
<td>AL</td>
<td>Alendronate</td>
<td>DADS</td>
<td>Diallyl disulfide</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
<td>DATS</td>
<td>Diallyl trisulfide</td>
</tr>
<tr>
<td>AR-S</td>
<td>Alizarin red staining</td>
<td>DCF</td>
<td>Dichlorofluorescein</td>
</tr>
<tr>
<td>Axin2</td>
<td>Axin-related protein</td>
<td>DM-22</td>
<td>H₂S-releasing AL</td>
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<tr>
<td>Bax</td>
<td>BCL2-associated X protein</td>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>BFR/BS</td>
<td>Bone formation rate per bone surfaces</td>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
<td>FACS</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
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<td>BMMSCs</td>
<td>m-MSCs</td>
<td>GABABR</td>
<td>Gamma-aminobutyric acidB receptor</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Trabecular bone volume over total volume</td>
<td>GAPDH</td>
<td>Glyceraldehydes-3-phosphate dehydrogenase</td>
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<tr>
<td>CAT</td>
<td>Cysteine aminotransferase</td>
<td>GSH</td>
<td>Glutathione</td>
</tr>
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<td>CBS</td>
<td>Cystathionine-β-synthase</td>
<td>GYY</td>
<td>GYY4137</td>
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<tr>
<td>CFU-ALP</td>
<td>Alkaline phosphatase positive colony forming unit-fibroblast</td>
<td>h-</td>
<td>Human</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
<td>HA</td>
<td>Hydroxylamine hydroxychloride</td>
</tr>
<tr>
<td>Col-1</td>
<td>Collagen type-1</td>
<td>HA</td>
<td>Haematoxylin-Eosin</td>
</tr>
<tr>
<td>CSE (protein)</td>
<td>Cystathionine-γ-lyase</td>
<td>HBF</td>
<td>High bone formation</td>
</tr>
<tr>
<td>CTH (gene)</td>
<td></td>
<td>H-CD</td>
<td>High calcium deposing phenotype</td>
</tr>
<tr>
<td>μCT</td>
<td>Micro-computed tomography</td>
<td>Hcy</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>CTRL</td>
<td>Control cells</td>
<td>H₂DCFDA</td>
<td>Dichlorodihydrofluorescein diacetate</td>
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<tr>
<td>CTRL+</td>
<td>Positive control cells</td>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>Ct.Th</td>
<td>Cortical thickness</td>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
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<td>Ct.V</td>
<td>Cortical volume</td>
<td>H₂S</td>
<td>Hydrogen sulphide</td>
</tr>
<tr>
<td>CTX</td>
<td>C-terminal telopeptide of type 1 collagen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Name</td>
<td>Abbreviation</td>
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<td>--------------</td>
<td>------------------------------------------------</td>
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<td>---------------------------------------------------</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischemia reperfusion injury</td>
<td>N.Oc/BS</td>
<td>Number of OCs per millimeter of bone surface</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt; channels</td>
<td>ATP-sensitive potassium channel</td>
<td>NQO1</td>
<td>NAD(P)H dehydrogenase, quinone 1</td>
</tr>
<tr>
<td>KEAP-1</td>
<td>Kelch-like ECH-associated protein 1</td>
<td>NON MIN</td>
<td>Non mineralizing h-MSCs</td>
</tr>
<tr>
<td>LBF</td>
<td>Low bone formation</td>
<td>NRF2</td>
<td>Nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
<tr>
<td>L-CD</td>
<td>Low calcium depositing phenotype</td>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
<td>NT</td>
<td>Non targeting</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
<td>OBs</td>
<td>Osteoblasts</td>
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<td>Lef-1</td>
<td>Lymphoid enhancer factor-1</td>
<td>Ob.S/BS</td>
<td>Percentage of bone surfaces covered by OBs</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
<td>Ocn</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>LTP</td>
<td>Long terminal potentiation</td>
<td>OCs</td>
<td>Osteoclasts</td>
</tr>
<tr>
<td>m-</td>
<td>Murine</td>
<td>Oc.S/BS</td>
<td>Percentage of bone surfaces covered by OCs</td>
</tr>
<tr>
<td>MAR</td>
<td>Mineral apposition rate</td>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
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<td>Modeling-based bone formation</td>
<td>Osx</td>
<td>Osterix</td>
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<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
<td>Ovx</td>
<td>Ovariectomy / Ovariectomized</td>
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<td>MIN</td>
<td>Mineralizing h-MSCs</td>
<td>Ovx Veh</td>
<td>Vehicle treated ovx mice</td>
</tr>
<tr>
<td>MS/BS</td>
<td>Percentage of bone surfaces covered by mineralized surfaces</td>
<td>Ovx GYY</td>
<td>GYY treated ovx mice</td>
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<td>MSCs</td>
<td>Mesenchymal stem cells</td>
<td>PAG</td>
<td>DL-Proparglycine</td>
</tr>
<tr>
<td>3-MST</td>
<td>3-Mercapto-pyruvate sulphur transferase</td>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetyl-cysteine</td>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>NaHS</td>
<td>Sodium hydrosulfide</td>
<td>PCR</td>
<td>Polimerase chain reaction</td>
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<td>N-CD</td>
<td>Non calcium depositing phenotype</td>
<td>PDLC</td>
<td>Periodontal ligament cells</td>
</tr>
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<td>Nkd2</td>
<td>Naked cuticle 2 homolog</td>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td>PLP</td>
<td>Pyridoxal phosphate</td>
</tr>
<tr>
<td>N.Ob/BS</td>
<td>Number of OBs per millimeter of bone surface</td>
<td>P1NP</td>
<td>N-terminal propeptide of type 1 procollagen</td>
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<tr>
<td>Abbreviation</td>
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<td>Abbreviation</td>
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<tr>
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<td>Peroxiredoxin-1</td>
<td>T-BHQ</td>
<td>tert-Butylhydroquinone</td>
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<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
<td>Tb. N</td>
<td>Trabecular number</td>
</tr>
<tr>
<td>RBF</td>
<td>Remodeling-based bone formation</td>
<td>Tb.SP</td>
<td>Trabecular space</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
<td>Tb.Th</td>
<td>Trabecular thickness</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
<td>Tcf</td>
<td>T cell factor</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
<td>Tgfβ3</td>
<td>Transforming growth factor β3</td>
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<td>Runx2</td>
<td>Runt related transcription factor-2</td>
<td>Thbs1</td>
<td>Thrombospondin 1</td>
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<td>SEM</td>
<td>Standard Error of the Mean</td>
<td>Twist1</td>
<td>Twist gene homolog 1</td>
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<td>SF</td>
<td>Silk fibroin</td>
<td>TRAP</td>
<td>Tartrate acid phosphatase</td>
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<tr>
<td>SFN</td>
<td>Sulphoraphane</td>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>Sham GYY</td>
<td>GYY treated sham operated mice</td>
<td>UN</td>
<td>Unstimulated</td>
</tr>
<tr>
<td>Sham Veh</td>
<td>Vehicle treated sham operated mice</td>
<td>VGCCs</td>
<td>Voltage-gated Ca2+ channels</td>
</tr>
<tr>
<td>SMCs</td>
<td>Smooth muscle cells</td>
<td>VK</td>
<td>Von Kossa</td>
</tr>
<tr>
<td>SSCs</td>
<td>Skeletal stem cells</td>
<td>WT mice</td>
<td>Wild Type mice</td>
</tr>
<tr>
<td>Tagln</td>
<td>Transgelin</td>
<td>Wisp 1</td>
<td>Wnt1 inducible signalling pathway protein 1</td>
</tr>
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</table>
2. INTRODUCTION

2.1. Hydrogen Sulphide (H$_2$S): foe but friend

All of us certainly have experienced or have been told about sulphurous thermal sites and sulphurous springs. Probably we have been advised for a visit for their healthy properties but we could have been downhearted for the unpleasant smell of rotten eggs. However, little of us probably know that what confer this particular smell is a small gaseous compound named Hydrogen Sulphide (H$_2$S).

Given its broad-spectrum toxicity which leads to death, H$_2$S has been traditionally considered only as a toxic agent for living organisms (H. Kimura 2013). H$_2$S poisonings are classically reported in occupational settings such as for sewer workers (Christia-Lotter et al. 2007) but also in domestic situations (Sastre et al. 2013) (Daldal et al. 2010). H$_2$S intoxications are lethal because high concentrations are odourless and heavier than air, therefore poisoning occurs also in colleagues, first aid helpers and professional rescue teams (Kage et al. 2002) (Kage et al. 2004) (Barbera et al. 2016). Acute intoxication causes an almost instantaneous cardiovascular failure, coma, and a rapid death, like in stroke of lightening (Oesterhelweg and Püschel 2008). The most affected organs are heart, brain and lungs (Shivanthan et al. 2013). Depending on the concentration of exposure, acute H$_2$S poisoning can cause: sore throat, dizziness, nausea, airway and eyes irritation, respiratory depression and paralysis, pulmonary edema, fatal central apnea, chronic neurological sequelae like memory problems, neurasthenic symptoms, rhabdomyolysis (Haouzi 2012). The molecular mechanisms underlying H$_2$S toxicity are still not clear, however it is widely believed that H$_2$S targets mitochondria via reversible inhibition of cytochrome c oxidase (Khan et al. 1990) (Reiffenstein, Hulbert, and Roth 1992), leading to inhibition of mitochondrial respiration, cell anoxia and cell damage. Recently, it has been emerged that H$_2$S toxicity may be due to interactions with cysteine residues of various protein such as ion channels (Haouzi, Sonobe, and Judenherc-Haouzi 2016).
Nowadays, **H₂S is regarded as the third endogenous gasotransmitter**, alongside nitric oxide (NO) and carbon monoxide (CO). These molecules are defined gasotransmitters as: 1) they are small lipophilic molecules of gas that can easily spread through the plasma membrane without requiring membrane receptors; 2) their endogenous synthesis is strictly regulated; 3) they play a relevant role in the physiology; 4) their function is mediated by many molecular targets (Kasparek et al. 2008). However, it must be underscored that the term gasotransmitter is not acknowledged by many Authors who consider more appropriate the terms messengers or signaling molecules as they 1) could not be stored in vesicles and released on demand like classic neurotransmitters and 2) act as paracrine and systemically active molecules (Boehning and Snyder 2003). The three gasotransmitters have been demonstrated to work together displaying an important role in different pathologic and physiologic states such as: inflammation (Lo Faro et al. 2014), myocardial ischemia/reperfusion injury (IRI) (Andreadou et al. 2015), haemostasis and thrombosis (Olas 2015), cardiovascular function (L. Li, Hsu, and Moore 2009), gastrointestinal regulation (Magierowski et al. 2015) (Farrugia and Szurszewski 2014), sexual function (Yetik-Anacak et al. 2015), hypothalamic-pituitary axis (Ruginsk et al. 2015) (Mancuso, Navarra, and Preziosi 2010).

### 2.2. Sources and metabolism of H₂S

The diet is the major source of H₂S. Drinking water provides inorganic sulphide while plants and animals provide the two sulphur-containing amino acids: methionine (an essential amino acid) and cysteine (a semi-essential amino acid) (Benjamin Lee Predmore, Lefer, and Gojon 2012). These amino acids are the substrates of enzymes which endogenously produce H₂S in mammalian cells (Szabó 2007). Sulphate-reducing bacteria (SRBs), belonging to the microbiota residing in the gastrointestinal tract (Rey et al. 2013), are another form of endogenous H₂S synthesis. Finally, H₂S can be non-enzimatically released from inorganic polysulphides in a redox or pH sensitive manner (it is released in the presence of a reducing agent and alkaline conditions) (Ishigami et al. 2009) (Kolluru et al. 2013).
The endogenous generation of H$_2$S is mainly mediated by the enzymes cystathionine-β-synthase (CBS; EC 4.2.1.22) and cystathionine-γ-lyase (CSE/CTH; EC 4.4.1.1), which catalyze the “transsulfuration” step within methionine / homocysteine (Hcy) metabolic pathway (Szabó 2007), shown in Figure 1. H$_2$S is also produced by L-cysteine aminotransferase (CAT; EC 2.6.1.3) and 3-mercapto-pyruvate sulphurtransferase (3-MST; EC 2.8.1.2) during the cysteine catabolic pathway. However 3-MST is considered more a sulphur carrier rather than a proper H$_2$S producer as it generates sulphate sulphur (Wallace and Wang 2015) which can release H$_2$S only after a redox reaction between RSSH and a biological thiol such as Thioredoxin (Trx). Similarly, CAT does not produce directly H$_2$S. Recently, some Authors have found another H$_2$S-producing enzyme: D-amino acid oxidase (DAO) (Shibuya and Kimura 2013), which produces H$_2$S from D-cysteine. CBS, CSE and CAT are pyridoxal phosphate (PLP)-dependent enzymes, contrary to 3-MST and DAO.

![Figure 1: Scheme showing the various H$_2$S generating reactions catalyzed by enzymes in the transsulfuration pathway (CBS and CSE) and the cysteine catabolic pathway (CAT and 3-MST). (Singh and Banerjee 2011)](image-url)
CBS has been thought to be the primary physiological source of H$_2$S in the brain (Abe and Kimura 1996). Conversely, CSE has been described to be predominantly expressed in peripheral tissues appearing to be the main H$_2$S producing enzymes in cardiovascular and respiratory system as well as in liver and kidney (R. Wang 2012). However, recent evidences demonstrated that CSE is expressed and plays a physiological role in brain (Paul et al. 2014). Similarly, CBS has been found to be modulated in uterine vasculature (Lechuga et al. 2015). Moreover, many recent publications showed the expression of both enzymes in the same organ, such as in liver (Mani et al. 2014) and kidney (Xia et al. 2009). Therefore, **we are still far from a comprehensive understanding of the differential expression of CBS and CSE as well as their role in the physiology and pathology.**

H$_2$S concentrations are tightly regulated by its metabolism through: 1) scavenging; 2) oxidation and 3) Methylation processes. H$_2$S in the circulation is actively scavenged by different proteins (methaemoglobin, neuroglobin, cytochrome c oxidase, metallo- or disulfide-containing proteins) and by erythrocytes and is converted to sulphone-sulfur, the bound-sulphate pools (R. Wang 2002). H$_2$S is methylated by thiol-S-methyltransferase within the cells and then converted to methanethiol and dimethylsulphide (R. Wang 2002) or oxidated in mitochondria by sulphide quinone oxidoreductase (SQR) which products of oxidation are thiosulfate (S$_2$O$_3^{2-}$); sulfite (SO$_3^{2-}$); sulfate (SO$_4^{2-}$) (Mishanina, Libiad, and Banerjee 2015). For many years these mechanisms have been thought to play only a role in disposing of excess sulphide; however, recently it has been postulated that the oxidation of H$_2$S may play a role in cellular signaling (Mishanina, Libiad, and Banerjee 2015).

Up to now, in the field of H$_2$S biochemistry and signaling there are more questions than answers. In particular, there is little understanding of 1) how the catalysts switch between the Hcy metabolism and H$_2$S production and 2) if and how the mitochondrial enzymes are regulated to increase or decrease H$_2$S or sulphone-sulfur pools (Kabil and Banerjee 2014).

H$_2$S concentration in blood and tissue still remains a highly debated topic. For many years it has been considered to be in the range of 20-100 μM, recently these measurements have been thought to
be overestimated as new measurements were in the order of nM (Furne, Saeed, and Levitt 2008). This observation raised two important questions: 1) Does H$_2$S exist in the circulation?; 2) Are the micromolar concentrations of H$_2$S employed in many studies physiologically relevant? (Olson 2009). In the absence of such a strong knowledge, many scientists think of being careful to assay physiological role of H$_2$S and of predicting in vivo potency of their in vitro findings obtained with administration of ‘high’ doses of H$_2$S (Wallace and Wang 2015). Some authors advanced that it may be conceivable that contrary to whole tissue or circulation where H$_2$S is rapidly metabolized and remains low (nM range), the intracellular microenvironment may increase H$_2$S concentration sufficiently to activate cellular function (μM range) (Furne, Saeed, and Levitt 2008). This may be a possible explanation for the discrepancy between H$_2$S levels measured in circulation and tissue and H$_2$S concentration necessary for induce an effect in vitro. Moreover, some authors think H$_2$S of being poisonous to cells and tissues even at low concentrations (Bouillaud and Blachier 2011). Therefore the demarcation between effects reflecting a putative physiological function and those related to H$_2$S poisoning remains, yet, to be established (Haouzi 2012). Therefore among the challenges facing the field is the accurate measurement of biologically active H$_2$S (P. Nagy et al. 2014) (Shen et al. 2012). The two most employed techniques for H$_2$S measurement are methylene blue assay (a colorimetric assay which hold many limitation such as low sensitivity and inability to distinguish between H$_2$S, HS$^-$, S$^{2-}$ species) and gas chromatography (high specificity and sensitivity but inability to perform real-time measurements) (Shen et al. 2011) (Kashfi and Olson 2013) (Nicholson et al. 2013). Growing research is currently under way to discover a sensitive technique which can allow real-time measurements of H$_2$S. Recently, new methodologies have been developed: amperometric analyses (L. Nagy et al. 2014) (Citi et al. 2014); fluorescent probes (Peng et al. 2014); zinc trap spectrophotometry (Matthew Whiteman et al. 2010); H$_2$S selective electrode (Mustafa et al. 2009).
2.3. Physiologic role of H₂S

H₂S has been described in mammalian tissues for many years, however only recently it has been appreciated as a critical molecule for the homeostasis of tissues and organs (Vandiver and Snyder 2012). Figure 2 shows a schematic representation of the wide range of tissue and organs in which H₂S plays an active role.

Figure 2: Schematic representation of the tissues and organs in which H₂S plays an active role.

In first instance, H₂S was identified as a neuromodulator (Abe and Kimura 1996). Indeed, H₂S is able to enhance N-methyl D-aspartate (NMDA) receptor-mediated excitatory post synaptic currents and stimulates hippocampal long-term potentiation (LTP) (Abe and Kimura 1996). Moreover, it stimulates calcium signaling in astrocytes (Nagai et al. 2004).

In the cardiovascular system it was found to exert an anti-hypertensive function acting both on vasodilatation (G. Yang et al. 2008) as well as affecting sodium renal handling (Ahmad et al. 2014). It has been defined both an endothelium derived releasing factor (EDFR) (R. Wang 2009) as an
endothelium derived hyperpolarizing factor (EDHF) (G. Tang et al. 2013). Moreover, it acts as an anti-atherogenic agent reducing lipid hydroperoxides to the less reactive lipid hydroxides (Muellner et al. 2009); it counteracts oxidation of low density lipoprotein (LDL) via hypochlorous acid (HOCl) scavenging, hydrogen peroxide (H$_2$O$_2$) scavenging, myeloperoxidase inhibition, inhibition of foam cells formation (Laggner et al. 2007) (Z.-Z. Zhao et al. 2011).

In liver, H$_2$S affects glucose metabolism, insulin sensitivity, lipoprotein synthesis, mitochondrial biogenetics and biogenesis (Mani et al. 2014). In pancreas, H$_2$S activates ATP-sensitive potassium channels (K$_{ATP}$ channels) in β-cells, increasing hyperpolarisation and decreasing insulin release (G. Yang et al. 2007). In kidney, H$_2$S prevents fibrosis (Song et al. 2014) and exerts diuretic, natriuretic, kaliuretic effects (Ge et al. 2014) (Xia et al. 2009) (Ahmad et al. 2014). H$_2$S regulates reproductive organs: mediates penile erection (d’Emmanuele di Villa Bianca et al. 2011) and decreases uterine contractility (Sidhu et al. 2001). H$_2$S has been found to modulate immune system through regulation of regulatory T cells differentiation and function (R. Yang et al. 2015).

Among various functions, H$_2$S has been reported to be an oxygen sensor: H$_2$S acts as an electron donor in the mitochondrial respiratory chain; promotes vasorelaxation and angiogenesis in hypoxic tissues or mediates hypoxic pulmonary vasoconstriction (allowing the diffusion of the blood stream from oxygen deprived areas to oxygen-supplied areas); increased lung ventilation through vasodilatation of airway smooth muscle cells (SMCs); mediates the response of carotid body to hypoxia through voltage-gated Ca$^{2+}$ channels (VGCCs) (Makarenko et al. 2015). Moreover, H$_2$S has been investigated for its ability to induce a hibernation-like metabolic state called 'suspended animation' (Blackstone, Morrison, and Roth 2005).

One of the most recognized roles of H$_2$S is as antioxidant. Indeed, H$_2$S has been found to inhibit reactive oxygen species (ROS)-induced levels by different stimuli: glutamate/peroxynitrite/HOCl-induced ROS production in neurons (Y. Kimura, Goto, and Kimura 2010) (Matthew Whiteman et al. 2004) (Matthew Whiteman et al. 2005); methylglyoxal-induced ROS production in vascular SMCs (T. Chang et al. 2010); cytokines or hydrogen peroxide-induced ROS production in
pancreatic β-cells (Taniguchi et al. 2011); rotenone-induced ROS production in microglia (Du et al. 2014); 1-methyl-4-phenylpyridinium/β-amyloid-induced ROS production in PC12 cells (Yin et al. 2009) (X.-Q. Tang et al. 2008). However, underlying mechanisms for the antioxidant effect of H₂S are still poorly comprehended.

A common identified mechanism is S-sulfhydration of key proteins. One example is H₂S-mediated S-sulfhydration of Kelch-like ECH-associated protein 1 (KEAP-1) (G. Yang et al. 2013) (Nguyen et al. 2003). KEAP-1 is the ubiquitine ligase-adaptor which drives Nuclear factor (erythroid-derived 2)-like 2 (NRF2) toward degradation (Itoh et al. 1999). NRF2 is the master regulator of the intracellular antioxidant response (Venugopal and Jaiswal 1996) (Calvert et al. 2009) and in normal conditions, its activity is primarily controlled at the level of protein stability through the interaction with KEAP-1. Indeed, in its KEAP-1-bound form, NRF2 has a short half-life thereby restraining the homeostatic activation of antioxidant genes such as NAD(P)H dehydrogenase, quinone 1 (NQO1) and peroxiredoxin-1 (PRDX1) (Kensler, Wakabayashi, and Biswal 2007). H₂S-mediated S-sulfhydration of KEAP-1 dislocates KEAP-1 driving NRF2 stabilization, its nuclear translocation and transcription of antioxidant genes. Another mechanism that has been identified is the intracellular restoration of glutathione (GSH) levels (Y. Kimura and Kimura 2004).

H₂S production in cells was recently proposed as the unifying mechanism by which different calories restriction regimens triggers increased lifespan in diverse organisms (Hine et al. 2015).

Furthermore, H₂S mediates cytoprotection to different insults in many cellular types. It is cardioprotective, as it inhibits myocardial IRI (Johansen, Ytrehus, and Baxter 2006). Interestingly, H₂S confers resistance to hypoxia in SMCs by increasing ATP synthesis: under hypoxic conditions, CSE may translocate to mitochondria, metabolize cysteine, produce H₂S, and increase ATP production (Fu et al. 2012). In brain, H₂S sulfhydrates the ubiquitin-ligase, parkin enhancing its catalytic activity (Vandiver et al. 2013); impairment in parkin activity is present in Parkinson's disease as well as in glioblastoma and other human malignancies (K. Liu et al. 2016). H₂S protects neurons against glutamate-induced toxicity through activation of Kₐ₅ channels. H₂S alleviates the
hippocampal damage increasing the expression of gamma-aminobutyric acid B receptor (GABABR) (Han et al. 2005). Moreover, recently H₂S has been found to promote wound healing in diabetic rats (G. Wang et al. 2015).

However, controversies continue to exist on what is the real nature of this gaseous transmitter: a friend or a foe? Indeed, high concentrations of H₂S induce β-cell apoptosis (G. Yang et al. 2007). Moreover, H₂S has provided evidence for both: 1) pro- (Stuhlmeier, Bröll, and Iliev 2009) and anti-inflammatory (Hu et al. 2007) (C. Yang et al. 2011) (Zanardo et al. 2006) (Mirandola et al. 2007) effects; 2) pro- (Matsunami et al. 2009) and anti-nociceptive (Distrutti et al. 2006) effects in particular in colonic mucosa; 3) pro-cancer (S. Zhang et al. 2016) and anti-cancer effects (Lu et al. 2014). The dual face of this molecule would suggest the existence of a threshold level able to discriminate between a beneficial and a detrimental effect (Martelli et al. 2012).

2.4. Relevance of H₂S in pathology

H₂S levels in humans declines with age (Benjamin L Predmore et al. 2010). Moreover, several correlations were established between low or high levels of plasmatic H₂S or H₂S-generating enzymes and diseases, as shown in Table 1.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>H₂S levels / H₂S generating enzymes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes type 1</td>
<td>↓ CSE activity</td>
<td>(Manna et al. 2014)</td>
</tr>
<tr>
<td>Diabetes type 2</td>
<td>↓ H₂S levels</td>
<td>(Jain et al. 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(M. Whiteman et al. 2010)</td>
</tr>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td>↑ H₂S levels</td>
<td>(Ran et al. 2014)</td>
</tr>
<tr>
<td>Chronic haemodialysis in diabetic nephropathy</td>
<td>↓ H₂S levels</td>
<td>(H. Li et al. 2014)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>↑ H₂S levels</td>
<td>(Zheng et al. 2011)</td>
</tr>
<tr>
<td>Congenital heart disease with pulmonary hypertension</td>
<td>↓ H₂S levels</td>
<td>(Ling Sun et al. 2014)</td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>↓ H₂S levels</td>
<td>(K. Wang et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>↓ CSE in placenta</td>
<td>(Eto et al. 2002)</td>
</tr>
<tr>
<td>Alzheimer disease</td>
<td>↓ H₂S levels in brain</td>
<td>(Eto et al. 2002)</td>
</tr>
</tbody>
</table>
Table 1: H<sub>2</sub>S, CBS or CSE levels in blood or tissues derived from patients with different disease.

Furthermore, H<sub>2</sub>S and H<sub>2</sub>S-generating enzymes were found to be modulated in different animal models mimicking human pathologies, as shown in Table 2.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>H&lt;sub&gt;2&lt;/sub&gt;S levels / H&lt;sub&gt;2&lt;/sub&gt;S generating enzymes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneously hypertensive rats</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels ↓ CSE expression</td>
<td>(Yan, Du, and Tang 2004)</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels ↓ CSE expression</td>
<td>(K. Wang et al. 2013)</td>
</tr>
<tr>
<td>Mouse model of atherosclerosis</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels</td>
<td>(Yanfei Wang et al. 2009)</td>
</tr>
<tr>
<td>Mouse model of cirrhosis</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels in liver ↓ CSE expression</td>
<td>(Fiorucci et al. 2005)</td>
</tr>
<tr>
<td>Rat model of hepatotoxicity, cirrhosis and portal hypertension</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels in serum and liver ↓ CSE expression in liver</td>
<td>(Tan et al. 2011)</td>
</tr>
<tr>
<td>Genetic diabetes type 1 rat models</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels in liver ↓ CSE expression in liver</td>
<td>(Manna et al. 2014)</td>
</tr>
<tr>
<td>Streptozotocin-induced diabetes rat models</td>
<td>↑ H&lt;sub&gt;2&lt;/sub&gt;S levels in pancreas and liver ↑ CSE, CBS in pancreas and liver</td>
<td>(Yusuf et al. 2005)</td>
</tr>
<tr>
<td>Non-obese diabetic mice</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S plasma Progressively decline as the severity of diabetes increases over-time.</td>
<td>(Brancaleone et al. 2008)</td>
</tr>
<tr>
<td>Rat model of ischemic vascular dementia</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels</td>
<td>(L.-M. Zhang, Jiang, and Liu 2009)</td>
</tr>
<tr>
<td>Mouse model of Parkinson disease</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels in substantia nigra and striatum</td>
<td>(Hu et al. 2010)</td>
</tr>
<tr>
<td>Mouse model of Huntington disease</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S levels in cell lines of Huntington disease ↓ CSE expression in different brain regions (progressing depletion with increases severity of the disease)</td>
<td>(Paul and Snyder 2014)</td>
</tr>
<tr>
<td>Mouse model of colitis</td>
<td>↑ H&lt;sub&gt;2&lt;/sub&gt;S levels at the site of ulceration</td>
<td>(Flannigan et al. 2013)</td>
</tr>
<tr>
<td>Animal model of chronic renal</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;S plasma levels</td>
<td>(Perna et al.)</td>
</tr>
</tbody>
</table>
Table 2: \( \text{H}_2\text{S, CBS or CSE levels in blood or tissue derived from animal models of human pathologies.} \)

These findings lead to the hypotheses that dysregulation of \( \text{H}_2\text{S} \) levels may be critical in the onset of different diseases. Knock out animal models for \( \text{H}_2\text{S} \)-generating enzymes have demonstrated worsening of various diseases, as shown in Table 3, with some exception.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Enhanced/Decreased pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE-knockout mice</td>
<td>Pronounced hypertension and diminished endothelium-dependent vasorelaxation</td>
<td>(G. Yang et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Accelerated atherosclerosis</td>
<td>(Mani et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>Reduced rate of gluconeogenesis</td>
<td>(Untereiner et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>Exacerbated myocardial and hepatic IRI</td>
<td>(King et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>Increased glucotoxicity in ( \beta )-cells</td>
<td>(Okamoto et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>Increased damage and mortality after renal IRI</td>
<td>(Holwerda et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>Greater cardiac dilatation and dysfunction after aortic constriction</td>
<td>(Kondo et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>Protection on caerulein-induced pancreatitis</td>
<td>(Ang et al. 2013)</td>
</tr>
<tr>
<td>CBS-knockout mice</td>
<td>Vascular and neurological complications</td>
<td>(Beard and Bearden 2011)</td>
</tr>
</tbody>
</table>

Table 3: \textit{Enhanced or decreased pathology in CSE or CBS knockout mice.}

Moreover, it must be underscored that the findings of dysregulated \( \text{H}_2\text{S} \) levels in many pathologies focused the attention on the need of obtaining accurate measurements of the absolute concentration of \( \text{H}_2\text{S} \) in the circulation and in tissues with the future perspective of using these levels as possible biomarkers for diagnosis, prognosis or therapies monitoring.

2.5. \( \text{H}_2\text{S} \)-based novel therapeutic agents

Given the emerging role of \( \text{H}_2\text{S} \) in human physiology, a number of pharmacological approaches have been developed to exploit the \textit{therapeutic potential of this molecule}, alone or in combination with existing drugs (Szabó 2007). Because of its cytoprotective, antifibrotic, anti-apoptotic,
angiogenic properties, H₂S has been proposed as a therapeutic choice in age-associated diseases (Y. Zhang et al. 2013). Moreover, H₂S is thought to be the next powerful therapeutic agent for preventing and ameliorating the symptoms of pathologies such as renal-associated diseases (Pan et al. 2015) and erectile dysfunction (d’Emmanuele di Villa Bianca et al. 2011).

H₂S replacement therapy may be based on different options: 1) hydroponic therapy with H₂S rich water; 2) H₂S donors; 3) H₂S-releasing molecules; 4) H₂S-releasing drugs (H₂S releasing molecules combined with pharmacologically active compounds). According to the nature of acute or chronic pathologies, rapidly-acting compounds or slow-releasing compounds might be developed. To this end, it is important to point out that one of the major challenges for prolonged H₂S treatments is its extremely short half-life.

**Hydroponic therapy** with H₂S-rich water (1) was found to improve the redox status in healthy subjects (Benedetti et al. 2009) and has been proposed as an innovative approach to slow down Alzheimer disease progression in humans (Giuliani et al. 2013).

Many **H₂S donors** (2), such as sodium hydrosulfide (NaHS) and GYY4137 (GYY), has been used in different animals models of human disease, as shown in Table 4. All these experiments highlighted the ability of H₂S in preventing and counteracting the progress of various diseases. Furthermore approaches aiming to induce CSE expression revealed protective effects; it is the case of anti-atherogenic effect due to CSE overexpression (Cheung et al. 2014).

<table>
<thead>
<tr>
<th>Animal model</th>
<th>H₂S treatments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE-knockout mice</td>
<td>NaHS prevented the progress of hypertension and vascular remodeling; decreased vascular damage.</td>
<td>(G. Yang et al. 2008)</td>
</tr>
<tr>
<td>Spontaneously hypertensive rats</td>
<td>• NaHS decreased blood pressure.</td>
<td>• (W. Zhao et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>• GYY improved myocardial fibrosis.</td>
<td>• (Meng et al. 2015)</td>
</tr>
<tr>
<td>Rat model of renovascular hypertension</td>
<td>NaHS prevented systemic hypertension and ameliorated endothelial dysfunction.</td>
<td>(Xue et al. 2015)</td>
</tr>
<tr>
<td>Preeclampsia mice and rat models</td>
<td>GYY restored fetal growth.</td>
<td>(X.-H. Wang et al. 2013)</td>
</tr>
<tr>
<td>Mice model of</td>
<td>• NaHS reduced thickening and stiffening of arteries.</td>
<td>• (Yanfei Wang et al.</td>
</tr>
</tbody>
</table>
Atherosclerosis

- GYY reduced vascular inflammation and oxidative stress, improved endothelial function and reduced atherosclerotic plaque.

2009)  
- (Z. Liu et al. 2013)

Atherogenic diet fed CSE knock-out mice

NaHS inhibited the accelerated atherosclerosis development

(Mani et al. 2013)

Mice model of cirrhosis

- NaHS induced relaxation of hepatic microcirculation.
- NaHS attenuates hepatotoxicity, liver cirrhosis and portal hypertension.

(Fiorucci et al. 2005)
- (Tan et al. 2011)

Genetic diabetes rat models

NaHS prevented high glucose dependent endothelial dysfunction and relaxation.

(Suzuki et al. 2011)

Mice model of type 2 diabetes

NaHS accelerated wound healing.

(G. Wang et al. 2015)

Rat model of ischemic vascular dementia

NaHS prevented neuronal injury; improved learning and memory.

(L.-M. Zhang, Jiang, and Liu 2009)

Mouse model of Parkinson disease

NaHS prevented neurodegeneration through inhibition of inflammation and apoptosis and activation of antioxidant defence mechanisms. Prevented the progression in movement dysfunction.

(Hu et al. 2010)

Rat model of colon-rectal distension

NaHS inhibited nociception.

(Distrutti et al. 2006)

Mice model of ethanol-induced gastric damage.

NaHS prevented gastric damage.

(Medeiros et al. 2009)

Mice model of oesophagitis

NaHS reduced the severity of the injury and inflammation.

(Zayachkivska et al. 2014)

Mice model of myocardial infarction

GYY preserves cardiac function, attenuates adverse remodeling and may exert post-ischemic cardioprotective (Lilyanna et al. 2015)

Mice xenografts of tumor cells

GYY exerts anti-cancer activity

(Z. W. Lee et al. 2011)

Table 4: H₂S treatments mediate beneficial effect in many animal models of different human diseases.

Among H₂S-releasing molecules (3) there are DATS (diallyl trisulfide) and DADS (diallyl disulfide). Both DATS and DADS are components of natural compound such as garlic (Powolny and Singh 2008). They both were found to induce apoptosis in different cancers cells: neuroblastoma cells (Filomeni et al. 2003), prostate cancer cell lines (D. Y. Shin et al. 2012), human gastric cancer cell lines (H. Tang et al. 2013), lung adenocarcinoma cancer cell lines (Wu et al.
2009), glioblastoma cells (Das, Banik, and Ray 2007). Furthermore DATS was found to inhibit migration, invasion, angiogenesis of colon cancer cell lines (Lai et al. 2015).

**H$_2$S-releasing drugs** (4) have been originally generated to prevent the adverse reactions associated with the use of the original drugs; however, they later found application in other pathologies than the ones indicated for the original drug. One of the first applications was in combination with nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, naproxen and diclofenac, as their use is often associated with a number of side effects including gastrointestinal irritation and damage, development of cardio-, cerebrovascular- and renal- pathologies which are a significant clinical concern and a considerable economic burden (Wallace, de Nucci, and Sulaiieva 2015). Therefore many hybrids have been developed to solve this clinical problem, as shown in Table 5, with the aim of deliver an endogenous, cytoprotective "rescue molecule" together with the original drug. More in general, the hybrids molecules demonstrated remarkable improvement in activity and tolerability as compared with the related parent compounds, suggesting an active pharmacological role for H$_2$S (Sparatore et al. 2011). In particular, H$_2$S-releasing-NSAIDs showed a greater anti-inflammatory effect, lower gastrointestinal toxicity, marked reduction in pancreatitis-associated lung injury and cardioprotection over the parent NSAID (Sparatore et al. 2011) (Rossoni et al. 2008). Moreover, H$_2$S-releasing-NSAIDs found an application in inhibiting the growth of a variety of cancer cells.

<table>
<thead>
<tr>
<th><strong>H$_2$S-releasing drugs</strong></th>
<th><strong>Application</strong></th>
<th><strong>Effect compared to that of the parental drug</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>NBS-1120 (H$_2$S-aspirin hybrid)</td>
<td>Chemoprevention of cancer</td>
<td>Similarly to aspirin: anti-inflammatory, analgesic, anti-pyretic, anti-platelet activities. Contrary to aspirin: did not cause stomach ulcers. Dose-dependently inhibited tumor growth and mass.</td>
<td>(Kodela et al. 2015)</td>
</tr>
<tr>
<td>ACS 14 (H$_2$S-aspirin hybrid)</td>
<td>Cardiovascular</td>
<td>Much stronger antithrombotic effects than aspirin.</td>
<td>(Pircher et al. 2012)</td>
</tr>
<tr>
<td>ATB-346 (H$_2$S-naproxen hybrid)</td>
<td>Gastric defence</td>
<td>Similar to naproxen: anti-inflammatory properties. Contrary to naproxen: did not cause toxicity on gastrointestinal tract and</td>
<td>(Wallace et al. 2010)</td>
</tr>
<tr>
<td>Disease/Medication</td>
<td>Effect</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>--------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Spinal cord trauma</td>
<td>Accelerated the healing of pre-existing gastric ulcers.</td>
<td>(Campolo et al. 2013)</td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td>Enhancement of the recovery of motor function and anti-inflammatory additional effects.</td>
<td>(Ekundi-Valentim et al. 2013)</td>
<td></td>
</tr>
<tr>
<td>ATB-337 or ACS 15 (H₂S-diclofenac hybrid)</td>
<td>Similar anti-inflammatory activity and reduction of edema and pain. Contrary to naproxen: no damage of gastrointestinal tract.</td>
<td>(Frantzias et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>IRI</td>
<td>Marked anti-inflammatory activity.</td>
<td>(Rossoni et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Acute pancreatitis and associated lung injury</td>
<td>Marked reduction in severity of pancreatitis-associated lung injury; greater anti-inflammatory activity; much lower gastrointestinal toxicity than diclofenac.</td>
<td>(Bhatia et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>GIC-1001 (H₂S-trimebutine hybrid)</td>
<td>Phase I clinical trial; Safe, well tolerated.</td>
<td>(Paquette et al. 2014)</td>
<td></td>
</tr>
<tr>
<td>Colonoscopy</td>
<td>Significantly reduced nociceptive response compared to trimebutine.</td>
<td>(Cenac et al. 2015)</td>
<td></td>
</tr>
<tr>
<td>ACS83, ACS84, ACS85, ACS86 (H₂S - L-DOPA hybrid)</td>
<td>Parkinson disease; Superior to L-DOPA as neuroprotectants, anti-inflammatory and antioxidant effects.</td>
<td>(M. Lee et al. 2010)</td>
<td></td>
</tr>
<tr>
<td>ACS67 (H₂S-latanoprost acid hybrid)</td>
<td>Retinal ischemia; Attenuated ischemic damage in retina.</td>
<td>(Osborne et al. 2010)</td>
<td></td>
</tr>
<tr>
<td>AVT-18A (H₂S-sulindac hybrid)</td>
<td>Familial adenomatous polyposis; Maintains the anti-inflammatory, analgesic, antipyretic, and antiplatelet properties. Inhibited the growth of cancer cells with higher potency. Contrary to sulindac: did not cause ulcers and bleeding.</td>
<td>(Kashfi, Chattopadhyay, and Kodela 2015)</td>
<td></td>
</tr>
<tr>
<td>ACS 6 (H₂S - sildenafil hybrid)</td>
<td>Urology; Promoted erection and had an anti-oxidant effect.</td>
<td>(Shukla et al. 2009)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: H₂S-releasing drugs demonstrated remarkable improvement in activity and tolerability as compared with the related parent compounds.
2.6. Bone homeostasis

Bone is a dynamic tissue continuously remodelled throughout adult life. Indeed, bone remodeling exerts different functions such as: 1) regulation of calcium homeostasis (Felsenfeld, Rodriguez, and Levine 2013); 2) maintenance of mechanical strength (Iolascon, Resmini, and Tarantino 2013); 3) regulation of acid/base balance (Arnett 2003); 4) reservoir of labile mineral (Confavreux 2011); 5) acting as an endocrine organ (Gonciulea and de Beur 2015). During bone remodeling, a finely regulated process referred to as coupling (Hattner, Epker, and Frost 1965) (Sims and Martin 2014) keeps in balance new bone formation by osteoblasts (OBs, the bone anabolic cells) and bone resorption by osteoclasts (OCs, the bone catabolic cells). This process occurs within microscopic, basic multicellular units lying at the interface of bone and bone marrow (BM) (Hauge et al. 2001) (Parfitt 2001). Recently, has been formulated the concept of modeling-based bone formation (MBF) opposed to remodeling-based bone formation (RBF). During this process, bone resorption and formation are thought to occur at different quiescent sites in an independent way (Ominsky et al. 2015). The biomolecular mechanism underlying MBF is still in its infancy, however, it has been proposed to occur primarily in response to changes in mechanical loading (Robling and Turner 2009). Both osteoblastogenesis and osteoclastogenesis are essential for correct bone development, modeling/remodeling and function. An imbalance in any steps of bone formation or resorption leads to a net loss or gain of bone tissue (Feng and McDonald 2011).

Excessive bone resorption by the OCs is the main cause of erosive diseases of bone, including osteoporosis, periodontal bone disease and inflammatory diseases of bone, thus making OCs the primary target of bone sparing therapies (Feng and McDonald 2011) (Haynes 2006) (Wiebe et al. 1996). OCs arise from circulating progenitor cells commonly known as osteoclasts precursors (pre-OCs), bearing monocytic phenotype and expressing, among others, CD11b as a distinctive marker (Tanaka et al. 2014). The pool of circulating pre-OCs as well as their mobility towards the bone surface was proven to be critically linked to the onset and the development of erosive bone diseases (Ritchlin et al. 2003) (Ishii et al. 2009). Genetically modified mouse models led to define receptor
activator of nuclear factor kappa-B ligand (RANKL) signaling as the crucial pathway in osteoclastogenesis (Edwards and Mundy 2011). The process, which results in MAPK, JNK, p38, ERK and NFκβ activation, is critically regulated by the redox balance (Wada et al. 2006). Indeed, RANKL induces a transient and rapid increase in ROS through activation of TRAF6, NOX1, RAC1 (N. K. Lee et al. 2005). ROS are critically involved in the signaling leading to h-OCs production, as in vitro treatment with antioxidants such as N-Acetyl-cysteine (NAC) prevents the signaling cascade induced by RANKL (Ha et al. 2004) (N. K. Lee et al. 2005), and results in decreased OCs differentiation. Strategies aimed at reducing ROS-mediated signalling lead to an inhibition of OCs differentiation and were proven to successfully decrease bone erosion in bone wasting diseases such as osteoporosis (Grassi et al. 2007) (Lean et al. 2003) or disorders associated with elevated ROS levels (Tsay et al. 2010). Another important molecule for osteogenic differentiation is macrophage colony-stimulating factor (M-CSF) which increases the pool of osteoclast precursors (Trouvin and Goëb 2010). Conversely, osteoclastogenesis and bone resorption is inhibited by osteoprotegerin (OPG), a decoy receptor of RANKL, which is produced by OBs, endothelial cells, vascular SMCs, and lymphoid cells. The balance of RANKL/OPG is thus essential to modulate osteoclastogenesis and bone remodeling.

In the last decade, increasing attention has been devoted also on the role of mesenchymal stromal cells (MSCs) due to their multipotent nature since they are able to differentiate toward osteogenic, adipogenic, chondrogenic lineages (Herzog, Chai, and Krause 2003) and due to their action as OCs supporting cells (D.-C. Yang et al. 2008) (Dalle Carbonare et al. 2009). Recently has been postulated the concept of skeletal stem cells (SSCs) which reside in the postnatal BM and have been proved to give rise to cartilage, bone, hematopoiesis-supportive stroma and marrow adipocytes in defined in vivo assays (Bianco and Robey 2015) (Bianco 2011). In healthy bone, osteogenic and adipogenic differentiation of MSCs is balanced in favour of osteogenic differentiation and bone formation. Aging and osteoporosis hamper this osteogenic potential in
favour of adipogenic differentiation (Rodríguez et al. 2008) and uncouple bone resorption and bone formation. Furthermore, aging decreased the BM-MSCs pool (H. Liu, Xia, and Li 2015).

Like h-MSCs, OBs actively regulates OCs formation and function as well as hematopoietic stem cells homeostasis (Capulli, Paone, and Rucci 2014). Similarly, osteocytes are increasingly recognized as significant sources of RANKL (the OCs differentiation factor), of sclerostin (osteoblast differentiation inhibitory factor) (J.-H. Kim et al. 2014) and mineralization-related genes (Sapir-Koren and Livshits 2014). They are now known to integrate mechanical, local, and hormonal signals and orchestrate bone remodeling (Noble 2008).

2.7. Postmenopausal osteoporosis

Osteoporosis is defined as a quantitative and qualitative degeneration of bone tissue leading to increased risk of fracture (Italiana et al. 2009). It is classically divided into primary, secondary and idiopathic forms (Mirza and Canalis 2015). In the elderly there are mainly primary forms; conversely, in the juvenile osteoporosis the primitive forms are relatively rare, but the secondary forms are emerging given the much longer survival in chronic diseases. Indeed, different drug therapies have been found to play a role in inducing osteoporosis: examples are methotrexate therapy for rheumatic diseases (Westhovens and Dequeker 2000); immunosuppressive drugs for organ transplantation (Kulak et al. 2012) (Stein, Ebeling, and Shane 2007) and chemotherapies for cancer (Pfeilschifter and Diel 2000) (Vestergaard 2008).

Postmenopausal osteoporosis is a common skeletal disease leading to fracture and disability that stems from the cessation of ovarian function at menopause and from genetic and non-genetic factors that heighten the impact of estrogen deficiency on the skeleton (Riggs and Melton 1983) (Riggs and Melton 1986). Etiopathogenetic mechanisms are estrogen depletion, aging of tissues, increased ROS levels and increased inflammatory factors (Sapir-Koren and Livshits 2013). Osteoporosis-related fractures are one of the major causes of morbidity and mortality in elderly people. Vertebral fractures are a source of significant pain and crippling, while hip fractures lead to
mortality rates of 24-30% in the first year alone. Furthermore, almost 50% of survivors suffer permanent disability (Johnell and Kanis 2006) (Burge et al. 2007) (Lewis et al. 2006) (Cummings and Melton 2002).

Declining estrogen levels results in a potent stimulation of bone resorption and, to a lesser extent, bone formation leading to a period of rapid bone loss (Zaidi 2007). This initial phase is followed by a slower but more prolonged period of bone loss that affects mostly the cortical compartment of the skeleton. The acute effects of menopause are modeled in animals by ovariectomy (ovx) that, like natural menopause, stimulates bone resorption by increasing OCs formation (Weitzmann and Pacifici 2006) (Li Sun et al. 2006) and lifespan (Nakamura et al. 2007) (Krum et al. 2008) (Martin-Millan et al. 2010). The net bone loss caused by ovx is limited by an increase in bone formation resulting from stimulated OBs formation (R L Jilka et al. 1998). This compensation is fueled by an expansion of the pool of MSCs, increased commitment of such pluripotent precursors toward the osteoblastic lineage (R L Jilka et al. 1998), and enhanced proliferation of early OBs precursors (Di Gregorio et al. 2001). Subsequent escalations in OBs apoptosis (Kousteni et al. 2001) (Almeida et al. 2007), extensions of OCs lifespan (Nakamura et al. 2007) (Krum et al. 2008), increased oxidative stress (Almeida et al. 2007) and increased secretion of inflammatory cytokines which suppress bone formation such as IL-7 and TNFα (Weitzmann and Pacifici 2006) contributes to explain why bone formation does not increase as much as resorption after ovx. However, the mechanism that prevents bone formation from increasing sufficiently to offset bone resorption is still largely unknown.

2.8. Current therapies for osteoporosis

OCs hold the unique property to resorb bone and strategies directed to specifically inhibit OCs function are the predominant approach to therapy for bone loss associated with high-bone turnover (Zaidi 2007). Today, the anti-resorptive therapy based on h-OCs inhibition is the first-line treatment of pathologies involving bone loss is. The gold standard treatment for post-menopausal
osteoporosis is bisphosphonates treatment. The mechanism that account for anti-resorptive action of bisphosphonates are: 1) suppressed OCs recruitment (Kimachi et al. 2011) (Hayami et al. 2004); 2) suppressed OCs survival (Cecchini et al. 1987) (Hughes et al. 1989); 3) direct suppressed OCs function (Neutzsky-Wulff et al. 2010); 4) indirect suppressed OCs function mediated by decrease of RANKL/OPG ratio in MSCs (Ohe et. Al. 2012). Among bisphosphonates, alendronate (AL) is one of the first-line treatment for primary and secondary osteoporosis (Drake, Clarke, and Khosla 2008). Due to its high trofism to bone and its potent anti-osteoclastic activity, AL increases bone mineral density (BMD) and bone quality and reduces the risk of bone fractures even if there are some contrasting evidences (Iwamoto et al. 2008). However, an important limitation of AL, as of other bisphosphonates, is that it do not restore the lost bone structure. Most importantly, AL has been reported to increase the risk for atypical bone fractures after long term administration probably due to severe suppression of bone turnover and inhibition of the coupling of bone formation and resorption (Odvina et al. 2005) (Armamento-Villareal et al. 2006). Another significant complication of AL and other bisphosphonates, is the osteonecrosis of the jaw (Paiva-Fonseca et al. 2014). This is a rare but painful complication of treatment characterized by infection, exposed bone, and poor wound healing (Faiman, Pillai, and Benghiac 2013). Another important clinical concern is the low adherence to therapy, resulting in a poor clinical outcome (Briesacher et al. 2007). Gastrointestinal adverse events, including severe events such as oesophageal ulcer, oesophagitis and erosive oesophagitis, are the primary reason for non-adherence (Segal, Tamir, and Ish-Shalom 2003) (Strampel, Emkey, and Civitelli 2007).

In addition to the firmly established role of AL on OCs, it is emerging a growing role on MSCs and OBs, although it is still a high debated topic. Many experimental evidences suggest that AL induces osteogenic gene expression in MSCs of different species and sources (Soares et al. 2016) (Duque and Rivas 2007) (H. K. Kim et al. 2009) (Yingjun Wang et al. 2010). Conversely, it was reported that high concentrations of AL alters cell viability and inhibits osteogenic differentiation of MSCs. The authors proposed that this disturbance of osteogenesis may lead to the
occurrence of atypical bone fractures and suggested both that anabolic bone-inducing agents may well be beneficial when prescribed with AL and that safer alternatives should be developed (Patntirapong, Singhatanadgit, and Arphavasin 2014). Currently, there is no explanation to account these opposing reports on AL function on MSCs; therefore it is mandatory to increase the shortcoming in such a knowledge for opening new therapeutic opportunities. Furthermore based on the important side effects of AL therapies, researchers are working to develop new formulations which could reduce adverse events and increase the adherence to therapy (Piscitelli et al. 2014) and increase the cost-effectiveness of drug therapy (Brandi and Black 2013).

2.9. Development of scaffolds for bone regenerative medicine

The field of regenerative medicine had steadily increased in the past few year as the need for replacing injured bone tissue has exponentially grown. Bone graft procedures are employed in a range of settings including dentistry, orthopaedics, and craniofacial medicine in order to regenerate large osseous defects caused by trauma, tumor resection, or congenital defects (Curry et al. 2016) (Larsson et al. 2016) (Gupta et al. 2015) (Baskin and Eppell 2013). Up to now there are only two alternatives to replace the damaged tissue: bone transplantation and prosthetic surgery. Bone transplantation include three procedures: autologous bone graft, which is the gold standard, and its alternatives, allogenic and xenogenic bone implants (Campana et al. 2014). Each of these approaches has its limits: 1) different surgeries and a lot of pain for patients for autologous bone graft; 2) high risk of pathologies transmission and the use of immunosuppressive agents for allogenic and xenogenic bone graft (Campana et al. 2014). Therefore bone substitutes are being increasingly used in surgery and the development of bone substitutes is become one of the major challenges in the field of bone tissue engineering. The development of a biomedical device has to fulfil specific requirements: (1) be biocompatible, (2) be biodegradable, (3) be osteoconductive, (4) be osteoinductive, (5) support angiogenesis and vasculogenesis and have (6) a shape that could fill relevant defects and (7) be resistant to mechanical load (Dumic-Cule et al. 2015) (Krishnan et al.
In this context, silk fibroin (SF) has gained increased attention in the field. It holds unique mechanical properties, manageable biodegradation rate and the ability to support the differentiation of MSCs along the osteogenic lineage. Furthermore it can easily be processed into different scaffold forms, combined synergistically with other biomaterials to form composites and chemically modified, providing many potential areas for future research (Melke et al. 2016) (Riccio et al. 2012).

2.10. MSCs and bone regenerative medicine

Many efforts have been attempted in the research for selecting MSCs population able to predict bone formation ability in vivo. Lineage specification has been defined as a highly complex hierarchical process (Phinney 2012). Different studies have evaluated the lineage differentiation potential and reported different percentage between the different progenitor frequencies (Phinney 2012) (Russell et al. 2010). It has been postulated that difference in the function of MSCs accounts for distinct sub-populations and that selective pressure (imposed for example by long term and large scale expansion) has a great impact on the composition and function of MSCs population. Minimal expansion may fail to enrich the sub-population with the desired functional attribute; on the contrary large scale expansion may select a particular sub-population thereby enhancing or reducing potency (Phinney 2012). MSCs manufactured by different clinical trial protocols have been demonstrated to be different as for cell yield and colony forming capacity (Seeger et al. 2007). Knowledge of self-renewal and lineage specification could be exploited to produce more homogeneous and potent cellular products for cellular biology (Phinney 2012). Donor-to-donor and intra-population heterogeneity has been widely described and has been defined as a critically important aspect of MSCs biology (Patricia Janicki et al. 2011) (Pevsner-Fischer, Levin, and Zipori 2011). Not all the cells maintained under standard culture conditions are capable of bone formation (Larsen et al. 2009). Moreover not all the clonally cells, obtained by clonally cultures of the same
MSCs population, are capable of bone formation. Larsen et. al. indentified, high-bone forming (HBF) and low-bone-forming (LBF) clones based on the amount of bone formed in vivo (Larsen et al. 2009). Moreover, they found a group of markers higher in HBF than in LBF which were able to prospectively identify different clones with different ability to form bone in vivo. Only few clones fulfilled the criteria of markers expression, evidence which limited the potential clinical application. Therefore a first step toward bridging the gap between basic research and clinical manufacturing is to understand heterogeneity of MSCs in order to find markers able to sort MSCs for their bone formation ability.

2.11. H2S and bone tissue

Up to now the role of H2S in the regulation of bone homeostasis has been scarcely investigated.

*Lessons from homocystinuria: May H2S be the link between CBS deficiency and bone loss?*

Homocystinuria or Hyperhomocysteinemia is a rare autosomal recessive disorder caused by CBS mutations (Mudd et al. 1985). This pathology is characterized by multisystem disorders including dislocated lenses, mental deficiency, premature atherosclerosis and thrombosis (Schedewie et al. 1973) (Brenton 1977). While the most striking cause of morbidity and mortality is thromboembolism, patients develop a marked osteoporosis at early age along with many other skeletal abnormalities (including kyphoscoliosis, temporal shortening of long bones due to impaired cartilage differentiation, arachnodactyly).

As CBS normally converts Hcy to cystathionine (Fig. 1), the result of CBS deficiency is an accumulation of Hcy. For many years Hcy accumulation, from which the pathology is named, has been proposed as the major determinant of the bone loss. Several lines of evidence indicated a role of Hcy on OCs compartment. *In vitro* treatments of Hcy on m-BM cells, m-BMMSCs and human peripheral blood mononuclear cells (h-PBMCs) increased OCs differentiation and function
(Herrmann et al. 2005) (Koh et al. 2006) (Y. Liu et al. 2014). Moreover experiments in the animal model of homocystinuria (CBS\(^-/-\) and CBS\(^+/+\) mice) further confirmed that Hcy levels may be associated with OCs activity (Y. Liu et al. 2014). Indeed, in both CBS\(^-/-\) and CBS\(^+/+\) mice, where Hcy levels were respectively 34 and 2 fold higher than in WT mice, the number of OCs were higher than WT mice and CBS\(^-/-\) exhibited a more significant increase in OCs than CBS\(^+/+\) mice (Y. Liu et al. 2014). Moreover Hcy has been proposed to cause defects in the collagen cross-linking (A. H. Kang and Trelstad 1973). However, the effect of high Hcy levels on OBs is still controversial. In vitro treatments of Hcy was shown to enhance apoptosis in h-MSCs and h-OBs cell line HS-5 (D. J. Kim et al. 2006) as in m-BMSCs (Cai et al. 2013). Conversely in vitro treatments of Hcy on h- MSCs and h-Ao-SMCs (aortic SMCs) was found to enhance osteogenic differentiation (Van Campenhout et al. 2009) and OBs function (Herrmann et al. 2008). **However, it seems to be conceivable that other mechanisms than high levels of Hcy cause the defect in bone homeostasis**, as betaine, the gold standard therapy for thromboembolism, is unable to completely prevent osteoporosis (Gahl et al. 1988). Notably, betaine is used as a Hcy-lowering therapy as it is a methyl donor in Hcy remethylation (Olthof and Verhoef 2005). Recent studies on CBS\(^-/-\) and CBS\(^+/+\) have demonstrated the relevance of H\(_2\)S for skeletal development and bone homeostasis. These models displayed a delayed endochondral ossification and an osteopenic phenotype reminiscent of the human inherited genetic disorder homocystinuria. Robert et. al., first proposed the transsulfuration pathway as a candidate mechanisms that might account for the delay in the endochondral ossification not Hcy accumulation-dependent (Robert et al. 2005).

**H\(_2\)S regulation of OBs differentiation**

The study of Liu et al. (Y. Liu et al. 2014), first indicated depleted of H\(_2\)S levels as a molecular mechanism driving osteoporosis in homocystinuric patients. They first reported a link between the activities of CBS, CSE; H\(_2\)S levels; the osteogenic differentiation of MSCs in vitro; and the stimulation of bone formation in vivo. Both CBS\(^-/-\) mice and CBS\(^+/+\) mice are characterized by
osteopenic phenotype. While CBS−/− mice and CBS+/+ have respectively 34 and 2 fold higher Hcy levels, they both have 50 % reduction of serum H2S levels. Therefore, Liu et al. hypothesized that H2S levels, but not Hcy, may account for the osteopenic phenotype and the aberrant differentiation of m-MSCs (BMMSCs) in CBS−/− and CBS+/+ mice (Y. Liu et al. 2014). In particular, when BMMSCs isolated from CBS−/− and CBS+/+ mice were characterized, they showed 1) increased proliferation rates and 2) reduced mineralization ability in vitro and in vivo. When BMMSCs isolated from WT mice were pharmacologically inhibited or silenced for CBS and CSE, aiming at reducing H2S levels, they displayed a similar phenotype to the one observed in CBS+/+. Coherently, the exogenous administration in CBS−/− and CBS+/+ BMMSCs of an H2S donor (NaHS) rescued both the proliferation rates and mineralization ability in vitro and in vivo. Moreover, m-MSCs isolated from H2S-treated CBS−/− and CBS+/+ mice showed similar proliferation rates and mineralization ability in vitro and in vivo similar to the ones of WT mice. Those data suggested that H2S actively regulates proliferation and function of BMMSCs.

Interestingly, beside the fact that patients affected by CSE deficiency seems not to have involvement on bone tissue, the pharmacological inhibition of CSE function, alongside the pharmacological inhibition of CBS, caused an osteopenic phenotype due to defective bone formation in mice, similar to that of CBS knockout mice (Y. Liu et al. 2014).

Moreover, they found that the mechanism underlying the H2S-deficiency-induced reduction of osteogenic differentiation is based on Ca2+-associated regulation of osteogenic differentiation. H2S sulfhydrates Ca2+ channels, inducing Ca2+ levels elevation and activation of pPKC and β–actin while inhibition of pERK.

Moreover, they demonstrated that H2S levels did not influence the BMMSCs adipogenic differentiation, as this capacity was shown to be similar between WT, CBS−/− and CBS+/+ mice, WT mice-inhibited for CBS and CSE, WT mice-silenced for CBS and CSE, H2S treated CBS−/− and CBS+/+ BMMSCs.
Currently, it is unknown whether pathologies with bone loss caused by mechanisms other than CBS deficiency display H$_2$S, CBS or CSE impairments. Further investigations will clarify whether H$_2$S may ultimately be the link between CBS, CSE expression and the osteogenic differentiation of MSCs and the unifying mechanism in different pathologies with bone loss.

**H$_2$S regulation of OCs differentiation**

Loss of estrogens or androgens has been proposed to accelerate the effects of aging on bone by decreasing defence against oxidative stress (Almeida et al. 2007) (Manolagas 2010). Conversely, antioxidants such as NAC, ascorbate or catalase was shown to prevent ovx-induced bone loss in rodents (Grassi et al. 2007) (Lean et al. 2003). As H$_2$S levels decrease with aging (Benjamin L Predmore et al. 2010) and H$_2$S prevents oxidative stress (Y. Kimura, Goto, and Kimura 2010) it is conceivable that H$_2$S decrease in both age and estrogen deficiency may intensify the effects of oxidative stress on bone tissues. However to our knowledge, this hypothesis has never been investigated. In this context, has been published that garlic oil can prevent the development of osteoporosis in mice (Mukherjee et al. 2004). However authors did not provided the mechanism of action of garlic oil on bone mass. Interestingly, recently garlic oil has been shown to be an H$_2$S-donor (Benjamin L Predmore et al. 2012).

To date, the effect of H$_2$S on OCs differentiation is still elusive and conflicting evidences have been reported. One day topical application of NaHS in a rat model of periodontal disease, was associated to higher number of OCs in the periodontal tissue (Irie et al. 2009). Additive effects were found with the concomitant treatment of lipopolysaccharide (LPS) (Irie et al. 2012). Analysis in the nearby gingival tissue, revealed higher expression of RANKL (Irie et al. 2009) (Irie et al. 2012). Conversely, 15 days of systemic administration of NaHS in a rat model of periodontitis (Toker et al. 2014) was associated to a dose-dependently decrease of OCs in alveolar bone. Moreover down-regulation of H$_2$S levels in CBS$^{+/-}$ and CBS$^{+-}$ mice, was associated to an higher number of OCs than WT mice (Y. Liu et al. 2014). Further *in vitro* experiments showed conflicting evidences on H$_2$S
role in regulating OCs differentiation. NaHS was reported to stimulate OCs differentiation in mouse macrophages (Ii et al. 2010). Conversely, several lines of evidence indicate that H2S can play a role in inhibiting OCs differentiation. NaHS inhibited (S. K. Lee et al. 2013) in vitro OCs differentiation in mouse macrophages; H2S inhibited nicotine- and LPS-induced mRNA expression of cytokines promoting osteoclastogenic differentiation (S. K. Lee et al. 2013); H2S increased the expression of OPG and the OPG/RANKL expression in human periodontal ligament cells (hPDLCs) (Liao and Hua 2013). Furthermore, whether NaHS can affect the osteoclastogenic differentiation of h-pre-OCs as well as the mechanisms underlying the regulation is still unknown.
3. AIMS

The main objective of this study was to increase the basic knowledge of the role of H$_2$S in bone tissue through *in vitro* and *in vivo* studies and to propose novel therapeutic approaches for bone diseases. In order to reach these objectives, we followed three specific aims:

1) **Elucidating the role of exogenous administration of H$_2$S in modulating bone remodelling.**

To this end, we aimed to investigate the *in vitro* effects of H$_2$S on the two main pathways involved in bone remodelling: osteoclastogenesis and osteoblastogenesis. In a second phase we aimed to a) investigate whether H$_2$S is able to modulate bone formation or resorption *in vivo* and to b) exploit the therapeutic potential of H$_2$S in a murine model of post-menopausal osteoporosis.

2) **Gaining new insights on the role of endogenous H$_2$S in bone tissues.**

In this section we aimed to investigate whether: a) CBS and/or CSE may be the major producers of H$_2$S in bone tissues (*ex vivo* analysis); b) osteogenic and estrogenic stimulations modulate CBS and CTH/CSE expression (*in vitro* analysis); c) CBS and CSE activities may in turn modulate osteogenic differentiation (*in vitro* analysis). In a second phase we aimed to verify whether: i) H$_2$S may be a molecule regulating bone homeostasis and ii) post-menopausal osteoporosis is associated to dysregulation of H$_2$S and H$_2$S generating enzymes.

3) **Translating *in vitro* and *in vivo* findings to possible applications of clinical interest.**

In this context, we considered two major clinical challenges: a) the development of improved drugs for post-menopausal osteoporosis and b) the development of more efficient bone substitutes. The study followed two lines of investigation: the development and analysis of the biological effects of a) DM-22, an H$_2$S-releasing drug based on alendronate (AL) and b) SF_GYY, an H$_2$S-releasing scaffolds based on silk fibroin (SF).
4. MATERIALS AND METHODS

4.1. Methods for cellular biology

**h-monocytes (CD11b+ cells) isolation**

h-monocytes were isolated from peripheral blood of healthy donors, by Ficoll gradient separation (Lympholite-H, Cederlane, Burlington, Ontario, Canada) followed by immunomagnetic positive selection of CD11b+ cells (MACS system, Miltenyi Biotech; Calderara di Reno, Italy), according to procedures well established in our laboratory (Grassi et al. 2011) and after having obtained informed consent by each donor. Contaminant, non-adherent, cells were washed off after the first medium change and the purity of the cell population was verified by Flow cytometry (FACS) analysis (please refer to ‘FACS analysis’ section).

**h-OCs differentiation**

For osteoclastogenesis assays, h-monocytes were seeded into 96 well-plates at a concentration of 1x10^6 cells/ml. For the functional assays, h-monocytes were seeded on synthetic hydroxyapatite-coated 16-well slides (Osteologic slides, BD Pharmingen, Franklin Lakes, NJ, USA), which mimics in vitro bone matrix, at the density of 5x10^5/cm^2.

h-pre-OCs and h-OCs were obtained by culturing h-monocytes respectively for 3 and 6 days in osteoclastogenic medium: α–MEM medium supplemented with 10% FBS and 1% penicillin/streptomycin, in the presence of M-CSF (10 ng/ml) and RANKL (75 ng/ml). α–MEM medium was purchased from Euroclone (Milan, Italy); Fetal bovine serum (FBS) Australian was purchased from Lonza (Basel, Switzerland); RANKL and M-CSF were purchased from Miltenyi Biotech.

Cells were cultured in osteoclastogenic medium in the presence or absence of different stimuli. NaHS working solution (500 mM stock solution) was prepared immediately before the use and added to cell cultures at concentrations ranging from 50 to 300 μM. NRF2 activators were added at...
medium culture in concentrations as following: sulforaphane (SFN; 0.2-1.5 μM) and tert-Butylhydroquinone (t-BHQ; 5-10-15 μM) (B. Y. Shin et al. 2012) (Vauzour et al. 2010). Both Alendronate (AL) and the H2S releasing AL (DM-22) were added at medium culture in concentrations 1-3,3-10-33 μM. In experiments of silencing during osteoclastogenic differentiation cells were treated with NT siRNA and NRF2 siRNA ± NaHS (please refer to the ‘RNA interference assay’ section). NaHS, t-BHQ, SFN, AL were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cells were cultured in 37°C, 5% CO2 and 95% O2 and medium and stimuli were replaced three times per week.

**Evaluation of h-OCs differentiation and function**

At the end of the osteoclastogenic differentiation (day 6), tartrate acid phosphatase (TRAP) assay (Acid Phosphatase, Leukocyte (TRAP) Kit, Sigma Aldrich) was performed to evaluate h-OCs differentiation. Mature h-OCs (TRAP positive cells containing at least three nuclei) were manually counted in duplicate using an inverted microscope; ten microscope fields at 20X magnification were considered for each well and the h-OCs count was expressed as average h-OCs number/fields.

Pit assay was performed as functional assays for h-OCs function. Osteologic slides were washed with bleach to eliminate cells. Afterwards, pits formed on the Osteologic slides upon matrix breakdown by OCs, were acquired at microscope Eclipse 90i and NIS software (Nikon Instruments Europe BV, Amstelveen, the Netherlands).

**ROS quantification in h-pre-OCs**

Macrophagic h-pre-OCs were obtained in black 96-wells plates by stimulating with M-CSF (25 ng/ml) alone for 3 days. Then, for evaluating ROS production during RANKL stimuli, cells were treated with RANKL (100 ng/ml) or H2O2 (100 μM), the positive control, for 30 minutes. NaHS (100-200 μM) was added 10 minutes before RANKL. Cells were then loaded with 5 μM 2’, 7’-
dichlorodihydrofluorescein diacetate (H$_2$DCFDA, Life Technologies, Carlsbad, CA, USA) and kept in the dark for 15 minutes for the formation of oxidized fluorescent 2′, 7’-dichlorofluorescein (DCF) molecule by ROS, according to manufacturer instructions. DCF fluorescence was measured with Spectramax Fluorimeter (Molecular Devices, Sunnyvale, CA, USA) at 520 nm and by FACS analysis.

**h-MSCs and h-OBs isolation and culture**

A total of 76 samples of h-MSCs and 28 samples of h-OBs were employed in this study. Surgical procedures were all performed at the Rizzoli Orthopedic Hospital (Bologna) after having obtained patients’ informed consent and approval form the Ethic Committee. Tybial plateau from patients undergoing surgical knee replacement was the source for both h-MSCs and h-OBs isolation (Manferdini et al. 2011). Briefly, bone fragments were mechanically removed from tybial plateau and fragmented into small pieces. The bone fragments were, subjected to enzymatic digestion in “Enzyme medium” (Roche, Basel, Switzerland) at 37°C in rotation. h-OBs were allowed to sprout from the small pieces of bone fragments and grow until confluence in “Medium complete”. h-MSCs were obtained throught a Ficoll-density (Lympholite-H, Cederlane) gradient isolation protocol as previously reported (Torreggiani et al. 2012) (Cristino et al. 2005) from two different sources: a) tybial plateau (we derived h-MSCs from the cell suspension produced during the mechanical isolation of h-OBs); b) iliac crest (we derived h-MSCs from bone marrow aspirated during ankle replacement surgery). After 1 week, non-adherent cells were removed and the adherent h-MSCs were expanded in vitro in α–MEM medium supplemented with 15% FBS and 1% penicillin/streptomycin. When stimulated with 17β-estradiol cells were cultured in α–MEM medium supplemented with 5% FBS and 1% penicillin/streptomycin without phenol red (Gibco). Cells were cultured in 37°C, 5% CO$_2$ and 95% O$_2$ and medium was replaced twice per week. Then, cells were harvested and seeded, depending on the experimental settings at different passage of
culture. In selected experiments they were treated with NaHS (6 μM-3,5 mM) and 17β-Estradiol (10^-8 to 10^-9 M; Sigma Aldrich).

“Enzyme medium” was prepared as following: 50:50 Ham’s F12 without calcium and DMEM without calcium, penicillin/streptomycin (100U/100 μg), L-glutamine 4 mM, calcium chloride dehydrate 2 mM supplemented with collagenase P (0,7 U/ml). “Medium complete” was prepared as following: 50:50 Ham’s F12 without calcium and DMEM without calcium, penicillin/streptomycin (100U/100 μg), L-glutamine 4 mM, 11% heat inactivated FBS, ascorbic acid 25 μg/ml. Ham’s F12 without Calcium, DMEM without Calcium and penicillin/streptomycin were purchased from Gibco (Life Technologies Italia, Monza MB, Italy). Calcium chloride dehydrate, L-glutamine and Ascorbic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Collagenase P was purchased from Roche (Basel, Switzerland).

**Viability and toxicity assays**

 Annexin V/propidium iodide (PI) staining assays (Annexin V-FITC apoptosis detection kit, Roche, Molecular Biochemicals, Mannheim, Germany) and lactate dehydrogenase (LDH) assays (Cytotoxicity detection kit (LDH), Roche) were performed according to manufacturer instructions, to test cellular apoptosis and acute toxicity respectively. While Annexin V/PI assay was performed only in h-OCs precursors, LDH assay was performed on both h-OCs and h-MSCs. h-monocytes were seeded into 96 well-plates at a concentration of 2x10^5 cells/ml; h-MSCs were seeded into 96 well-plates at a concentration of 1x10^4 cells/ml. h-monocytes were cultured in unstimulated medium (α–MEM 10% FBS for Annexin V/PI assays and α–MEM 5% FBS depleted of phenol-red for LDH assays), h-MSCs were cultured in α–MEM 7,5% FBS depleted of phenol-red for LDH assays. h-OCs and h-MSCs were cultured for 24-72 h in the presence or absence of increasing concentrations of NaHS (50-300 μM); AL and DM-22 (1-33 μM). Briefly, apoptotic cells were identified as Annexin V_+ /PI_+ by FACS analysis. LDH measurements on supernatants of cell culture medium were performed at 492-620 nm on TECAN Infinite® 200 PRO (Tecan Italia S.r.l.,
Cernusco Sul Naviglio, Italy). Photos of morphology were taken using Nikon Instruments Europe BV (Amstelveen, the Netherlands) after having performed Toluidine blue staining. To this end, cells were firstly fixed with formalin 10% for 20 min and then stained with toluidine blue for few seconds. Toluidine blue positive cells were measured as absorbance at 560 nm on TECAN (Tecan Italia Srl).

**Cell proliferation assay**

h-MSCs were seeded in quadruplicates in 96-well plates at 1x10^4 cells/well in α-MEM 15% FBS. Eighteen hours before each time point, 5 µCi of ³H-thymidine (Perkin Elmer, Boston, MA, USA) was added to each well and radioactivity was then measured using a beta-counter (Perkin Elmer).

**Osteogenic differentiation of h-MSCs in static condition**

For osteogenic assays and quantification of mineralization capacity, 2x10^5 h-MSCs were plated on 12-well plate in osteogenic medium and cultured for 7, 14, 21 and 28 days. Osteogenic medium was prepared with α-MEM 20% FBS in the presence of 0,1 μM Dexamethasone, 100 μM Ascorbic Acid and 10 mM β-glicerolphosphate (Sigma Aldrich). In specific experimental settings were performed additional treatments with NaHS (6-200μM); CBS, CTH, NT siRNA (please refer to “RNA interference assay” section); DL-propargylglycine (PAG), Hydroxylamine hydroxychloride (HA) which were purchased from Sigma Aldrich and administered in combination respectively at 10 mM and 100 μM concentration; AL and DM-22 (1-3.3-10-33 μM).

**Evaluation of h-OBs differentiation in vitro**

To assess the extent of mineralization we performed Alizarin Red S (AR-S) staining (Sigma Aldrich). Briefly, cells were firstly fixed with 10% buffered formalin formaldehyde (Kaltek, Padova, Italy) for 20 minutes at room temperature (RT), washed twice with phosphate buffered saline (PBS), and then stained with 40 mM AR-S for 20 minutes at RT. AR-S-positive nodules
were measured as absorbance at 510 nm using spectrophotometric analysis with TECAN Infinite® 200 PRO (Tecan Italia S.r.l.). This technique allowed us to evaluate a great number of case studies obtained over time and discriminate cells with different behaviour in response to osteogenic stimuli.

**Osteogenic differentiation of h-MSCs in dynamic condition**

1x10⁶ cells were seeded in scaffolds placed in the U-CUP perfusion bioreactor device (CELLEC BIOTEK AG, Basel, Switzerland) in α–MEM 15% FBS at 1,2 ml/min. The day after the seeding cells were induced with osteogenic medium and were cultured for 14 days at 0,3 ml/min. Medium was replaced twice per week; cells were cultured in 37°C, 5% CO₂ and 95% O₂.

**4.2. Methods for molecular biology**

**RNA interference Assay**

RNA interference was used to down regulate the expression of CBS, CSE in h-MSCs and of NRF2 in h-OCs.

In h-MSCs gene silencing was achieved by transfecting with control, non-targeting siRNA (NT siRNA, ON-TARGETplus, Non-targeting pool, FE5D0018101005) or CTH siRNA (ON-TARGET plus SMARTpool siRNA, J-003481) or CBS siRNA (ON-TARGET plus SMARTpool siRNA, J-008617), with a pool of four sequences to ensure high level of silencing. Each transfection was performed according to manufacturer instructions and using 20 nM siRNA (purchased from Dharmacon, ThermoScientific) and 6 µl INTERFERin, the siRNA transfection reagent (Polyplus Transfection, Illkirch France, 409-10). The first transfection was performed in h-MSCs reaching 60% confluency in α–MEM 15% FBS. Afterwards, cells were induced with osteogenic medium. Transfection was repeated at every medium change until mineralization was observed in control cells. Afterwards AR-S and RT-PCR analyses for CBS and CSE were performed.
Macrophagic h-pre-OCs were obtained in 96-wells plates by stimulating with M-CSF (25 ng/ml) alone for 3 days before transfection with NT siRNA (ON-TARGETplus Non-targeting pool) and NRF2 siRNA (ON-TARGET plus SMART pool Human NFE2L2), both purchased by Dharmacon (Thermo Scientific). Each transfection was performed according to manufacturer instructions and using 10 nM siRNA (Dharmacon) and 0.75 μl INTERFERin. The day after transfection, cells were induced with osteoclastogenic medium in presence or absence of 200 μM NaHS. Transfection was repeated every 3 days of culture to ensure maximal suppression of NRF2, until the end of osteoclastogenic differentiation, when TRAP assays, RT-PCR and WB analyses for NRF2, NQO1, PRDX1 were performed. The percentage of silencing was measured by comparative CT method (ΔΔCT) according to the following formula: ΔΔCT = ΔCT- NRF2 siRNA - ΔCT- NT siRNA.

Reverse transcription-Polymerase chain reaction (RT-PCR)

RNA was extracted with RNApure (Euroclone), and purified from DNA with DNA-free™Kit (Ambion, Life Technologies) according to manufacturer instructions. RNA concentration was measured by Nanodrop 2000c (Thermo Scientific, Rockford, IL, USA) and RNA quality was assessed through 260/280 and 260/230 nm absorbance ratio. Only samples showing a 260/280 ratio >1.8 were sent to the transcription step. Reverse transcription (SuperScript® VILO™ cDNA Synthesis Kit; Invitrogen, Life Technologies) was performed utilizing 0.5-1 μg of RNA according to manufacturer instruction and as following: 25°C for 10 minutes, 42°C for 60 minutes, 85°C for 5 minutes and 4°C for 30 minutes on 2720 Thermal cycler (Applied Biosystem, Life Technologies). Polymerase chain reaction (PCR) (SYBR Premix Ex Taq, TaKaRa Biomedicals, Tokyo, Japan; LightCycler Instrument, Roche) was performed on 20 ng cDNA as following: one cycle at 95°C for 10 seconds and 45 cycles at 95°C for 5 seconds and at 60°C for 20 seconds.

All primers designed by us were purchased from Life Technologies Italia (primers sequences are reported in Table 6 and 7, respectively for human and mouse). The other murine primers were designed by our collaborators using Primer Express Express® Software v2.0 (PE Biosystems) and

The specificity of the PCR products was confirmed by standard melting curve analysis with the following thermal cycling profile: 95°C for 10 seconds, 65°C for 15 seconds and 95°C in one-degree increments. Relative quantification of PCR products was obtained with the comparative CT method, comparing to the housekeeping mRNA expression of glyceraldehyde-3 phosphate dehydrogenase (GAPDH).

<table>
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<tr>
<th>Approved Name</th>
<th>Approved symbol</th>
<th>5'-Sequence-3'</th>
<th>Product size (bp)</th>
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<td>Glyceraldehyde-3 phosphate dehydrogenase</td>
<td>GAPDH</td>
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<td></td>
<td></td>
<td>Reverse CCTGGAAGATGGTGATGG</td>
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<td>Cystathionine-β synthase</td>
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<td>Peroxiredoxin 1</td>
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<td></td>
<td></td>
<td>Reverse CACAAGGTAAGTCAAGAGGG</td>
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<td>Receptor activator of nuclear factor kappa-B ligand</td>
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<td>Reverse GACTCACTTATGGGAAACCAG</td>
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<td>Osteoprotegerin</td>
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Table 6. The human primer sequences for RT-PCR
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<td>187</td>
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*Table 7. The murine primer sequences for RT-PCR*

Levels of murine mRNA expression were also analyzed at Atlanta according to an established protocol. Briefly, RT-PCR was performed using an ABI Prism 7000 or One Step Plus Sequence Detection System and SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA). Changes in relative gene expression between groups were calculated using the 2 −ΔΔCT method with normalization to 18S rRNA as previously described.

**PCR Arrays**

mRNA expression in mice whole BM lysates was analyzed for a cluster of pathway-focused Wnt-signaling related genes by means of PCR Array (Mouse Wnt signaling pathway Kit, Qiagen, Milan, Italy), according to the manufacturer's instructions. Briefly, total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Milan, Italy) and contamination of genomic DNA was removed from total RNA samples by treating with DNase I (DNA-free Kit, Ambion, Austin, TX, USA). Complementary DNA (cDNA) synthesis was performed from 0.8 μg RNA using the RT2 PCR array First Strand Kit (Qiagen, Milan, Italy). Amplification was carried out on a Rotor Gene Thermal Cycler (Corbett Research, Qiagen) equipped with a 100-well rotor, under cycling and thermal conditions suggested by the manufacturer. The data were analyzed using the web-based RT2 PCR array Data Analysis tool provided by the manufacturer.
**Gene expression profiling of h-MSCs and h-OBs**

A subset of 6 h-MSCs and 6 h-OBs obtained from tibial plateau were screened for CBS and CSE expression within a database of Gene Array obtained in previous analysis performed in our laboratory (Lisignoli et al. 2009). Total RNA from h-MSCs and h-OBs at passage 1 in culture were hybridized to biotin labeled cRNA on GeneChip® Human Genome U133A Arrays (Affimemrix, Inc., Santa Clara, CA, USA). Scanning of the chip was carried out according to manufacturer instruction (GeneChip® Scanner 3000 7G, Affimetrix Inc.). Probe level data were converted to expression values using the Bioconductor function for robust Multi-array average procedure. dChip software (DNAChip analyzer) and Significant Analysis of Microarrays software version 3.00 were used respectively to cluster and generate dendograms and for a supervised analysis as described in Lisignoli et. al. 2009 (Lisignoli et al. 2009). In the analysis, genes with at least a 1.5 average change in the expression from the mean across the whole panel were selected (2AVEFC).

**FACS analysis**

FACS analysis was performed on FACS canto II (BD bioscience, San Jose, California, USA) to assess: 1) the purity of CD11b+ enriched population obtained in the procedure of isolation of h-monocyte. CD11b+ cells were on average 92% of the total cell population; 2) the surface markers expression of h-MSCs and h-OBs as detailed in Manferdini et. Al. (Manferdini et al. 2011); 3) The measurement of h-OCs positive to NRF2 after 24 h of 100-200 μM NaHS stimulation in osteoclastogenic culture medium. Briefly, cells were fixed with paraformaldehyde (PFA) 4%, permeabilized with PBS 0,2% tween (only for NRF2 measurement), resuspended at 1x10^6 cells/ml in FACS buffer (0.1% NaN3, 2% di FBS in PBS 1X) and incubated with primary antibody and secondary antibody anti-rabbit-FITC (DAKO, Milan, Italy), which are shown in Table 8. The analysis and measurement of signal intensity was performed by FACS analyses.
<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Antibody Code and Producer</th>
<th>Application / Concentration</th>
</tr>
</thead>
</table>
| Anti-Nrf2 IgG   | H-300, Santa Cruz Biotechnology, Santa Cruz, CA, USA. | WB (2,9 μg/ml)  
|                 |                            | Immunocytochemical analysis (2,9 μg/ml)  
|                 |                            | FACS analysis (2,9 μg/ml)          |
| Anti PRDX1 IgG  | ab109498, Abcam, Cambridge, UK | WB (0,06 μg/ml)  |
| Anti Nqo1 IgG   | Ab28947, Abcam              | WB (1 μg/ml)            |
| Anti β–actin IgG| A228, Sigma Aldrich         | WB (0,5 μg/ml)          |
| Anti CBS IgG    | H00000875-A01 (polyclonal), Abnova, Taipei City, Taiwan | WB (dilution 1:300)  
|                 |                            | Immunocytochemical analysis (dilution 1:250)  
|                 |                            | Immunohistochemical analysis (dilution 1:400) |
| Anti CSE        | H00001491-M02, Abnova      | WB (1,7 μg/ml)  
|                 |                            | Immunocytochemical analysis (2,5 μg/ml)  
|                 |                            | Immunohistochemical analysis (5 μg/ml)  |
| Anti CD11b      | a-human CD11b PE, Beckton Dickinson, Milan, Italy | FACS analysis (2,9 μg/ml)  |
| Anti-rabbit and Anti-mouse IgG HRP | 211-032-171,115-035-174, Jackson ImmunoResearch, West Grove, PA, USA | WB (0,03 μg/ml)  |
| Anti-rabbit-FITC| F0054 DAKO, Milan, Italy   | FACS analysis (10 μg/ml) |

*Table 8. List of antibodies used in the study, for each antibody the code and application of use is reported.*

**Immunocytochemical analysis**

h-pre-OCs were cultured in osteoclastogenic medium in 8 wells Permanox chamber slides and treated as occurs with NaHS (100-200 μM) or NRF2 activators (0,2-1-5 μM SFN and 5-10-15 μM t-BHQ). For NaHS two treatments were compared: 1. standard treatments, performed for the whole duration of the culture (treatments were renewed during each medium change); 2. single dose treatment, 2h before the analysis. h-MSCs and h-OBs were cultured respectively in α–MEM
15%FBS and Medium complete. h-pre-OCs, h-MSCs and h-OBs were then fixed with 4% PFA/PBS. Only for NRF2 staining h-pre-OCs were permeabilized 0.25% Triton X-100 (Sigma Aldrich)/PBS. Cells then were incubated with primary antibody to NRF2, CBS or CSE as listed in Table 8 and revealed with Universal AP Detection kit (Biocare Medical, Concord, CA, USA). Slices were counterstained with haematoxylin, subjected to tap-water activation and finally mounted in glycerol gel. Negative and isotype-matched controls were performed. CBS, CSE and NRF2 staining were evaluated with Eclipse 90i microscope and NIS-Elements Software (Nikon).

For NRF2 staining we performed additional immunocytochemical quantification. As osteoclastogenic cultures of h-monocytes give rise to a mixed population of cells at a different stage of differentiation, we pursued the quantification of nuclear translocation of NRF2 by manually counting nuclear NRF2 staining in cells showing homogenous phenotypical features of h-pre-OCs. The frequency of nuclear translocation was manually counted, in duplicates, by an investigator blind as for the nature of the specimen, on 16 pictures at 200X magnification for each duplicate, captured by NIS software (Nikon).

**Western blot (WB) analyses**

30 μg of total proteins were separated under denaturing conditions in NuPAGE® Novex® 4-12% Bis-Tris Gels (Invitrogen, Life Technologies) mounted on XCell SureLock® Mini-Cell device (Invitrogen) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA) through iBlot® Gel Transfer Device (Invitrogen). The immunodetection was performed by SNAP i.d.™ Protein Detection System (Millipore) using as occurred the antibody reported on Table 8.

Signal detection was revealed using Amersham ECL Select WB detection reagent (RPN2235, GE Healthcare Italia, Milan, Italy) and acquired through KODAK Image Station 4000R Digital Imaging System (Kodak, Rochester, NY, USA). CARESTREAM and Image J software were used respectively to confirm the correct size of bands and to relatively quantify bands’ intensities.
**Immunohistochemical analysis**

Tybial plateau biopsies were obtained from 10 osteoarthritic patients undergoing surgical knee replacement. Biopsies were fixed in a freshly prepared 9:2 mixture of B2% solution (mercuric-chloride containing fixative)/40% formaldehyde (Kaltek) at RT for 2 h. The biopsies were then decalcified in 0.1M EDTA-bisodic salt (Sigma), dehydrated and embedded in paraffin as previously described (Lisignoli et al. 2002) and sectioned with microtome in 3-4 μm thick slices. Immunostainings for CBS and CSE were performed on deparaffinized and rehydrated slices for the primary antibody listed in Table 8, as well as for negative and isotype-matched controls. Positive staining was revealed with Universal AP Detection kit (Biocare Medical). Slices were counterstained with haematoxylin and mounted in glycerol gel. The staining was evaluated on Eclipse 90i Microscope (Nikon) and pictures at 200X or 400X magnification were captured by NIS software (Nikon, Firenze, Italy).

**Histological analysis**

At D0, D7, D14 scaffolds placed in the U-CUP perfusion bioreactor device, were harvested and fixed with PFA 4%, processed, embedded in paraffin and sectioned with microtome in 3-4 μm thick slices for histological assessments. Then, Haematoxylin-Eosin (H/E) and Von Kossa (VK) staining were performed on deparaffinized and rehydrated sections. Briefly, for H/E staining, slices were stained with CAT haematoxylin (BioCare Medical, Concord, USA), activated with tap-water and then stained with eosin (Honeywell Riedel de haen, Seelze, Germany), rinsed in water and dehydrated. For VK staining, slices were incubated in silver nitrate solution 1% (Carlo Erba, Milan, Italy) for 30 min under UV light, rinsed in water, incubated in sodium thiosulphate 5% (Sigma) for 5 min, rinsed in water, counterstained with haematoxylin (BioCare Medical), rinsed with water and dehydrated. For both staining, slices were then mounted with Entellan (Merk, New York, USA). The staining was evaluated on Eclipse 90i Microscope (Nikon) and pictures at 200X or 400X magnification were captured by NIS software (Nikon, Firenze, Italy).
4.3. Methods for *in vivo* murine study

It should be noted that the experiments which methods are reported in this section have been performed by our collaborators (Prof. Pacifici and Dr Tyagi) at Emory University, Atlanta.

All animal procedures were approved by the Institutional Animal Care and Use Committee of Emory University. Adult female C57BL/6 mice (9–10 weeks old) were used for this study. All mice were housed at 25°C, in 12-hour light: 12-hour dark cycles. Normal chow diet and water were provided ad libitum. Mice were ovariectomized (ovx) or sham operated (surgically operated as for ovariectomy –ovx- while leaving ovary intact). In mice ovx induces rapid bone loss since 4 weeks. The treatment was given as intraperitoneal injections of 1mg/mice of the slow-releasing H$_2$S donor, GYY, or of vehicle every other day. Two types of study were performed: 1) a preventive and 2) a therapeutic study. In the former, mice were treated with vehicle or GYY for 4 weeks starting the day of surgery; in the latter vehicle or GYY were administered for 4 weeks starting 4 weeks after surgery. Therefore, in both experiments we compared vehicle treated sham operated mice (SHAM Veh), GYY treated sham operated mice (SHAM GYY), vehicle treated ovx mice (OVX Veh) and GYY treated ovx mice (OVX GYY).

*H$_2$S* measurements

0.1 mL of serum was collected after sacrifice. H$_2$S and bound sulfur levels were measured by gas chromatography, according to previously described methods (Nicholson et al. 2013). The concentrations of free H$_2$S and H$_2$S released by bound sulfur in the samples were calculated using a standard curve of Na$_2$S as a source of H$_2$S. Chromatographs were captured and analyzed with Agilent ChemStation software (B.04.03).

*μCT* measurements

μCT scanning and analysis was performed as reported previously (Terauchi et al. 2009) (Tawfeek et al. 2010) using a Scanco μCT-40 scanner (Scanco Medical, Bassersdorf, Switzerland). Voxel sizes
were 8 μm³. For the femoral trabecular region we analyzed 140 slices from the 50 slices under the distal growth plate. Femoral cortical bone was assessed using 80 continuous CT slides located at the femoral midshaft. X-ray tube potential was 70 kVp, and integration time was 200 ms. Percentage trabecular bone volume over total volume (BV/TV %), trabecular thickness (Tb.Th: μm), trabecular number (Tb.N: 1/mm), trabecular space (Tb.Sp: μm), cortical volume (Ct. V: mm³), cortical thickness (Co.Th: μm) were measured.

**Quantitative bone histomorphometry**

Mice were injected subcutaneously with calcein (25 μg/g) 10 and 3 days before sacrifice. Vertebrae and femurs were fixed in 10% neutral-buffered formalin for 48 hours, dehydrated and defatted at 4°C, and embedded in methyl methacrylate resin. 5-mm non-consecutive longitudinal sections were cut using a Leica RM2155, stained with Goldner’s trichrome stain and used for analysis of static indices of bone formation: number of OCs per millimeter of bone surface (N.Oc/BS), percentage of bone surfaces covered by OCs (Oc.S/BS), number of OBs per millimeter of bone surface (N. Ob/BS), percentage of bone surfaces covered by OBs (Ob.S/BS).

Additional sections were cut at 10 μm, and left unstained for dynamic (fluorescent) measurements. Longitudinal sections of L4 were obtained in the frontal midbody plane. Measurements were obtained in an area of cancellous bone that measured ~2.5 mm² and contained only secondary spongiosa, which was located 0.5–2.5 mm proximal to the epiphyseal growth cartilage of the femurs or of the L4 vertebrae. Measurements of single-labeled and double-labeled fluorescent surfaces, and interlabel width were made in the same region of interest using unstained sections. Mineral Apposition Rate (MAR: mm/day), Bone Formation Rate (BFR/BS: mm³/mm²/day) and percentage of bone surfaces covered by mineralized surfaces per bone surfaces (MS/BS: %) were calculated by the software by applying the interlabel period. Histomorphometry was done using the Bioquant Image Analysis System (R&M Biometrics). Measurements, terminology and units used
for histomorphometric analysis, were those recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research (Parfitt et al. 1987).

**Markers of Bone Turnover**

C-terminal telopeptide of type 1 collagen (CTX) and N-terminal propeptide of type 1 procollagen (P1NP) were measured, according to manufacturer instructions, by a rodent specific ELISA assay and a Rat/Mouse ELISA kit (Immunodiagnostic Systems, Scottsdale, AZ) respectively.

**BM harvesting and murine stromal cells (m-SCs) purification**

BM was harvested at the end of the treatment period and was investigated for expression of a cluster of 17 Wnt ligands (Wnt1, Wnt2, Wnt3, Wnt5a, Wnt7a, Wnt 7b, Wnt8b, Wnt9, Wnt10a, Wnt10b, Wnt11, Wnt16, Wnt2b, Wnt3a, Wnt4, Wnt5b, Wnt6, and Wnt8a) by using an approach based on PCR Array technology. m-SCs were purified from BM as previously described (Gao et al. 2007) (Bedi et al. 2012). Briefly, BM was cultured for 7 days in α-MEM medium containing 10% FBS, to allow the proliferation of m-SCs. After discarding non-adherent cells, adherent macrophages were eliminated by positive immunoselection by MACS Microbeads (Miltenyi Biotec, Auburn, CA) coupled to anti-CD11c antibody. m-SCs were then purified and utilized to assess the levels of CBS and CSE mRNAs; of osteoblastic genes mRNAs (Runx2, Col1, Osx and Ocn); of WNT target genes mRNAs (Ahr, Axin2, Cyr61, Nkd2, Tagln, Tgfβ3, Thbs1, Twist1, Wisp1, Tcf, Lef-1).

**CFU-ALP Assays**

BM was harvested at the end of the treatment period and cultured for 1 week to assess the formation of alkaline phosphatase positive colony forming unit-fibroblast (CFU-ALP), an index of m-SCs commitment to the osteoblastic lineage, as previously described (Gao et al. 2008). Briefly, BM m-SCs were cultured in α-MEM medium containing 10mM β-glycerophosphate and 50 μg/mL
ascorbic acid at a density of $2 \times 10^6$ cells/cm$^2$. After 7 days, the cells were fixed and stained for ALP and the number of colonies positive for ALP was counted.

4.4. Methods for scaffolds production and characterization

It should be noted that the experiments which methods are reported in this section have been performed by our collaborators (Prof. Motta and Dr Raggio) at University of Trento.

Preparation of silk fibroin (SF) and $H_2S$ releasing based on silk fibroin (SF_GYY) scaffolds

A certain amount of silk cocoons, produced by poly-hybrids silkworms of the species Bombyx mori, were opened, reduced to small pieces and manually defoliated. Boiling in aqueous alkaline solutions in $Na_2CO_3$ allowed the solubilization of the external coating of the fibers of silk, composed by sericin. Then, the fibers were degummed in concentrated aqueous solutions of LiBr, under a slight heating, for several hours. Afterwards, LiBr was completely removed dializing in water for 3-4 days. This procedure allowed obtaining an aqueous solutions of a single skein of fibroin to 6-8% (w/V). Then porous scaffolds were obtained through the method of salt leaching, using NaCl as a porogen agent sieved in grain size in the range between 425 and 1180 pM (weight ratio of porogen to silk 25:1). This protocol has been developed and optimized gradually, over the years, at the Department of materials engineering and industrial technology (Univesity of Trento) and ensures the formation of pores of size suitable for host and promote the growth and proliferation of h-MSCs. NaCl was then removed with water and the solid dry products were achieved by freeze-drying. The layer of scaffold produced was conveniently punched for obtaining small cylinders of 10 mm in diameter, 3 mm thick and around 20 mg in weight.

Different methods were evaluated and tested for the adhesion GYY to the scaffolds. Finally, the method selected was dipping in dimethyl sulfoxide (DMSO; Sigma Aldrich) which avoided the use of aqueous environment that can induce an early GYY decomposition, and the $H_2S$ release.
Solutions of GYY in DMSO were prepared and dropped onto the scaffolds till sponge saturation. To remove the solvent, sponges were immediately frozen at -50°C and freeze-dried over night and stored in desiccator for the analysis. The native (SF) and modified fibroin scaffold (SF\_GYY) were characterized for their morphology by SEM (Scanning Electron Microscope analyses) analyses and for indentifying components of the scaffold by FTIR (Fourier Transform Infrared Spectroscopy).

**H₂S release measurements**

H₂S release from SF\_GYY was measured by methylene blue assay as detailed elsewhere (Shen et al. 2011). Each sample was immersed in an eppendorf tube containing 1 ml of bidistilled water, and the release of H₂S in aqueous solution was monitored at established time points. A calibration curve for H₂S was obtained measuring different concentration of NaHS (between 0 and 80 μM). Spectrophotometric analysis measuring absorbance at 670 nm detected the methylene blue generated by the reaction of H₂S with sulphate of N, N-dimethyl-p-phenylenediamine.

**4.5. Statistical analyses**

GraphPad Prism 5 software (La Jolla, CA, USA) and IBM SPSS Statistics (New York, USA) were used for statistical analyses. All values are expressed as mean ± Standard error of the mean (SEM). Kolmogorow Smirnow Test was performed for the analysis of normality in each data set. Depending on the experimental set data were analyzed: 1) two-way Anova and Bonferroni posttest; 2) Kruskal-Wallis test and Dunns positive test; 3) one-way ANOVA and Tukey or Dunnett multiple comparison tests; 4) ANOVA for repeated measures; 5) Mann-Whitney t-test and non parametric Wilcoxon test (exact method for small samples) for simple comparisons; 6) two-tailed unpaired Student’s t test. Values of p<0.05 were considered significant. Correlation between marker expression and levels of mineralization was performed with Spearman correlation and represented with Loess curves.
5. RESULTS

5.1. Elucidating the role of exogenous stimulation of H$_2$S in modulating bone remodelling.

5.1.1. In vitro H$_2$S exogenous administration regulates osteoclastogenesis

*NaHS dose-dependently inhibits h-OCs differentiation and function in vitro*

To investigate the hypothesis that H$_2$S regulate h-OCs differentiation, h-monocytes were differentiated into mature h-OCs in the presence of increasing concentrations of NaHS a rapid-releasing H$_2$S donor able to readily generate solutions containing known quantities of H$_2$S. Functional assays for osteoclastogenic differentiation revealed that NaHS dose-dependently decreases the total number of TRAP positive mature h-OCs, resulting into a statistically significant inhibition at the concentrations of 100-300 μM (***) p<0.001) (Fig. 3A). Besides this effect, we found that h-OCs function was also regulated. In fact, the ability of mature h-OCs to break down a mineral substrate as tested *in vitro* by a ‘pit assay’, was strongly prevented by NaHS treatments and virtually completely inhibited at the concentration of 200 μM (Fig. 3B f-j). Fig. 3B compares the number of TRAP-stained h-OCs with the amount of matrix actively resorbed by h-OCs. Both the number of h-OCs and the number of pits formed, were dose-dependently inhibited in treated samples (Fig. 3B, b-e and g-j, respectively) compared to control untreated cells (Fig. 3B, a; f).
Figure 3: **NaHS dose-dependently inhibits h-OCs differentiation and function in vitro.** h-OCs were differentiated from CD11B+ h-monocytes and were grown for 6 days on either plastic well-plates or osteologic slides in osteoclastogenic medium in the presence or absence of increasing concentrations of NaHS. A, Histogram showing the average of h-OCs number/field. Data are expressed as mean ± SEM of N=9 independent experiments. ANOVA and Dunnett’s test were performed for statistical analyses (*** p<0.001 vs control cells). B, Representative pictures of TRAP staining (a-e) and pit assay (f-j) showing the effects of NaHS on h-OCs differentiation (a-e) and function (f-j). Magnification 20X.

As the low number of h-OCs and their inhibited function may be the result of NaHS-dependent impairment in the viability of h-OCs precursors, we assayed the potential cytotoxicity and apoptosis induction of NaHS within a time-range of 72 h from the stimulation. Annexin V/PI assays showed absence of apoptosis induction even at the highest dose employed in our experiments (300 μM) (Fig. 4A-B). Furthermore, quantification of LDH release indicated no significant cytotoxicity throughout the concentrations range of NaHS employed (Fig. 4B-C). Taken together these data demonstrate that NaHS-dependent inhibition on h-OCs differentiation and function was not
dependent on impaired viability of h-OCs precursors, under the culture conditions used in this study.

![Figure 4](image)

**Figure 4: Lack of toxicity of NaHS on h-OCs precursors.** h-OCs precursors were cultured in unstimulated medium (UN) in the presence or absence of increasing concentrations of NaHS for 24 (A,C) or 72 h (B,D). **A-B.** Histograms showing the percentage of Annexin V<sup>+</sup>/PI<sup>-</sup> cells obtained by FACS analysis. Data are expressed as mean ± SEM of N=3 independent experiments. ANOVA and Dunnett’s test were performed for statistical analyses (ns = non significative). **C-D.** Histograms showing LDH measurement; data are expressed as fold increase compared to unstimulated cells (UN) and refers to arbitrary units obtained by colorimetric detection of LDH activity. Data are expressed as mean ± SEM of N=3 independent experiments. One sample t-test was performed for statistical analyses (ns = non significative).

**NaHS dose-dependently inhibits RANKL-induced ROS production in macrophagic h-pre-OCs**

To investigate potential mechanisms of action of H<sub>2</sub>S donors in h-OCs development, we focused on 100 and 200 µM NaHS, the two concentrations showing anti-osteoclastogenic activity most closely resembling physiologic and pharmacologic levels of H<sub>2</sub>S. Since ROS-mediated signaling is one of the potential targets of H<sub>2</sub>S (Y. Kimura, Goto, and Kimura 2010), we performed FACS analysis by
DCF staining to analyze whether short-term NaHS treatment affects RANKL-dependent intracellular ROS generation in macrophagic h-pre-OCs. As expected, unstimulated cells showed the same lack of positivity as unstained cells (Fig. 5A, a-b); on the contrary, 30 minutes RANKL-stimulation was able to increase intracellular ROS levels compared to unstimulated cells (Fig. 5A, b-c). Interestingly, the RANKL-dependent increase in ROS levels was actively inhibited in a dose-dependent fashion when cells were pre-treated for 10 minutes with 100 or 200 μM NaHS (Fig. 5A, c-e). Fluorimetric quantification of intracellular ROS intensity confirmed that RANKL induced a significant increase in intracellular ROS (*** p<0.001), which was dose-dependently inhibited by NaHS treatment (* p<0.05, *** p<0.001; Fig. 5B). As expected, H₂O₂ treatment, used as a positive control, caused a marked increase in intracellular ROS levels (Fig. 5B).

**Figure 5: NaHS dose-dependently inhibits RANKL-induced ROS production in macrophagic h-pre-OCs.**

To generate macrophagic h-pre-OCs, h-monocytes were grown in M-CSF for 3 days. DCF staining was then performed after treating cells with RANKL (30 minutes) and 100-200 μM NaHS 10 minutes before RANKL stimulation. **A**, Representative dot-plots obtained by FACS analysis showing the frequency of DCF-positive cells as detected by DCF staining. The percentage of DCF-positive cells is shown above the gate. **a**,
unstained cells; b, unstimulated cells (UN); c, cells stimulated with RANKL alone (CTRL); d-e, cells stimulated with RANKL and treated with 100-200 μM NaHS. B, Histogram showing fluorimetric quantification of ROS production as detected by DCF staining comparing unstained cells (UN), cells stimulated with RANKL alone (CTRL), and cells stimulated with RANKL and treated with 100-200 μM NaHS; H₂O₂ was used as positive control (CTRL +). Data are expressed as mean ± SEM of triplicates of N=3 independent experiments. ANOVA and Tukey’s multiple comparison test were performed for statistical analyses (* p<0.05, *** p<0.001 vs unstimulated cells and control cells).

**NaHS-dependent inhibition of osteoclastogenic differentiation is mediated by NRF2 nuclear translocation and transcription of antioxidant target genes**

Next we investigated whether H₂S can induce long-term modifications of redox-buffering proteins related to h-OCs differentiation. In this context, a master regulator of the intracellular antioxidant response is NRF2 (Venugopal and Jaiswal 1996) which has been recently found to affect osteoclastogenic differentiation in mice (Kanzaki et al. 2013).

Therefore, to investigate whether ROS inhibition by NaHS is associated to the activation of NRF2, we analyzed the expression of NRF2 at both mRNA and protein level in h-pre-OCs treated with NaHS. RT-PCR showed that 24 h of NaHS treatment did not significantly increase NRF2 mRNA levels (Fig. 6A). Similarly, NaHS did not affect the expression of KEAP-1 (the ubiquitine ligase-adaptor which drives NRF2 toward degradation; data not shown) or the NRF2/KEAP-1 ratio at the transcriptional level (Fig. 6B). On the contrary, NRF2 protein expression at 24 h by FACS analysis in h-pre-OCs revealed that both 100 and 200 μM NaHS induced a significant increase in the percentage of cells positive to NRF2 (** p<0.05, Fig. 6C-D). These data indicate that NaHS promotes accumulation of NRF2 in h-pre-OCs by a mechanism that does not involve up-regulation of NRF2 mRNA or transcriptional down-regulation of KEAP-1.
Figure 6: NaHS increases NRF2 protein expression in h-pre-OCs. h-pre-OCs were obtained culturing h-monocytes in osteoclastogenic medium in the presence or absence of increasing concentrations of NaHS for 24 h. A, Histogram showing NRF2 mRNA expression. B, Histogram showing NRF2/KEAP1 mRNA expression ratio. Data are expressed as fold increase compared to control sample and as the mean ± SEM of N=7 independent experiments. One sample t-test was performed for statistical analyses (ns = non significative). C, Representative dot-plots obtained by FACS analysis showing the frequency of h-pre-OCs positive to NRF2 protein intracellular staining. D, bar graph showing the average frequency of NRF2-positive h-pre-OCs as measured by FACS analysis. Data are expressed as fold increase compared to control sample and as the mean ± SEM of N=3 independent experiments. One sample t-test was performed for statistical analyses (** p<0.05 vs control cells).
To validate the hypothesis of KEAP-1 dislocation, NRF2 stabilization and NRF2 nuclear translocation (Nguyen et al. 2003) under NaHS treatments, we performed immunocytochemical analysis in h-pre-OCs. In these experiments, h-pre-OCs were grown for 72 h in osteoclastogenic medium and standard NaHS treatment (100-200 μM) was compared to a short-term 2 h stimulation. The NRF2 nuclear localization was then evaluated. When the frequency of nuclear NRF2-positive cells was counted over three independent experiments, we found that in control cells approximately 14% of h-pre-OCs showed positive nuclear staining to NRF2. This frequency was dose-dependently increased by NaHS during standard treatments. In particular, 100 μM and 200 μM NaHS induced, respectively, a significant, 43% and 86% increase (** p<0.05; *** p<0.01) relative to control samples (Fig. 7A). Moreover, for the highest dose of 200 μM, a short term pre-treatment of 2 h was sufficient to induce a significant nuclear translocation of NRF2 (*** p<0.01; Fig. 7A). These findings show that 200 μM NaHS significantly increase NRF2 nuclear translocation as soon as 2h after the stimulation, while 100 μM NaHS required, in our experimental model system, repeated stimulations to induce NRF2. Fig. 7B compares NRF2 nuclear translocation to h-OCs differentiation. Interestingly, the higher is NRF2 nuclear translocation the lower is the number of h-OCs formed.
Figure 7: NaHS dose-dependently increases NRF2 nuclear translocation in h-pre-OCs. h-pre-OCs were obtained from h-monocytes in osteoclastogenic medium for 72 h in the absence or presence of NaHS. 100-200 µM NaHS was added throughout the experiment or only for 2h before the assay. h-OCs were obtained from h-monocytes in osteoclastogenic medium for 6 days in presence or absence of 100-200 NaHS. A, Histogram showing the average frequency of cells with positive nuclear staining for NRF2 (red staining). Data are expressed as mean ± SEM of N=3 independent experiments of the percentage of nuclear NRF2-positive cells vs total cell per microscope field. ANOVA and Dunnett’s test were performed for statistical analyses (*** p<0.05; *** p<0.001 vs control cells). B, Representative pictures showing NRF2 nuclear translocation on h-pre-OCs at 3 days of differentiation (a-c, magnification 600X) and TRAP positive staining at 6 days of differentiation (d-f, magnification 20X).

To further confirm the strong relationship between NRF2 translocation and inhibition of h-OCs differentiation, we performed additional experiments treating h-OCs precursors with two NRF2 inductors (SNF and t-BHQ) reported to induce NRF2 nuclear translocation (K. W. Kang et al.)
2002). Quantification of TRAP positive h-OCs showed that SFN significantly inhibited h-OCs formation at 5 μM (** p<0.01), while t-BHQ inhibited h-OCs at all the concentrations tested (* p<0.05) (Fig. 8A). Immunocytochemical analysis confirmed that NRF2 translocation was increased in h-pre-OCs treated with SFN and t-BHQ compared to control cells (Fig. 8B d-f). Comparison of the number of h-OCs (by TRAP assay; Fig. 8B a-c) with NRF2 nuclear translocation (by immunocytochemical analysis; Fig. 8C d-f) showed that higher is the NRF2 nuclear translocation lower is the number of h-OCs formed. These data showed that, similar to NaHS, treatment with two prototypical NRF2 activators (SFN and t-BHQ) inhibited h-OCs differentiation.
Figure 8: *SFN and t-BHQ increase NRF2 nuclear translocation in h-pre-OCs and inhibit h-OCs differentiation.* h-monocytes were differentiated in osteoclastogenic medium in the presence or absence of increasing concentrations of NRF2 activators. A, Histogram showing average h-OC-s number/well for the indicated concentrations of NRF2 activators. Data are expressed as mean ± SEM of N=3 independent experiments. ANOVA and Dunnett’s t test were performed for statistical analyses (* p<0.05; ** p<0.01 vs control cells). B, Representative pictures showing TRAP positive staining at day 6 of differentiation (a-c, magnification 20X) and NRF2 nuclear translocation on h-pre-OCs at day 3 of differentiation (d-f, magnification 600X).

NRF2 controls the activation of over 200 antioxidant genes through the interaction with antioxidant response elements (ARE). To further validate that NRF2 accumulation and translocation to the nucleus results in increased expression of ARE-responsive genes, we analyzed the expression of NQO1 and PRDX1 genes on h-pre-OCs. As expected, SFN, used as a positive control, caused a significant increase in NQO1 and PRDX1 mRNA levels (** p<0.01; Fig. 9A,B). Similarly, NaHS
stimulation induced a significant up-regulation of the two genes at both 100 and 200 μM (* p<0.05; Fig. 9A,B). At the protein level, WB analysis revealed that, while NQO1 was dose-dependently up-regulated by NaHS (Fig. 9C), PRDX1 did not undergo to a detectable up-regulation in our experimental conditions (Fig. 9D). These findings evidenced that NaHS treatment up-regulates the transcription of endogenous antioxidant genes downstream of NRF2 activation in h-pre-OCs while differentially regulating their protein expression levels.

**Figure 9: NaHS induces NRF2-target genes expression in h-pre-OCs.** h-pre-OCs were obtained from h-monocytes cultures in osteoclastogenic medium for 72 h in the presence or absence of NaHS or NRF2 activator SFN (5μM). Cells were harvested 6h after the latest stimulation. A-B, Histograms showing NQO1 and PRDX-1 mRNA expression. Data are expressed as fold increase compared to control sample and as the mean ± SEM of N=3 independent experiments. Wilcoxon Test was performed for statistical analyses (* p<0.05, ** p<0.01 vs control cells); C-D, WB analyses of NQO1, PRDX1; upper panel shows representative WB analysis for target proteins and control β-actin; lower panels shows quantification by Image J software where target proteins were normalized by β-actin band intensity.
NaHS fails to inhibit h-OCs differentiation under NRF2-silencing conditions in h-OCs

To address the question of whether NaHS inhibition of h-OCs differentiation is mechanistically linked to NRF2 activation, we investigated the effects of NaHS stimulation on h-OCs silenced for NRF2.

First, we validated the NRF2 silencing at both mRNA and protein level, as shown in Fig. 10. Analyses of mRNA and protein expression, by means of RT-PCR and WB analyses, confirmed the silencing of NRF2 in our system. NRF2 silencing resulted into a 70% suppression of NRF2 mRNA expression as compared to control NT siRNA (** p<0.05; Fig. 10A), which resulted into an approximately 35% down-regulation at the protein level (Fig. 10B). Consistent with NRF2 silencing we found a marked and significant down-regulation of NQO1 mRNA (** p<0.05; Fig. 10C) confirmed at the protein level (Fig. 10D). On the contrary, PRDX1 was not significantly regulated at both mRNA and protein level after NRF2 silencing (Fig. 10E-F). These data validate our protocol of silencing and suggest that two different ARE target genes, NQO1 and PRDX1, are differentially regulated by NRF2 in our cell culture system.
Figure 10: Validation of NRF2 silencing during h-OCs differentiation. h-OCs precursors were treated with NT and NRF2 siRNA during osteoclastogenic differentiation. Panels A-C-E: Histograms showing mRNA expression for NRF2 (A), NQO1 (C), PRDX1 (E) in control (NT) vs NRF2-silenced samples. Data are expressed as mean ± SEM of N=6 independent experiments. Paired t-test was performed for the statistical analyses (** p<0.05 vs NT siRNA). B-D-F: WB analyses of NRF2 (B), NQO1 (D), PRDX1 (F) protein expression; upper panel shows representative WB analysis for target proteins and control β-actin; lower panels shows quantification by Image J software where target proteins were normalized by β-actin band intensity.

Further analyses of mRNA and protein expression, performed during NaHS treatments, revealed that NaHS failed to induce NRF2, NQO1 or PRDX1 mRNA (Fig. 11A-C) and protein (Fig. 11D-F) expression under conditions of NRF2 silencing. Altogether, these data suggest that NRF2 is necessary for NaHS to induce NQO1 and PRDX1 during h-OCs.
Figure 11: NaHS fails to induce NRF2 target genes expression under NRF2-silencing conditions in h-OCs. h-OCs precursors were treated with NRF2 siRNA during osteoclastogenic differentiation in the presence or absence of NaHS. Panels A-C: Histograms showing mRNA expression for NRF2 (A), NQO1 (B), PRDX1 (C) in NRF2-silenced samples treated or untreated with NaHS. Data are expressed as mean ± SEM of N=6 independent experiments. Paired t-test was performed for the statistical analysis (ns = non significative). D-F: WB analyses of NRF2 (D), NQO1 (E), PRDX1 (F) protein expression; upper panel shows representative WB analysis for target proteins and control β-actin; lower panels shows quantification by Image J software where target proteins were normalized by β-actin band intensity.

TRAP staining was found to be in agreement with findings obtained in Fig. 3, as NT silenced cells showed an inhibition of h-OCs formation by 200 μM NaHS (Fig. 12A a,b). However, NaHS completely failed to inhibit h-OCs formation in cells silenced for NRF2 (Fig. 12A c,d). Coherently, h-OCs counts confirmed a significant inhibition of h-OCs in NT transfected cells during NaHS treatments in comparison to both NT untreated cells as well as both NaHS-treated and untreated NRF2 transfected cells (*** p<0.0001).
As a result, we can state that NRF2 expression in h-pre-OCs cells is essential for H2S-mediated inhibition of h-OCs differentiation.

**Figure 12:** Silencing NRF2 mRNA in h-pre-OCs prevents inhibition of h-OCs differentiation by NaHS. h-monocytes precursors were treated with NT siRNA or NRF2 siRNA during osteoclastogenic differentiation in the presence or absence of 200 μM NaHS. A, Representative pictures showing TRAP positive staining at day 6 of differentiation (magnification 20X). B, Histogram showing average h-OCs number/field. Data are expressed as mean ± SEM of N=3 independent experiments. ANOVA and Tukey’s multiple comparison test were performed for statistical analyses (*** p<0.0001 vs NT siRNA or NRF2 siRNA ± NaHS).

**NaHS downregulates the RANKL/OPG mRNA ratio in h-MSCs**

To understand whether NaHS inhibits h-OCs formation also via indirect mechanisms, we sought to analyze the RANKL/OPG mRNA expression ratio in h-MSCs, one of the key OCs supporting cells (D.-C. Yang et al. 2008) (Dalle Carbonare et al. 2009). RANKL mRNA expression was not modulated by NaHS treatments in our culture system. Conversely, a trend of increase of OPG was shown by RT-PCR after 12 h of treatment, resulting in a significant down-regulation of RANKL/OPG mRNA ratio (Fig. 13 A-C). Therefore, NaHS not only directly inhibits h-OCs but it is also capable of an indirect inhibition of osteoclastogenesis through down-regulation of the RANKL/OPG mRNA ratio in h-MSCs.
Figure 13: NaHS downregulates RANKL/OPG mRNA ratio in h-MSCs. h-MSCs were cultured in α-MEM 15 %FBS in the presence or absence of 100-200 μM NaHS for 12 h. A-C, Histograms showing RANKL (A) and OPG (B) mRNA expression and RANKL/OPG (C) mRNA expression ratio. Data are expressed as fold increase compared to control sample and as the mean ± SD of N=3 independent experiments. One sample t-test was performed for statistical analyses (* p<0.05 vs control cells).
5.1.2. *In vitro* H2S exogenous administration regulates osteoblastogenesis

**AR-S staining reveals heterogeneous response of h-MSCs to osteogenic stimulation**

h-MSCs are known to be an heterogeneous population with high variability both in terms of subpopulation and of donor-dependence (Rada et al. 2012) (Phinney 2012) (Russell et al. 2010). In an attempt to describe the donor-dependent variability during osteogenic differentiation of h-MSCs, we performed AR-S quantification of mineral deposition through spectrophotometric measurement at 510 nm. According to a statistical multivariate analysis, cell source was not an independent factor for the differentiation of h-MSCs, therefore data obtained from bone marrow and tybial plateau h-MSCs were pooled in these analyses. h-MSCs displayed on average a significant mineralization capacity starting at D14 in culture (Fig. 14A; *** p<0.001). However, h-MSCs from an approximate 30% of the donors failed to display any positive staining to AR-S at the end of the culture, revealing a heterogeneous response to osteogenic stimuli *in vitro*. Based on their differential AR-S quantification, we segregated h-MSCs into ‘mineralizing’ or ‘non mineralizing’ groups. Mineralizing h-MSCs displayed a mineralization capacity sufficient to raise AR-S absorbance levels above the threshold of 0.3 nm, at least at D28 of differentiation. h-MSCs with no mineralization capacity showed absorbance levels below 0.3 nm anytime during the osteogenic culture and were defined as non mineralizing h-MSCs (Fig. 14B). Comparing the two groups, the mineralizing population showed significantly higher AR-S absorbance at the time points D21 and D28 compared to the non mineralizing group (° p<0.05; Fig. 14C). Moreover, the most significant increase in AR-S values occurred between D14 and D21 (as compared to D7-D14 and D21-D28) within the mineralizing group (*** p<0.01; Fig. 14C). These data suggest that the most relevant time points for evaluating the osteogenic differentiation are D0, D14, D21.

When we investigated whether there was a difference in the age between the two groups we found no significant difference (47±24 and 48±24, respectively, for mineralizing and non mineralizing
group). In an attempt to further describe the heterogeneous time course of mineral deposition in the mineralizing group, we segregated h-MSCs based on their phenotype of calcium deposition, through AR-S quantification. Non calcium depositing cells (N-CD), low calcium depositing cells (L-CD) and high calcium depositing cells (H-CD) showed, respectively, absorbance values below 0.3; between 0.3 and 1; and higher than 1. Non mineralizing donors exhibited N-CD phenotype throughout all the time course of differentiation; conversely, mineralizing donors exhibited N-CD, L-CD and H-CD phenotype depending on the time point of the analysis (Fig. 14E). Interestingly, among mineralizing group we found a donor-dependent variability in the response to osteogenic stimuli both in the time of beginning of mineralization and in the amount/time of calcium produced. As a results, while all mineralizing cells, regardless to the time points, were changing from a N-CD to a L-CD and/or a H-CD phenotype, the percentage of donors at each time point with a specific phenotype of mineralization resulted very different: N-CD phenotype (89% at D7, 61% at D14 and 13% at D21), a L-CD phenotype (11% at D7, 27% at D14, 53% at D21, 33% at D28) and an H-CD phenotype (12% at D14, 34% at D21, 67% at D28) (Fig. 14F).
Figure 14: AR-S staining identifies different mineralization behaviour of h-MSCs during osteogenic differentiation. h-MSCs were cultured in osteogenic medium for 28 days. A, Histogram shows the overall quantification of AR-S in the whole population of MSCs. Data are expressed mean ± SD of 60 independent experiments. Kruskal-Wallis test + Dunns multiple comparison test was performed for statistical analyses (*** p<0.0001). B, Table representative of how we segregated h-MSCs in mineralizing and non mineralizing group, based on absorbance values. C, Histogram showing the different mineralization
behaviour of h-MSCs selected for mineralization behaviour. Data are expressed mean ± SD of 42
mineralizing and 18 non mineralizing cells. Two-way Anova + Bonferroni post-test (*** p<0.001) and
Kruskal-Wallis test + Dunns multiple comparison test (° p<0.05) were performed for statistical analyses. D,
Table representative of how we segregated h-MSCs in N-CD, H-CD, L-CD cells, based on absorbance
values. E, Representative picture showing the different mineralization behaviour between the two
populations. F, Table representative of the percentage of donors at N-CD, L-CD or H-CD phenotype at D7,
D14, D21, D28.

NaHS promotes osteogenic differentiation in vitro

Recently, H₂S has been found to maintain MSCs function and bone homeostasis via regulation of
Ca²⁺ channel sulfhydration. In particular, NaHS rescued impaired osteogenic differentiation of
CBS⁻/⁻ and CBS⁺/⁻ m-SCs both in vitro and in vivo (Y. Liu et al. 2014). Here, we addressed the
question of whether NaHS treatment modulates osteogenic differentiation in h-MSCs.

When h-MSCs were differentiated toward h-OBs lineage in the presence of NaHS, we first found
that treatments with micromolar concentrations of NaHS did not appear to cause a significant
decrease in cellularity. Quantification of LDH release indicated no significant cytotoxicity
throughout the concentrations range of NaHS employed in this study (6-200 µM NaHS).
Interestingly, we found that LDH release was lower in NaHS-treated h-MSCs compared to control
cells both at 24 and 72 h (* p<0.05) (Fig. 15A-B). Moreover, in a restricted set of experiments, we
tested millimolar (1-3,5 mM NaHS) concentrations of NaHS, in order to define whether
“supraphysiologic” concentrations impaired h-MSCs viability. Toluidine blue staining (Fig. 15C)
and quantification (Fig. 15D), confirmed that higher levels of NaHS did not cause a significant
decrease in cellularity. All these data taken together demonstrate that micromolar and millimolar
NaHS treatment did not affect h-MSCs viability under the culture conditions used in this study.
Figure 15: Lack of toxicity of NaHS on h-MSCs precursors. h-MSCs were cultured in α–MEM 15% FBS in the presence or absence of increasing concentrations of NaHS for 24 (A) or 72 h (B). A-B, Histograms showing LDH measurement; data are expressed as fold increase compared to control cells (CTRL) and refers to arbitrary units obtained by colorimetric detection of LDH activity. Data are expressed as mean ± SEM of N=3 independent experiments. One sample t-test was performed for statistical analysis (* p<0.05; *** p<0.001 vs control cells); C, Representative pictures of toluidin blue staining comparing 200 μM and 3,5 mM NaHS to control cells; D, Bar graph showing toluidine blue staining quantification. Data are expressed as mean ± SEM of N=4 independent experiments. One sample t-test was performed for statistical analysis (ns = non significative).
When we performed osteogenic differentiation of h-MSCs during NaHS treatments, we first observed that the treatment contributed to increase the amount of calcium deposition by h-MSCs. In particular, while untreated h-MSCs displayed a L-CD phenotype, NaHS-treated h-MSCs displayed H-CD phenotype, as shown by a representative figure of AR-staining (Fig. 16A). AR-S quantification on a large set of h-MSCs further confirmed a significant induction of mineralizing activity in the range between 25-200 μM NaHS (* p<0.05; Fig. 16B). Second, we found that NaHS treatment induced calcium deposition in N-CD h-MSCs. In particular, while untreated h-MSCs displayed a N-CD phenotype, NaHS-treated h-MSCs displayed H-CD phenotype, as shown by a representative figure of AR-staining (Fig. 16C). AR-staining quantification in h-MSCs cells yet non-responsive to stimuli present in osteogenic medium (N-CD h-MSCs) confirmed that NaHS significantly induced osteogenic differentiation at all the concentrations tested, between the range of 6-200 μM (* p<0.05) (Fig. 16D). Finally, the ability to induce mineralization was further confirmed by mRNA expression of BSP, a marker of osteogenic differentiation. NaHS dose-dependently increased BSP expression (Fig. 16E), confirming NaHS –induced osteogenic differentiation. Taken together these data demonstrate that NaHS is able on one hand to induce mineralization in N-CD h-MSCs and moreover, it can increase the amount of mineralization in L-CD h-MSCs. Although the setting of these experiments did not allow us to clarify this hypothesis, our data argue in favour of a role of NaHS in anticipating the mineralization.
Figure 16: NaHS induced and increased h-MSCs osteogenic differentiation. h-MSCs were cultured in osteogenic medium in the presence or absence of NaHS stimulation. A, Representative figure of AR-staining in L-CD h-MSCs treated with NaHS; B, Histogram showing AR-staining quantification. Data are expressed as fold increase compared to control sample and as the mean ± SD of N=18 independent experiments. One sample t-test was performed for statistical analyses (* p<0.05 vs control cells); C, Representative figure of AR-staining in N-CD h-MSCs treated with NaHS; D, Histogram showing AR-staining quantification. Data are expressed as fold increase compared to control sample and as the mean ± SD of N=15 independent experiments. One sample t-test was performed for statistical analyses (* p<0.05 vs control cells); E, Histogram showing BSP mRNA expression. Data are expressed as fold increase compared to control sample and as the mean ± SD of N=23 independent experiments. One sample t-test was performed for statistical analyses (* p<0.05 vs control cells).
5.1.3. \textit{In vivo} $\text{H}_2\text{S}$ administration improved bone formation preventing bone loss

Our \textit{in vitro} data indicated a possible $\text{H}_2\text{S}$-mediated modulation of bone formation \textit{in vivo}. Therefore, we took advantage of the established expertise of our collaborator, Prof. Pacifici (Emory University, Atlanta) (Ryan et al. 2005) (Gao et al. 2007) (Grassi et al. 2007) for testing this hypothesis in ovx mice, an animal model of post-menopausal osteoporosis. As we modeled a chronic pathology, a slow-releasing compound, as GYY, was preferable to a rapid-releasing donor, as NaHS, for testing potential targets for the therapy. Most of these data were obtained at the Emory University.

\textit{Effect of GYY treatment on the H$_2$S serum levels}

In order to monitor the effectiveness of $\text{H}_2\text{S}$ administration we evaluated serum $\text{H}_2\text{S}$ levels at the end of the preventive study (please refer to materials and methods section 4.3).

This can be revealed as free $\text{H}_2\text{S}$ or bound sulfur, the stored form of sulfur, which can release free $\text{H}_2\text{S}$ in physiological conditions (Ishigami et al. 2009). Interestingly, we found that vehicle treated ovx mice had lower serum free $\text{H}_2\text{S}$ (~67%, ** $p<0.01$) and bound sulfur levels (~52%, * $p<0.05$) than sham operated mice (Fig. 17A,B). Treatment with GYY increased serum $\text{H}_2\text{S}$ (~129%, * $p<0.05$) and bound sulfur levels (~95%, * $p<0.05$) in ovx mice. As a result, GYY treated ovx mice and vehicle treated sham operated mice had similar $\text{H}_2\text{S}$ and bound sulfur levels. Those data demonstrate that the regimen used for GYY administration was able to normalize/balance the sulfur deficiency caused by ovx (Fig. 17A,B), realizing a pharmacological replacement of $\text{H}_2\text{S}$ levels.

Moreover treatment with GYY in sham mice increased above the baseline free $\text{H}_2\text{S}$ levels (~32%, * $p<0.05$). As a result, GYY treated sham operated mice displayed the highest $\text{H}_2\text{S}$ serum levels, resulting in the highest significant increase compared to vehicle treated ovx mice (~303%, **** $p<0.0001$; Fig. 17A). Interestingly, this implied that there was still a significant difference in the
H₂S levels between GYY treated sham operated mice and GYY treated ovx mice (~76% higher, **p<0.01; Fig. 17A). On the contrary, treatment with GYY in sham mice did not significantly increase bound sulfur levels, although it increased the significance in the difference between GYY treated sham operated mice and vehicle treated ovx mice (*** p<0.001; Fig. 17B).

**Figure 17: Effect of GYY treatment on the serum levels of free H₂S and bound sulfur in sham and ovx mice.** Mouse serum was collected at the end of preventive study and measurements of H₂S and bound sulfur were performed by gas chromatography. **A,** Histogram showing serum levels of free H₂S. **B,** Histogram showing bound sulfur serum levels. Data are expressed as mean ± SEM. N=10 mice per group. Two way anova + Tukey’s multiple comparison test were performed for statistical analyses (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs the indicated group).

**GYY-treatment prevents Ovx-induced bone loss and increases bone mass in mice**

To understand the preventive value of GYY administration on ovx-induced bone loss we first performed μCT analysis in femur harvested at sacrifice at the end of the preventive study.

Representative μCT images of femur trabecular bone from all the four groups are shown in Fig. 18A. Our data first confirmed that 4 weeks after ovx were sufficient for inducing bone loss in mice. Indeed, vehicle treated ovx mice had lower BV/TV (~30%, **** p<0.0001; Fig. 18B), Tb.N (~13%, **** p<0.0001; Fig. 18C), Tb.Th (~8%, * p<0.05 in femur; Fig. 18D), Ct.V (10%, **
p<0.01; Fig. 18F), Ct. Th (9%, ** p<0.01; Fig. 18G) and higher Tb. SP (~18%, **** p<0.0001; Fig.18E) than vehicle treated sham operated mice.

Conversely, GYY treatment prevented ovx-induced changes in bone volume and structure. Indeed, GYY treated ovx mice had higher BV/TV (~32%, ***p<0.001; Fig. 18B), Tb.N (~14, **** p<0.0001; Fig. 18C), Tb.Th (~9%, * p<0.05; Fig. 18D), Ct.V (7%, *p<0.05; Fig. 18F), Ct.Th (5%, **p<0.01; Fig. 18G) and lower Tb. SP (~15%, ** p<0.01; Fig. 18E) than vehicle treated ovx mice. As a result, GYY treated ovx mice and vehicle treated sham operated mice had similar BV/TV, Tb. N., Tb. Th., Tb. SP, Ct. V and Ct.Th values (Fig. 18B-E).

When we investigated GYY effect in sham operated mice we found significantly higher BV/TV (~18%, * p<0.05; Fig. 18B) and Tb.Th (~9%, * p<0.05; Fig. 18D) in GYY treated mice compared to vehicle treated sham operated mice. As a result, GYY treated sham operated mice displayed the highest BV/TV (~68%, **** p<0.0001; Fig. 18B) and Tb. Th (18%, * p<0.05; Fig. 18D) compared to vehicle treated ovx mice. Those data demonstrated that GYY exerted an anabolic role in sham operated mice.

Importantly, we noticed that GYY treated ovx mice had still lower BV/TV, Ct. V., Ct. Th and higher Tb. SP compared to GYY treated sham operated mice. This data could imply that ovx continued to cause significant changes in bone volume and structure even after GYY administration. However, we assumed that this difference accounts arise from the difference in H₂S levels. Interestingly, higher are H₂S levels (Fig. 17A), higher is BV/TV (Fig.18B). In particular, GYY treatment simulated a sulfur replacement therapy in ovx mice but increased H₂S levels above the baseline in sham operated mice (Fig. 17A). These data shed important light on the physiological and therapeutic value of H₂S in bone tissue.
Figure 18: GYY-treatment prevents Ovx-induced bone loss and increases bone mass in mice. µCT analysis was performed on femur harvested at sacrifice at the end of the preventive study in order to assess femoral trabecular and cortical bone volume. A, Representative images of 3D image reconstruction of 1 representative femur per group. B-G, Histograms showing (B) Trabecular bone volume over total volume (BV/TV), (C) Trabecular number (Tb.N), (D) Trabecular thickness (Tb.Th), (E) Trabecular space (Tb.Sp), (F) Cortical bone volume (Ct.V) and (G) Cortical thickness (Ct.Th) in femur. Data are expressed as mean ± SEM. Two way anova + Tukey’s multiple comparison test were performed for statistical analyses (* p<0.05, ** p<0.01, *** p<0.001 **** p<0.0001 vs the indicated group). N=10 mice per group.
**GYY-treatment reverses Ovx-induced bone loss and increases bone mass in mice**

To further verify the therapeutic value of GYY on ovx-induced bone loss we performed μCT analysis of femur harvested at sacrifice at the end of the therapeutic study.

Ovx induced a sustained change over time in femoral volume and structure: at 8 weeks vehicle treated ovx mice had lower BV/TV (~30%, * p<0.05; Fig. 19A), Tb.N (24%, **** p<0.0001; Fig. 12B), Ct.V (8%, ** p<0.01; Fig. 19E) and Ct. Th (9%, **** p<0.0001; Fig. 19F) and an higher Tb. SP (~36%, **** p<0.0001; Fig. 19A, D). Conversely, GYY treated ovx mice had higher BV/TV (~47 %, ** p<0.01; Fig. 19A), Tb.N (~22%, * p<0.05; Fig. 19B), Ct.V (8%,*p<0.05; Fig. 19E), Ct.Th (7%, **p<0.01; Fig. 19F) and reduced Tb. SP (~17%, ** p<0.01; Fig. 19D) than in vehicle treated ovx mice. As a result, GYY treated ovx mice and vehicle treated sham mice had similar values of BV/TV, Tb. N., Tb. SP, Ct. V and Ct.Th values. Unexpectedly, neither ovx nor GYY treatment affected Tb.Th (Fig. 19C) in this experiment. Moreover, GYY treated sham operated mice had significantly higher BV/TV (~27%, * p<0.05; Fig. 19A), Tb. N (~27%, * p<0.05; Fig. 19B) and Ct.V (7%, *p<0.05; Fig. 19E) than vehicle treated sham mice. As a result, GYY treated sham operated mice displayed the highest BV/TV (~82%, **** p<0.0001; Fig. 19A), Tb. N (~47%, **** p<0.0001; Fig. 19B) and Ct. V (~16%, **** p<0.0001; Fig. 19E) compared to vehicle treated ovx mice. Those data demonstrated that the regimen of GYY administration was able not only to prevent but to reverse the femoral trabecular bone loss and confirmed the anabolic role of H₂S in sham mice.
Figure 19: GYY-treatment reverses Ovx-induced bone loss and increases bone mass. \textit{\textmu}CT analysis was performed on femur harvested at sacrifice at the end of the therapeutic study in order to assess femoral trabecular and cortical bone volume. A-F, Histograms showing (A) Trabecular bone volume over total volume (BV/TV), (B) Trabecular number (Tb.N), (C) Trabecular thickness (Tb.Th), (D) Trabecular space (Tb.Sp), (E) Cortical bone volume (Ct.V) and (G) Cortical thickness (Ct.Th) in femur. Data are expressed as mean ± SEM. Two way anova + Tukey’s multiple comparison tests were performed for statistical analyses (* \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \), **** \( p<0.0001 \) vs the indicated group). \( N=10 \) mice per group
**GYY-treatment partially prevents and reverses Ovx-induced bone loss**

To investigate whether GYY exerts a differential effect at different bone sites, we performed μCT analysis of spine.

Preventive and therapeutic studies confirmed that ovx induced, respectively, in 4 weeks or 8 weeks spinal trabecular bone loss in mice. Indeed, vehicle treated ovx mice had lower BV/TV (~28%, **** p<0.0001, Fig. 20A; ~40%, * p<0.05, Fig. 20B), Tb.N (~9%, ** p<0.01, Fig.20C; 13%, *** p<0.001, Fig. 20D), Tb.Th (~15%, **** p<0.0001, Fig.20E; 20%, **** p<0.0001, Fig. 20F) and higher Tb. SP (~13%, ** p<0.01, Fig.20G; 17%, **** p<0.0001, Fig. 20H) than vehicle treated sham operated mice respectively in preventive and therapeutic study. GYY treated ovx mice had higher BV/TV (~11%, ** p<0.01, Fig.20A; ~59%, * p<0.05, Fig. 20B), Tb. N (~7%, * p<0.05, Fig.20C; ~16%, * p<0.05, Fig. 20D), Tb. Th (~9%, ** p<0.01, in therapeutic, Fig. 20F) and lower Tb. SP (~12%, * p<0.05 in therapeutic, Fig. 20H) than vehicle treated ovx mice. However, GYY treated ovx mice still had less BV/TV (~20%, **** p<0.0001, in preventive, Fig. 20A) and Tb.Th (~21%, **** p<0.0001, Fig.20E; ~5%, ** p<0.01, Fig. 20F) than vehicle treated sham operated mice respectively in preventive and therapeutic study. Moreover it still had high Tb. SP (~9%, * p<0.05, Fig.20H) than vehicle treated sham operated mice, in therapeutic study. Conversely, Tb. N and Tb.SP (in preventive study) were found to be similar in GYY treated ovx mice and vehicle treated sham operated mice (Fig. 20C,G). Spine cortical bone volume was not considered in our analyses as the cortical compartment in spine is thinner and less homogeneous than in femur. These findings demonstrate a differential effect of GYY at different bone sites, as μCT analysis of spine revealed that GYY treatment only partially prevented and reversed Ovx-induced changes in bone volume and structure.
Figure 20: GYY-treatment partially prevents and reverses Ovx-induced bone loss. A-H, Histograms showing (A-B) Trabecular bone volume over total volume (BV/TV), (C-D) Trabecular number (Tb.N), (E-F) Trabecular thickness (Tb.Th), (G-H) Trabecular space (Tb.Sp) in spine after preventive (A,C,E,G) or therapeutic study (B,D,F,H). Data are expressed as mean ± SEM. Two way ANOVA + Tukey’s multiple comparison tests were performed for statistical analyses (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs the indicated group). N=10 mice per group.
GYY stimulates bone formation in mice

Serum CTX and P1NP have been designated as reference standard markers respectively of bone resorption and formation (Vasikaran, Chubb, and Schneider 2014). As they provide an estimate of the rate of bone remodeling, they have been increasingly recognized to monitor treatments of osteopenia and osteoporosis (Inaba 2014). Therefore, we measured P1NP and CTX in serum to verify how GYY modulates bone turnover.

As expected, ovx induced a negative bone turnover balance due to an increase in bone resorption (~70% increase in CTX serum levels, p<0.05, Fig. 21A) and a low/ineffective compensatory increase in bone formation (~49% increase in P1NP serum levels, p<0.01, Fig. 21B).

GYY did not modulate serum CTX levels, although it displayed a downward trend (Fig. 21A). Conversely, treatment with GYY induced a further increase in P1NP in ovx mice (~23%, * p<0.05, Fig. 21B). As a result, GYY treated ovx mice had higher levels of P1NP than all the other groups of sham operated and ovx mice. In particular, P1NP was found to be increased by ~23% (* p<0.05) compared to vehicle treated ovx mice; by ~43% (** p<0.001) compared to GYY treated sham operated mice; by ~84% (**** p<0.0001) compared to vehicle treated sham operated mice (Fig. 21A). As a result, P1NP/CTX ratio increased both in sham and ovx mice after GYY treatment confirming a shift in the bone turnover balance toward bone formation in sham mice and a decrease in the negative bone turnover balance in ovx mice, thus reducing the gap between bone resorption and formation (Fig. 21C).
Figure 21: GYY stimulates bone formation in mice as evidenced by measurements of biochemical indices of bone turnover. Measurement of serum markers of bone turnover was performed at the end of the preventive study. Histograms showing: (A) serum levels of CTX, a marker of bone resorption; (B) serum levels of P1NP, a marker of bone formation; (C) P1NP/CTX ratio. Data are expressed as mean ± SEM. Two way anova + Tukey’s multiple comparison test (A,B) and simple comparison by Unpaired t-test with Welch’s correction (C) were performed for statistical analyses. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs the indicated group). N=10 mice per group.

Histomorphometric analysis in spinal cancellous bone further evidenced that ovx increased static indices of bone resorption, N.Oc/BS (~153%, **** p<0.0001; Fig. 22A) and Oc.S/BS (~106%, **** p<0.0001; Fig. 22B), and of bone formation, N.Ob/BS (~19%, ** p<0.01; Fig. 22C) and Ob.S/BS (~12%, * p<0.05; Fig. 22D), while did not increase dynamic indices of bone formation BFR/BS (Fig. 22E), MAR (Fig. 22F), and MS/BS (Fig. 22G).

Treatment with GYY increased both static and dynamic indices of bone formation: N.Ob/BS (~16%, ** p<0.01; Fig. 22C), Ob.S/BS (~13%, ** p<0.01; Fig. 22D), BFR/BS (~83%, ** p<0.01; Fig. 22E), MAR (~27%, ** p<0.01; Fig. 22F), and MS/BS (~57%, * p<0.05; Fig. 22G) in ovx mice.
Conversely, GYY did not modulate N.Oc/BS and Oc.S/BS. Taken together these data evidenced a role of GYY in modulating bone formation but not bone resorption *in vivo*.

Moreover treatment with GYY increased N.Ob/BS (~31%, **** p<0.0001; Fig. 22C), Ob.S/BS (~34%,**** p<0.0001; Fig. 22D) and MAR (~21%, ** p<0.01; Fig. 22F) in sham operated ovx mice, confirming the anabolic action of GYY in control animals.
Figure 22: GYY stimulates bone formation in mice as evidenced by spinal histomorphometric indices of bone turnover. Histomorphometry was performed at the end of the preventive study. Histograms showing: (A) Number of OCs per millimeter of bone surface (N.Oc/BS); (B) Percentage of bone surface covered by OCs (Oc.S/BS); (C) Number of OBs per millimeter of bone surface (N.Ob/BS); (D) Percentage of bone

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**Figure 22: GYY stimulates bone formation in mice as evidenced by spinal histomorphometric indices of bone turnover.** Histomorphometry was performed at the end of the preventive study. Histograms showing: (A) Number of OCs per millimeter of bone surface (N.Oc/BS); (B) Percentage of bone surface covered by OCs (Oc.S/BS); (C) Number of OBs per millimeter of bone surface (N. Ob/BS); (D) Percentage of bone
surfaces covered by OBs (Ob.S/BS); (E) Bone formation rate/bone surface (BFR/BS); (F) Mineral apposition rate (MAR); (G) Percentage of bone surfaces covered by mineralized surfaces (MS/BS). Data are expressed as mean ± SEM. Two way anova and Uncorrected Fisher’s LSD were performed for statistical analyses (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs the indicated group). n=10 mice per group.

Differently to spine, analysis of femoral cancellous bone by histomorphometry revealed that ovx did not alter most of the indices of bone resorption and bone formation. Indeed, N.Oc/BS (Fig. 23A), Oc.S/BS (Fig. 23B), N.Ob/BS (Fig. 23C), Ob.S/BS (Fig. 23D), BFR/BS (Fig. 23E) and MS/BS (Fig. 23F) were not modulated by ovx. Ovx increased only MAR by 32% (**** p<0.0001) in vehicle treated mice (Fig. 23G). GYY treated ovx mice had increased N.Ob/BS (~18%, * p<0.05; Fig. 23C) and Ob.S/BS (~19%, * p<0.05; Fig. 23D) as compared to vehicle treated ovx mice. Although GYY did not increase BFR/BS compared to vehicle treated ovx mice, GYY treated ovx mice had significant higher BFR/BS, as compared to vehicle (~25%, * p<0.05) and GYY treated (~34%, ** p<0.01) sham-operated mice (Fig. 23E). GYY did not modulate any of these indices in sham operated mice (Fig. 23A-G). These data indicate that GYY induced bone formation in femur during ovx.
Figure 23: GYY stimulates bone formation in mice as evidenced by femur histomorphometric indices of bone turnover. Histograms showing: (A) Number of OCs per millimeter of bone surface (N.Oc/BS); (B) Percentage of bone surface covered by OCs (Oc.S/BS); (C) Number of OBs per bone surface (N. Ob/BS); (D) Percentage of bone surfaces covered by OBs (Ob.S/BS); (E) Bone formation rate/bone surface.
(F) Mineral apposition rate (MAR); (G) Percentage of bone surfaces covered by mineralized surfaces (MS/BS). Data are expressed as mean ± SEM. Two way anova and Uncorrected Fisher’s LSD were performed for statistical analyses (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs the indicated group). n=10 mice per group.

**GYY inhibits apoptosis in m-SCs**

OBs apoptosis (Kousteni et al. 2001) (Almeida et al. 2007) is one of the key factors which limits the compensatory increase in bone formation (R L Jilka et al. 1998) (Di Gregorio et al. 2001) contributing to explain why bone formation does not increase as much as resorption after ovx. Here, we investigated whether GYY treatment was able to prevent ovx mediated apoptosis in m-SCs. Caspase-3 activity, which plays a central role in the execution-phase of cell apoptosis, and mRNA expression of BCL2-associated X protein (Bax), a pro-apoptotic gene, and B-cell CLL/lymphoma 2 (Bcl2), an anti-apoptotic gene, were measured as indices of apoptosis. We found that ovx increased Caspase-3 activity (Fig.24A) and Bax expression (Fig. 24B) while had no effect on Bcl2 expression (Fig. 24C) in m-SCs. GYY treatment inhibited caspase-3 activation and ovx-induced expression of Bax, reducing their activity/expression to a level similar to that of the sham mice (Fig. 24A-B). Moreover, GYY was found to increase the expression of Bcl2 compared to both ovx mice and sham operated mice (Fig. 24C). Overall, we showed with three different assays that the treatment with GYY decreases the rate of apoptosis induced by ovx in m-SCs.
**Figure 24: GYY inhibits apoptosis in m-SCs.** Histograms showing: **A**, Caspase-3 activity. **B-C**, Bax and Bcl2 mRNA expression. Data are expressed as mean ± SEM. Two way anova + Tukey’s multiple comparison test were performed for statistical analyses (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs sham operated control). N=5 mice per group.

**GYY increases m-SCs commitment to the osteoblastic lineage**

Based on the evidences that GYY induced *in vivo* bone formation and NaHS induced h-MSCs mineralization *in vitro*, we further investigated *ex vivo* m-SCs differentiation after GYY treatment in ovx and sham mice. m-SCs differentiation was evaluated by CFU-ALP formation and mRNA expression of osteogenic markers. Ovx induced in vehicle treated mice both CFU-ALP formation (~57 %, **** p<0.0001; Fig. 25A) and mRNA expression of each of the four analyzed genes (Fig. 25B; * p<0.05 for Runx2, Osx, Ocn; ** p<0.01 for Col-1).

Moreover, we found that GYY treatment in ovx mice further increased CFU-ALP (~28%, **** p<0.0001; Fig. 25A) and the mRNA expression of all the osteogenic genes analyzed (*p<0.05 for Runx2, Osx; ** p<0.01 for Ocn; *** p<0.001 for Col-1; Fig.25B). Accordingly, GYY treated ovx mice displayed the highest number of CFU-ALP per plate (Fig.25A), as well as the highest mRNA levels of Runx2, Col-1, Osx and Ocn (Fig.25B). Together, these findings demonstrate that increasing m-SCs commitment to the osteoblastic lineage is one of the mechanisms by which GYY treatment prevented ovx-induced bone loss. Moreover, GYY significantly increased the number of CFU-ALP (~43%, **** p<0.0001; Fig.25A) and the expression of osteogenic genes in sham-
operated (* p<0.05; Fig.25B) mice. Further confirming that the anabolic action of GYY is mediated by increased osteoblastogenic differentiation of m-SCs.

**Figure 25:** *GYY increases m-SCs commitment to the osteoblastic lineage.* At the end of the preventive study CFU-ALP and mRNA expression of osteogenic markers were performed on m-SCs. Histograms showing: A, CFU-ALP, B, mRNA expression levels of markers of osteogenic differentiation in m-SCs. Data are expressed as mean ± SEM. Two way anova + Tukey’s multiple comparison test were performed for statistical analyses (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs sham operated control). N=10 mice per group.

**GYY increases Wnt targets in m-SCs and up-regulates Wnt ligands in the BM**

Since OBs differentiation is induced by Wnt activation, we sought to investigate the effects of ovx and GYY treatment on Wnt signaling. Analysis of Wnt signaling in purified m-SCs revealed that the levels of mRNA for the 11 tested genes were all increased by ovx as compared to sham operated
controls (* p<0.05 for Ahr, Axin2, Cyr61, Nkd2, Tgfβ3, Thbs1, Wisp1, Tcf, Lef1; ** p<0.01 Tagln, Twist1; Fig. 26A). Moreover, treatment with GYY caused a further increase in the mRNA levels of the 11 measured genes in the ovx group (* p<0.05 Tagln, Thbs1, Wisp1, Tcf, Lef1; ** p<0.01 for Ahr, Axin2; *** p<0.001, Twist1; **** p<0.0001 Cyr61, Nkd2, Tgfβ3), and increased the mRNA expression of 8 genes in the sham operated group (* p<0.05 Ahr, Tagln, Tgfβ3, Wisp1, Lef1; ** p<0.01 Axin2, Cyr61, Twist1), suggesting that GYY stimulate bone formation by activating Wnt signaling in m-SCs (Fig. 26A).

Next we investigated WNT ligands in whole BM. Regulatory effects of ovx and GYY were detected for four Wnt ligands (Wnt16, Wnt2b, Wnt6 and Wnt10b; Fig. 26B-E). In vehicle treated mice ovx decreased the mRNA levels of Wnt10b (** p<0.01; Fig. 26E), while had no effects on Wnt16, Wnt2b, and Wnt6. Moreover, we found GYY treatment to increase the BM mRNA levels of Wnt16, Wnt2b, and Wnt10b in both sham operated (* p<0.05 for Wnt16 and Wnt2b; **** p<0.0001) and ovx mice (* p<0.05 for Wnt10b; *** p<0.001 for Wnt16 and Wnt2b) (Fig. 26B-C-E). The mRNA level of Wnt6 was increased by GYY in ovx (** p<0.01; Fig. 26D) but not sham mice. The largest changes in the expression of Wnt ligands in response to GYY were noted for Wnt16 and Wnt2b, as shown in Fig. 26B-C. The levels of these 2 ligands were in fact found to be higher in GYY treated ovx mice than in vehicle treated sham operated mice (respectively ~120% and ~67%; **** p<0.0001).
**Figure 26: GYY increases Wnt targets in m-SCs and up-regulates Wnt ligands in the BM.** At the end of the preventive study BM was harvested and m-SCs were isolated. mRNA analysis was then performed on both BM and m-SCs. **A,** Histogram showing mRNA expression of Wnt-signaling target genes in m-SCs. **B-E,** Histograms showing mRNA expression of Wnt ligands in BM. Data are expressed as mean ± SEM. Two way anova and un-corrected Fisher’s LSD were performed for statistical analyses (*p < 0.05,** p<0.01,** p<0.001,** p<0.0001 vs sham operated control). N=5 mice per group.
5.2 Gaining new insights in the role of endogenous H$_2$S in bone tissues

**CBS and CSE are differently expressed in bone biopsies**

Firstly, we aimed to verify H$_2$S-generating enzymes expression in bone tissue. We performed immunohistochemical analysis for CBS and CSE in human bone biopsies. Representative pictures are reported in Fig. 27. We found that both antigens were expressed broadly in bone. However, CBS and CSE showed a markedly different pattern of expression; CBS showed intense positive staining in bone lining cells, osteocytes, BM cells as well as vascular cells (Fig. 27a,b). Coherently with existing evidence from vascular biology, CSE was found to be mainly present in the perivascular cells while it was only occasionally positive in bone lining cells and it was mainly negative in osteocytes (Fig. 27d,e). Matched isotype control confirmed the specificity of the staining (Fig. 27c,f). These findings suggest that H$_2$S is enzymatically generated in bone and BM.

Figure 27: **CBS and CSE are differently expressed in bone biopsies**: Immunohistochemistry showing CBS and CSE expression in bone tissue. Figure shows representative pictures at low magnitude - 200x (a,c,d,f) or high magnitude - 600x (b,e) for CBS (a,b), CSE (d,e) or matched isotype control (c,f).
CBS and CTH/CSE expression and activity regulates h-MSCs osteogenic differentiation

Experiments in CBS⁻/⁻ mice and WT mice pharmacologically treated with CBS and CSE inhibitors showed impaired m-SCs osteogenic differentiation (Y. Liu et al. 2014). Here, we investigated whether endogenous H2S production regulates h-MSCs differentiation into h-OBs. CBS and CTH siRNA decreased CBS and CTH mRNA expression by ~75% as compared NT siRNA (Fig. 28A; *** p<0.0001). When subjected to osteogenic stimulation, h-MSCs silenced for CBS and CTH showed a dramatic reduction in mineralized nodule formation in vitro (~53%, ** p<0.01; Fig. 28B). Similar decrease in mineralization was found after treatment with CBS inhibitor (HA) and CSE inhibitor (PAG) (~56%, * p<0.05; Fig. 28C). These data confirmed that endogenous H2S is necessary for osteogenic differentiation and mineralization of h-MSCs.
**Figure 28:** CBS and CTH/CSE expression and activity regulates osteogenic differentiation of human h-MSCs. h-MSCs were cultured in osteogenic medium in the presence or absence of siRNA CBS/CTH (A-B) or CBS inhibitor (HA) / CSE inhibitor (PAG) (C). A, Histogram showing CBS and CTH mRNA expression after CBS and CTH silencing (N=5); B, Histogram showing AR-S staining quantification after CBS and CSE silencing (N=5); C, Histogram showing AR-S quantification after CBS and CSE functional inhibition (N=3). Data are expressed as mean ± SEM. One way Anova + Tukey’s multiple comparison test and Mann-Whitney test were performed for statistical analyses. * p<0.05, ** p<0.01, *** p<0.001 vs the respective NT siRNA or CTRL cells.
CBS and CTH expression is modulated by osteogenic stimuli and CBS and CTH are selectively up-regulated in mineralizing h-MSCs

Based on our observation suggesting that H₂S levels promotes osteogenic differentiation (Fig. 16), we aimed to first verify whether CBS and CTH, the gene codifying for CSE, were modulated by osteogenic process. In a second instance we aimed to verify whether their expression was differentially modulated in mineralizing and non mineralizing cells (based on data shown in Fig. 15). As a reference marker for osteogenic differentiation, we analyzed ALP expression in the same populations. When analyzed in the whole, unsegregated population of h-MSCs, CBS mRNA expression significantly increased at D14 and D21 compared to baseline levels (***p<0.0001; Fig. 29A); CTH mRNA expression significantly increased at D14 (*p<0.05; Fig. 29B), while ALP mRNA expression increased both at D14 and D21 (***p<0.0001; Fig. 29C). However, when h-MSCs were divided into mineralizing and non mineralizing, CBS and CTH showed a different pattern of expression. Mineralizing h-MSCs showed a time-dependent up-regulation of CBS, CTH and ALP (Fig. 29D,E,F). In particular, CBS mRNA expression was up-regulated both at D14 and D21 (***p<0.0001; Fig. 29D), CTH was up-regulated at D14 and D21 (*p<0.05; **p<0.01; Fig. 29E). Similarly, ALP expression retained the up-regulation shown in the unselected population, although at a lower significance (*p<0.05, **p<0.01; Fig. 29F). However, in non mineralizing h-MSCs, osteogenic stimulation failed to induce any up-regulation of CBS or CTH (Fig. 29G,H), while ALP expression was still significantly up-regulated at both D14 and D21 (**p<0.01, *p<0.05; Fig. 29I). Therefore, CBS and CTH were found to be selectively up-regulated during osteogenic differentiation only in the subset of h-MSCs undergoing complete differentiation toward osteogenic lineage.
Figure 29: CBS, CTH, ALP mRNA expression in h-MSCs during osteogenic differentiation. h-MSCs were cultured in osteogenic medium for 21 days. A-I, Histograms showing mRNA expression on 26 independent experiments and divided in 17 mineralizing cells (D-F) and 9 non mineralizing cells (G-I). Kruskal-wallis test + Dunns multiple comparison test was performed for statistical analyses (* p<0.05; ** p<0.01; *** p<0.001).

Increased expression of CBS and CSE in mineralizing h-MSCs was further confirmed at the protein levels by WB analysis. A representative WB obtained from a mineralizing h-MSCs is reported in Fig. 30.
Figure 30: CBS and CSE protein expression in h-MSCs during osteogenic differentiation. h-MSCs were cultured in osteoblastogenic medium for 21 days. A-B, Representative pictures of WB analyses showing CBS, CSE and β-actin protein expression in h-MSCs during osteogenesis.

CBS expression is differentially expressed in mineralizing and non mineralizing cells and correlates to mineralization

A cross-sectional statistical analysis of gene expression was performed for data obtained at D14 and D21 (Fig.31), it was found that CBS expression was higher in the mineralizing group of h-MSCs as compared to non mineralizing h-MSCs at both time points (*p<0.05; Fig. 31A,D). On the contrary, CTH and ALP expression were not significantly different between the two groups (Fig. 31B,C,E,F).

Figure 31: Cross-sectional analysis of CBS, CTH, ALP mRNA expression in mineralizing vs non mineralizing h-MSCs. h-MSC were cultured in osteogenic medium for 21 days. A-F, Histograms showing
CBS (A-D), CTH (B-E), ALP (C-F) mRNA expression levels in h-MSCs at D14 (A,B,C) and D21 (D,E,F) during osteogenic stimulation. Data compare 17 mineralizing h-MSCs to 9 non mineralizing h-MSCs. Mann-Whitney test was performed for statistical analyses (* p<0.05).

To further investigate if the expression profile of CBS and CTH is associated with the progression of h-MSCs through the osteoblastic phenotype, we performed analysis of correlation between mRNA expression values and mineralization as expressed by AR-S absorbance values, independent of time. Again, ALP expression was used as a reference. As shown in Table 9, CBS expression was positively correlated (R_s 0.358, p<0.0001) with in vitro mineralization. Moreover, CBS strongly correlated with CTH but not ALP expression (R_s 0.735, p<0.0001). ALP expression positively correlated with CTH (R_s 0.261, p < 0.01).

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>P value</th>
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<tr>
<td>Min vs CBS</td>
<td>0.358**</td>
<td>0.0001</td>
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<tr>
<td>N=97</td>
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<tr>
<td>Min vs CTH</td>
<td>0.199</td>
<td>0.05</td>
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<tr>
<td>N=97</td>
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<tr>
<td>Min vs ALP</td>
<td>0.086</td>
<td>0.403</td>
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<td>N=97</td>
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<tr>
<td>CBS vs CTH</td>
<td>0.735**</td>
<td>0.0001</td>
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<td>N=97</td>
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<td>CBS vs ALP</td>
<td>0.176</td>
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<td>N=97</td>
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<tr>
<td>CTH vs ALP</td>
<td>0.261**</td>
<td>0.01</td>
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<td>N=97</td>
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Table 9: Table shows correlation coefficient and P value for correlation between AR-S absorbance value and CBS, CTH or ALP mRNA expression or the correlation between CBS, CTH and ALP mRNA expression.
In Fig. 32, a graphical representation of the correlation between CBS, CTH and ALP mRNA expression to AR-S absorbance value is reported through the non-parametric regression curve obtained with the Loess model.

![Graphical representation](image)

**Figure 32**: Graphical view of the correlation of CBS, CTH, and ALP mRNA expression with AR-S absorbance value during osteogenesis. Correlation is drawn through Loess curves for non-parametric data.

**Ex vivo analysis of CBS and CTH mRNA and protein expression reveals higher expression in h-OBs than h-MSCs from same patients**

To gain further insights into in vivo relevance of our in vitro findings, we analyzed the expression of CBS and CTH in ex-vivo specimens constituted by h-MSCs and h-OBs isolated from the same tibial plateau in different donors. We performed this analysis by multiple approaches: first, we analyzed the expression at passage 0 in culture, a condition where cells undergo minimal manipulation and therefore are closer to the in vivo profile of markers expression. As shown in
Fig. 33A,B the paired analysis of data from 6 patients confirmed that CBS expression is significantly higher in h-OBs than in h-MSCs (*p<0.05; Fig. 33A), while CTH mRNA expression revealed high donor variability and did not show any significant difference between the two cell types (Fig. 33B). Second, to expand the pool of donors we analyzed the expression of CBS and CTH comparing h-MSCs and h-OBs at passage 1 in culture. Data obtained from 16 patients revealed that both CBS and CTH displayed significantly higher expression in h-OBs compared to h-MSCs (***p<0.001 for both, Fig. 33C,D). Finally, CBS and CTH expression levels were analyzed within a dataset of whole genome microarray analysis that we previously generated in our laboratory (Lisignoli et al. 2009). Further confirming our previous findings, CBS and CTH were found to be between the genes significantly up-regulated in mature h-OBs as compared to h-MSCs. In particular, CBS and CTH were expressed, respectively, 4.9 and 1.7 fold higher as compared to h-MSCs (Fig. 33E, F; °p<0.0001).
Figure 33: CBS and CTH mRNA expression is higher in h-OBs than in h-MSCs. A, B, Box plots (Whiskers min to max) showing CBS and CTH expression by RT-PCR of cells at passage 0 isolated from tibial plateau of 6 donors. Wilcoxon matched paired test was performed for statistical analyses (* p<0.05). C, D, Box plots (Whiskers min to max) showing CBS and CTH expression by RT-PCR of cells at passage 1 isolated from tibial plateau of 16 donors. Wilcoxon matched paired test was performed for statistical analyses (*** p<0.0001). E, F, Box plots (Whiskers min to max) showing CBS and CTH expression of cells by microarray analyses at passage 1 isolated from tibial plateau of 6 donors. Mann Whitney test was performed for statistical analyses (*** p < 0.05).
Then, we performed immunocytochemistry and WB analyses for CBS and CSE in h-MSCs and h-OBs isolated from tybial plateau. As shown in Fig. 34A, h-MSCs cultured in monolayer broadly expressed both CBS and CSE; moreover, h-OBs displayed a marked increase in the positive staining to both antigens. This protein expression was further confirmed in pairs of h-MSCs and h-OBs isolated from the same bone biopsy obtained from 3 different donors by WB analysis (Fig. 34B). While basal expression of CBS varied in h-MSCs among different donors, h-OBs consistently showed a much stronger expression levels compared to their respective h-MSCs counterpart, which resulted into a significant difference upon densitometric quantification (3.3 fold higher, ** p<0.01; Fig. 34C). CSE expression showed a similar trend of increased expression in h-OBs, which resulted into a significant difference upon densitometric quantification (2.4 fold higher, ** p<0.01; Fig. 34D). However, contrary to CBS, CSE showed higher variation among different donors in h-OBs counterpart (Fig. 34C).

Altogether these ex-vivo findings reveals that CBS and CTH/CSE are indeed differentially expressed in bone tissue between the osteoprogenitor cells and the terminally differentiated osteoblastic cells confirming increased expression during transition toward mature osteogenic phenotype revealed by in vitro data.
Figure 34: Immunocytochemical staining and WB analyses of CBS and CSE in h-MSCs and h-OBs. A, Representative pictures showing immunocytochemical staining for CBS (a,c) and CSE (b,d) in h-MSCs (a,b) and OBs (c,d) isolated from the tibial plateau of different donors; B, WB analyses showing CBS and CSE protein expression in h-MSCs vs h-OBs obtained from the tibial plateau of the same patient; data were obtained from 4 different donors. C, Histogram shows densitometric quantification of WB protein expression showed in panel 6B; quantification was performed by Image J software and target proteins were normalized by β-actin band intensity. Data were analysed by Un-paired t-test. (* p<0.05; ** p<0.01).

Ovx is associated with impaired H₂S-generating enzymes expression

Based on the evidences that a) ovx is associated with aberrant OCs activity and limited compensatory increase in OBs formation; b) H₂S inhibited OCs function in vitro and induced OBs differentiation both in vitro and in vivo; c) ovx blunted H₂S levels; d) GYY administration prevented ovx-induced bone loss, we prompted to investigate whether estrogen depletion is associated to a down-regulation of H₂S-generating enzymes.

Coherently to results previously shown in Fig. 17, in this experiment ovx blunted both free H₂S and bound sulfur serum levels indicating a physio-pathological role of H₂S levels in the development of osteoporosis due to estrogen deficiency. We further extended this investigation to the expression of
H₂S generating enzymes. We found that mRNA expression of CBS and CTH were significantly down regulated in both m-SCs (Fig. 35C,D) and total BM cells of ovx mice (Fig. 35E,F) compared to sham-operated mice. Therefore, ovx blunts the expression levels of CBS and CTH mRNA in bone confirming a physio-pathological role of transsulfuration pathway in bone homeostasis.

**Figure 35:** Effects of ovx on serum levels of H₂S and mRNA expression of CBS, CTH in BM m-SCs and total BM cells. Mouse serum was collected at the end of preventive study and measurements of H₂S and bound sulfur were performed by gas chromatography. mRNA expression was evaluated by RT-PCR in whole BM and m-SCs. Histograms showing: (A) Serum levels of free H₂S and (B) bound sulfur measured 4 weeks after surgery. (C-D) mRNA expression of CBS and CTH in total BM cells. (E-F) mRNA expression of CBS and CTH in BM m-SCs. Mann-Whitney (A-B) and unpaired t test (C-F) were performed for statistical
analysis. Data are expressed as mean ± SEM. N=10 mice per group. ** p<0.01, *** p<0.001 vs sham-operated mice.

**CBS and CTH mRNA expression is up-regulated by estrogen**

Furthermore, to investigate whether estrogen directly regulates CBS and CTH in human bone cells, we stimulated h-MSCs with 17-β estradiol. After 24 hours stimulation, 17-β estradiol significantly up-regulated CBS and CTH expression (** p<0.01; * p<0.05; Fig. 36A,B) suggesting that the endogenous pathway leading to H2S production is, at least in part, directly induced by estrogen.

Figure 36: **17-β estradiol regulates CBS and CTH mRNA expression.** h-MSCs were cultured in α-MEM 5% FBS without phenol red for 24 h. A-B, Histograms showing CBS and CTH mRNA expression. One sample t test was performed for statistical analysis. ** p<0.01 * p<0.05 vs CTRL cells. Data are expressed as mean ± SEM of N=5 independent experiments.
5.3 Translating *in vitro* and *in vivo* findings into possible applications of clinical interest

5.3.1 Development of DM-22, an H\(_2\)S-releasing drug for post-menopausal osteoporosis

Our data demonstrated an anabolic action of H\(_2\)S-donors *in vitro* and *in vivo* and an anti-catabolic action *in vitro*. Based on this dual action exerted by H\(_2\)S on bone homeostasis, we sought to exploit these findings into a pharmaceutical tool aiming at treating post-menopausal osteoporosis. Our strategy has been to develop an hybrid drug, based on the molecular structure of alendronate (AL), an anti-catabolic drug widely used in the clinic but with several limitations (Armamento-Villareal et al. 2006) (Piscitelli et al. 2010) (Faiman, Pillai, and Benghiac 2013) (Paiva-Fonseca et al. 2014). To achieve the manufacture of DM-22, a novel H\(_2\)S-releasing AL, we established a collaboration with the group of professor Calderone and Dr Rapposelli (University of Pisa), who has a great expertise in developing NO-hybrids drugs for cardiovascular pathologies (Breschi et al. 2006) (Calderone et al. 2009) (Digiacomo et al. 2015). By *in vitro* study comparing DM-22 to AL function we expected DM-22 to be more cytocompatible, exerts both anti-catabolic and anabolic action compared to the parent drug.

*DM-22 is a slow-releasing H\(_2\)S donor*

Fig. 37 shows that DM-22 is a compound capable of releasing H\(_2\)S in the presence of organic thiols such as L-cysteine. It exhibited a slow-H\(_2\)S-release reaching a maximum concentration of 45 μM H\(_2\)S after 20 min. Conversely, incubation of DM-22 without organic thiols supplementation caused
a slow and modest release of H₂S. The concentration of H₂S detected after 20 minutes of incubation was equal to about 1.5 μM.

**Figure 37: H₂S release from DM-22 as detected by amperometric measurements.** 1 mM DM-22 in phosphate buffer was incubated with (blacks squares) or without (white squares) 4 mM L-cysteine which allows H₂S release.

**DM-22 inhibits h-OCs differentiation and function without inducing cytotoxicity and preserving a residual h-OCs**

AL has been widely used in medical practice to treat osteoporosis (Piscitelli et al. 2014) for its inhibition on OCs differentiation and function (Azuma et al. 1995) (Rogers et al. 1996).

Here, we first aimed to investigate whether DM-22 retained the AL-dependent inhibition on osteoclastogenesis and AL toxicity on h-OCs. To investigate whether DM-22 and AL differentially regulate of h-OCs differentiation, h-monocytes were differentiated into mature h-OCs in the presence of increasing concentrations of the two compounds. Functional assays for osteoclastogenic differentiation revealed a different pattern of inhibition of DM-22 compared to AL. Fig. 38A compares the number of TRAP-stained h-OCs after AL or DM-22 treatment. DM-22 showed a less
potency in inhibiting h-OCs differentiation. Fig. 38B-C show quantification of the number of h-OCs obtained after 5 days of AL (Fig. 38B) or DM-22 (Fig. 38C) treatment. AL dose-dependently decreases the total number of TRAP positive mature h-OCs, resulting into a high statistically significant inhibition at all the concentrations tested 1-33 μM (***, p<0.001) (Fig. 38B). In particular AL decreased the number of h-OCs by 62% and 77% respectively in 1 and 3.3 μM; and virtually abolished h-OCs differentiation starting from the 10 μM concentration. Conversely, DM-22 was found to highly inhibit h-OCs differentiation only at the highest dose, 33 μM (~37%, ***, p<0.001); while in the lowest doses (1-10 μM) slightly inhibited h-OCs differentiation (~15%; ** p<0.01, *** p<0.001). These data suggested that DM-22 could preserve a residual h-OCs, important for OCs-OBs communication.

Coherently, h-OCs function was also differentially regulated by the two molecules. Fig. 38D compares the amount of matrix actively resorbed by h-OCs during DM-22 or AL treatment. The ability of mature h-OCs to break down a mineral substrate as tested in vitro by a ‘pit assay’, was strongly prevented by 1 μM AL treatments and virtually completely inhibited by 3,3-10-33 μM AL. Conversely, h-OCs function was dose-dependently inhibited by 1-3,3-10-33 μM DM-22.

When we performed LDH assay for evaluating the cytotoxicity at the end of differentiation, we observed that DM-22 displayed lower absorbance values than AL (* p<0.05, ** p<0.01, *** p<0.001). The similarity of effect between NaHS and DM-22 further confirm the specificity of our in vitro data on H₂S-mediated h-OCs inhibition. Based on these data, it is tempting to propose that H₂S moiety in DM-22 could have protected cells from toxicity, contrary to AL, and could have retained H₂S-dependent h-OCs inhibition ability previously reported in Fig. 1.
Figure 38: DM-22 inhibits h-OCs differentiation and function without inducing cytotoxicity and preserving a residual h-OCs. h-OCs were differentiated from h-monocytes and grown for 5 days on either
plastic well-plates (A,B,C,E) or osteologic slides (D), in osteoclastogenic medium in the presence or absence of increasing concentrations of AL or DM-22. A, Representative pictures of TRAP staining (magnification 20X), showing the effect of DM-22 and AL on h-OCs differentiation. B-C, Histograms showing average h-OCs number/field. Data are expressed as mean ± SEM of triplicates of N=3 independent experiments. ANOVA and Dunnett’s test were performed for statistical analyses (** p<0.01, *** p<0.001 vs control cells). D, Representative pictures of pit assay (magnification 20X), showing the effect of DM-22 and AL on h-OCs function. E, Histogram showing LDH measurement. Data are expressed as percentage of cytotoxicity and refers to arbitrary units obtained by colorimetric detection of LDH activity. Data are expressed as mean ± SEM of triplicates of N=3 independent experiments. Two way Anova + Bonferroni post-test was performed for statistical analysis (* p<0.05, ** p<0.01, *** p<0.001 vs CTRL).

**DM-22 is devoid of AL-like cytotoxicity and inhibition of proliferation on h-MSCs**

Given the absence of such a firmly established knowledge of AL function on MSCs (Soares et al. 2016) (Duque and Rivas 2007) (H. K. Kim et al. 2009) (Yingjun Wang et al. 2010) (Patnirapong, Singhanadagtit, and Arphavasin 2014), we aimed to perform viability assay on bone derived h-MSCs treated with AL or DM-22.

Treatments with micromolar concentrations of AL appeared to cause a sustained significant decrease in cellularity since 72 h of treatment. Fig. 39A, shows representative pictures of toluidin blue assay stained h-MSCs at 72 h of treatment. The highest dose of AL tested (33 μM) induced a noticeable decrease in cellularity and a change toward globular-like appearance in h-MSCs morphology, similar to those of Tryton 10X treated samples (the positive control of cellular toxicity, CTRL+). On the contrary the morphology of DM-22 treated cells was similar to those of control cells. Quantitative measurements of toluidin blue intensities evidenced the statistical decrease in cellularity for AL, but not DM-22, treatment (Fig. 39B; *** p < 0.001). We next assayed the potential acute toxicity within a time-range of 72 h from the stimulation. Quantification of LDH confirmed the absence of toxicity up to 10 μM concentration at both 24h and 72h (Fig. 39C,D) in both compounds. Conversely, 33 μM AL induced cellular toxicity starting from 72 h after the treatment (Fig. 39D). DM-22 significantly lowered LDH release at both 24 h (13%; Fig.
and 72 h (22%, Fig. 39D) compared to AL. It is important to note that at 24 h both DM-22 and AL did not induce cytotoxicity; conversely, at 72 h AL displayed high cytotoxicity compared to both control and DM-22 samples. It is tempting to therefore propose that H₂S moiety in DM-22 could have protected against AL-dependent toxicity in h-MSCs as in h-OCs (Fig. 38).

Recently, has been emerged a role of AL in inhibiting MSCs proliferation (Patntirapong, Singhatanadgit, and Arphavasin 2014). Here we aimed to compare DM-22 and AL effects on the proliferation of bone derived h-MSCs within a time-range of 72 h from the stimulation. We found that at both 24h and 72h neither AL nor DM-22 affected cells proliferation at 1-3,3-10 µM concentrations (Fig. 39E,F). Conversely, the concentration of 33 µM AL inhibited the proliferation by 22% at 24h and by 47% at 72h. Although DM-22 induced a significant inhibition of cell proliferation at the highest concentration, it showed a markedly lower inhibition of cell proliferation compared to AL (19%, 72 h; Fig. 39G).
Figure 39: DM-22 is devoid of AL-like cytotoxicity and inhibition of proliferation on h-MSCs. h-MSCs were cultured in α-MEM 15% FBS in the presence of increasing concentrations of AL and DM-22 (1-3,3-10-33 µM). A, Representative pictures of toluidin blue staining showing the morphology of AL/DM-22 treated h-MSCs (33 µM) compared to untreated cells (CTRL) and cells treated with Triton 10x (CTRL+). Magnification 20X. B, Histogram showing toluidin blue staining quantification (absorbance 560 nm). Data are expressed as mean ± SEM of N=3 independent experiments (each one in quadruplicate). One way Anova and Tukey’s multiple comparison test were performed for statistical analyses. C-D, Histograms showing LDH measurement; data are expressed as percentage of cytotoxicity and refers to arbitrary units obtained
by colorimetric detection of LDH activity. Data are expressed as mean ± SEM of N=3 independent experiments (each one in quadruplicate). Two way anova + Bonferroni post test was performed for statistical analyses. E-F, Histograms showing $^3$H-thymidine detection for DM-22 and AL compared to CTRL cells at 24 h (E) and 72 h (F). Data are expressed as fold increase compared to control sample and are expressed as mean ± SEM of N=3 independent experiments (each one in quadruplicate). ANOVA and Tukey’s multiple comparison test were performed for statistical analyses.

DM-22 stimulates mineralization both compared to AL as CTRL cells

The role of AL in modulating the differentiation of MSCs toward the OBs lineage is still a highly debated topic (C. H. Chang et al. 2014) (Lindtner et al. 2014) (Patntirapong, Singhatanadgit, and Arphavasin 2014) (Zhou et al. 2013). Therefore, we aimed to investigate the role of AL, compared to that of DM-22, in bone resident h-MSCs during osteogenic differentiation. We found that treatments with micromolar concentrations of AL during osteogenic differentiation, led to a significant decrease in cellularity since 72 h of treatment, similar to that shown in Fig. 39A in unstimulated medium. When AR-S quantification was performed for evaluating the amount of mineral matrix produced, we observed that concentration higher than 1 μM AL abolished the mineralization, while all concentrations tested for DM-22 were able to induce mineralization compared to control cells. Fig. 40A shows representing pictures of each concentration at D21 of mineralization. We first found that AL dose-dependently inhibited the mineralization of h-MSCs (Fig. 40B). 1 μM AL slightly decreased the mineralization (19%), without statistical significance. Higher concentrations of AL abolished the mineralization both at non toxic concentrations (3,3 and 10 μM; * p<0.05) and at the toxic concentration (33 μM; *** p<0.001). On the contrary, DM-22 not only did not inhibit mineralization, but also highly increased the mineralization at each concentration tested in respect to control cells (72, 91, 110, 106% respectively; * p<0.05 for 3,3 μM; ** p<0.01 for 1, 10, 33 μM). As a results DM-22 displayed higher AR-S quantification compared to AL (* p<0.05 for 1 μM; *** p<0.001 for 3,3-10-33 μM). We next evaluated mRNA expression of osteogenic markers in order to validate AR-staining data. We limited the analysis to
the concentration with the highest difference at AR-S levels (33 μM). AL significantly inhibited the mRNA expression of ALP (Fig. 40C) and BSP (Fig. 40D). Moreover, DM-22 treated cells and control cells had similar mRNA expression values. Taken together these data evidenced that AL, but not DM-22, inhibited osteogenic differentiation of h-MSCs.
Figure 40: Contrary to AL, DM-22 stimulates mineralization compared to CTRL cells. h-MSCs were cultured in osteogenic medium in the presence of increasing concentrations of AL and DM-22 (1-3,3-10-33...
µM). **A**, Representative pictures of AR-S staining. Magnification 20X. **B**, Histogram showing AR-S staining quantification. Data are expressed as mean ± SEM of N=8 independent experiments. Mann Whitney for simple comparison was used for statistical analyses. **C-D**, Histograms showing ALP and BSP mRNA expression. Data are expressed as mean ± SEM of fold increase of N=3 independent experiments. One sample t-test was performed for statistical analyses.

### 5.3.2. Development of SF_GYY, a novel H₂S-releasing scaffold based on silk fibroin

Based on the dual action exerted by H₂S on bone homeostasis, we sought to exploit these features for the development an innovative tool aiming at treating critical bone defects. Our strategy has been to develop an H₂S-based scaffold for bone regenerative medicine. To achieve the manufacture of silk fibroin (SF) H₂S releasing scaffold (SF_GYY), we established a collaboration with the Laboratory of Prof. Motta (University of Trento) who has a great expertise in developing SF scaffolds (Jones et al. 2009) (Riccio et al. 2012). Here, we present some preliminary results on scaffolds’ characterization and cells integration into the scaffolds comparing SF_GYY to the native SF.

#### Scaffolds’ characterization

Scanning Electron Microscope (SEM) analysis revealed a porous structure suitable for OBs growth and migration. Fig. 41A,B compares representative pictures of SF scaffold (Fig. 41A) and SF_GYY (Fig. 41B). The images revealed that the incorporation of GYY did not alter the native structure of the scaffold. Both SF (Fig. 41A) and SF_GYY (Fig. 41B) shows high porosity and the presence of interconnected pores. Pore sizes were found to be in between 200 to 400 μm. Fourier transform infrared spectroscopy (FTIR) spectroscopy further confirmed the presence of GYY in SF_GYY scaffold (Fig. 41C). Indeed, FTIR curves related to sample SF_GYY, showed, together with the typical peaks of silk fibroin, peaks with strong absorption of GYY, which are not present in the
spectrum of pure SF sponge (Fig. 41C). In order to preliminary evaluate H$_2$S release from the scaffolds spectrophotometric measurement of methylene blue generation was performed (Fig. 41D).

![Figure 41](image-url)

**Figure 41:** Characterization of SF and SF-GYY scaffolds. **A-B,** Representative pictures of SEM analysis. **C,** Representative FTIR curves obtained by FTIR spectroscopy analyses. **D,** Representative curves of H$_2$S release obtained by methylene blue assay.
Both SF and SF_GYY demonstrate osteoconductive properties

h-MSCs derived from tybial plateau were allowed to colonize and growth within the scaffolds. Cells were cultured in perfusion in U-cup bioreactor. Fig. 42a,c and b,d, respectively shows representative pictures of haematoxylin-eosin and Von Kossa stains, performed for evaluating cells integration and osteoconductivity of the scaffold. We found that GYY incorporation did not interfere with cell colonization and we did not evidence any signs of toxicity in cells (Fig. 42a,c). Moreover, cells grown both in SF and SF_GYY scaffold were able to differentiate toward osteogenic lineage confirming that both scaffolds are osteoconductive (Fig. 42b,d).

Figure 42: SF and SF_GYY are osteoconductive scaffolds. Figure shows representative pictures at 4X (a,c) or 10X (b,d) magnification of haematoxylin-eosin (a,c) staining or Von Kossa (b,d) staining. Pictures compares native scaffold (SF; a,b) to the scaffold incorporating GYY (SF_GYY; c,d).
6. DISCUSSION

We hypothesized a role of H$_2$S in bone physiology and in the pathophysiology of post-menopausal osteoporosis based on two important considerations. Firstly, H$_2$S has been found to exert important organ-specific functions such as regulation of vasodilatation (G. Yang et al. 2008) (Weimin Zhao and Wang 2002), neuronal excitability (Nagai et al. 2004), lipid metabolism (Namekata et al. 2004) or insulin production (Yusuf et al. 2005). However, whether it plays a role in the physiology of bone tissue was yet to be established. Secondly, patients affected by homocystinuria, the disorder caused by CBS deficiency, show severe osteoporosis (Mudd et al. 1985) and increased fracture risk (van Meurs et al. 2004) (McLean et al. 2004). As the pathophysiologic mechanisms underlying this bone phenotype were still largely unknown, we hypothesized a correlation between depleted H$_2$S levels and bone loss. Therefore, within this study we took several independent but connected approaches for investigating the role of H$_2$S in bone tissue evaluating: 1) CBS and CSE expression in human bone tissue and bone derived cells *ex vivo*; 2) exogenous and/or endogenous role of H$_2$S in bone cells *in vitro*; 3) CBS, CSE and H$_2$S levels in homeostatic condition or in post-menopausal osteoporosis *in vivo*; 4) the effect of H$_2$S exogenous administration in homeostatic condition or in post-menopausal osteoporosis *in vivo* and *ex vivo*. In a second phase we aimed to translate the gain of knowledge on H$_2$S regulation in bone tissue in order to: 1) validate the efficacy of H$_2$S treatments in the prevention or therapy post-menopausal osteoporosis; 2) develop H$_2$S-based drugs and scaffolds for the treatment of bone erosive diseases or traumatic bone lesions.

CBS and CSE are known to be the major mediators of H$_2$S production in mammalian cells (Szabó 2007). However the state-of-the-art highlights a poor understanding of the pattern of CBS and CSE expression in tissues and cells. It had long been assumed that CBS activity and CSE activity are, respectively, the primary source of H$_2$S in the brain and in peripheral tissues (R. Wang 2012). However, new findings (Paul and Snyder 2014) (Mani et al. 2014) (Xia et al. 2009) demonstrated that both enzymes are expressed and produce H$_2$S in many tissues. Moreover, to the best of our
knowledge the expression of CBS and CTH/CSE in bone tissue has never been investigated in deep. Our immunohistochemical analyses revealed, in agreement with the recent evidences in other tissues, that both CBS and CSE produces H$_2$S in human bone tissue. Moreover we found a **peculiar pattern of expression**: CBS is broadly expressed, while CSE expression seem to be more confined to perivascular region and negligibly expressed in bone lining cells. Interestingly, bone lining cells have been described as the region that harbours late osteoprogenitor cells and mature osteoblasts (Everts et al. 2002), while the perivascular area of BM microvasculature has been described as the region which gives rise to the earliest bone stem cells (Sacchetti et al. 2007). Recent evidences found CBS and CSE to be expressed in h-MSCs and m-SCs (Y. Liu et al. 2014). Our data confirmed that bone derived h-MSCs express both CBS and CTH/CSE. Furthermore a combination of data derived from RT-PCR, immunohistochemical and WB analyses first revealed that CBS and CTH/CSE not only are expressed in mature h-OBs but are highly expressed compared to h-MSCs. Moreover analyses of CBS and CTH/CSE expression during osteogenic differentiation of h-MCSs revealed how **CBS and CTH/CSE up-regulation appears to be a distinctive feature of the h-MSCs transition towards the osteogenic phenotype**. To our knowledge this is the first study reporting that during osteogenesis H$_2$S-producing enzymes are regulated at the transcriptional and translational level, suggesting a role of both enzymes in the differentiation process. Further analysis of correlation suggested that CTH may be mostly involved in the early phases of osteogenic differentiation (initiation phase), as it tightly correlates with ALP expression a marker of early osteogenesis (Koch, Jadlowiec, and Campbell 2005); while CBS may be involved in both early and late phases of osteogenic differentiation (late maturation phase) as it correlates to both CTH and mineralization levels.

One finding of special interest is that CBS and CTH mRNA expression was differentially regulated during osteogenic stimulation in two groups of h-MSCs segregated for their heterogeneous response to osteogenic medium and hereby named as mineralizing or non mineralizing. This donor-to-donor
heterogeneity was not unexpected as it has been widely reported and described as a critical important aspect of MSCs biology (Phinney 2012) (P Janicki et al. 2011) (Pevsner-Fischer, Levin, and Zipori 2011). Interestingly, the ratio we observed between mineralizing and non-mineralizing cells is similar to that reported by others (Siddappa et al. 2009). Intense research is currently under way to discover mechanisms leading to ineffective osteogenic differentiation, aiming to find a way to select h-MSCs with greater bone formation ability. In keeping with findings reported by others (Hoemann, El-Gabalawy, and McKee 2009), we found ALP up-regulation during osteogenic differentiation not proportional to the extent of mineralization. Conversely, we found that the lack of up-regulation of CBS and CTH mRNA expression is typical of non-mineralizing cells, first suggesting that may exist a threshold of CBS-CSE-H$_2$S levels above which mineralization occurs. This hypothesis was further validated by loss-of-function experiments (CBS and CTH gene silencing or CBS and CSE enzyme pharmacological inhibition) on h-MSCs during osteogenic differentiation, which revealed a marked inhibition of h-MSCs ability to produce mineralized matrix. Finally, we first found that exogenous administration of low micromolar concentrations of NaHS, a common H$_2$S donor in vitro was sufficient to promote mineral apposition by h-MSCs. Of special interest is the finding that the same range of NaHS concentrations induced the mineralization in N-CD h-MCSs. This observation led us to consider the possibility that NaHS exogenous administration could have restored H$_2$S intracellular levels in N-CD h-MSCs changing their fate of differentiation. Interestingly, other authors independently showed that NaHS exogenous administration in CBS$^+$ and CBS$^{+/−}$ m-SCs partially rescued their impaired mineralization ability both in vitro and ex vivo (Y. Liu et al. 2014).

Taken together these data revealed that H$_2$S plays an active role on osteogenic differentiation of h-MSCs. Interestingly, we found that H$_2$S exogenous administration modulates another important h-MSCs function. As, h-MSCs are known to be h-OC-supporting cell population in vivo (D.-C. Yang et al. 2008) (Dalle Carbonare et al. 2009), we evaluated the effect of NaHS treatments on regulation
of RANKL and OPG mRNA expression. Our data first revealed an ability of NaHS to suppress the osteoclastogenic potential in the bone microenvironment. While a similar effect was described in cells from other tissues (Liao and Hua 2013), these are the first evidence of down-regulated RANKL/OPG expression ratio in bone derived h-MSCs.

In an attempt to draw a comprehensive picture of the effect of exogenous H₂S on bone cells, we conducted a series of investigations on h-OCs aimed to clarify the conflicting evidences existing in the literature (Irie et al. 2012) (Irie et al. 2009) (Li et al. 2010) (S. K. Lee et al. 2013) (Y. Liu et al. 2014) (Frantzias et al. 2012) and to identify downstream targets of H₂S. To this regard, we first demonstrated that high micromolar concentration of NaHS were necessary to induce an inhibitory signal of h-OCs differentiation and function in vitro, as revealed by TRAP and Pit assays. After having assessed that this inhibition was not due to apoptosis and/or toxicity processes, we took into consideration the role of ROS as a potential target of H₂S since they play a prominent role in determining the fate of h-OCs differentiation (Reddy 2004). Despite H₂S was found to mediate the down-regulation of ROS levels induced by many stimulation in different cell types (Xu et al. 2011) (Hourihan, Kenna, and Hayes 2013), to our knowledge our results represent the first evidence suggesting that short-term treatment with NaHS is able to dose-dependently prevent the increase in ROS induced by RANKL-stimulation. As NaHS is known to have a rapid disappearance from culture medium (Hu et al. 2009), the scavenging effect of NaHS on ROS can be short-lasting. We therefore have been interested in verifying whether the H₂S inhibition was dependent on a sustained activation of NRF2, the master regulator of the antioxidant defence system (Kensler, Wakabayashi, and Biswal 2007). We took in consideration NRF2 as a potential target of H₂S based on two recent evidences. First H₂S has been demonstrated to displace NRF2 from KEAP-1 by inducing S-sulfhydration of specific cysteine residues on KEAP-1, triggering NRF2 stabilization and increasing the expression of its antioxidant target genes (Hourihan, Kenna, and Hayes 2013) (G. Yang et al. 2013). Second, NRF2 up-regulation has been recently reported to
actively inhibit mouse macrophage OCs differentiation in vitro and inhibit LPS-mediated RANKL-dependent osteoclastogenesis in vivo (Kanzaki et al. 2013). We showed, through a combination of data obtained by FACS, RT-PCR and immunocytochemical analyses, that NaHS stimulation on h-pre-OCs caused NRF2 increased protein expression without any modulation at the gene transcription level but rather by an increase of nuclear translocation, suggesting that H₂S is increasing NRF2 protein stability in our system. Further attesting the activation of NRF2 we evidenced the up-regulation of two key target genes, NQO1 and PRDX1, which are known to be activated by NRF2 through the ARE sequences (Taguchi, Motohashi, and Yamamoto 2011) (Wakabayashi et al. 2010) (Bergstrom et al. 2011). Further experiments performed with two molecules (SFN and t-BHQ) which were shown to increase the nuclear translocation of NRF2 through electrophilic modification of KEAP1 (Abiko et al. 2011) (B. Y. Shin et al. 2012), evidenced that they actively inhibited the differentiation of h-OCs. Thus, it appears that the stabilization of NRF2 in h-pre-OCs, induced by various compounds, is sufficient to suppress their ability to differentiate into h-OCs. Finally, we demonstrated that NRF2 is necessary to H₂S for mediating h-OCs inhibition. Notably, NRF2 silencing abrogates both H₂S-mediated inhibition of h-OCs and its up-regulation of NQO1 and PRDX1.

Altogether, these findings demonstrated that NaHS micromolar concentrations are required for directly and indirectly inhibit h-OCs differentiation on one hand, and promoting osteoblast differentiation on the other.

Taking in consideration a possible physiological role of H₂S we can speculate that in BM niche a local increase in H₂S concentration may be important in directing the fate of differentiation of bone cells. However, we did not further investigated this hypothesis our in vivo data revealed that CBS, CSE and H₂S levels play a role in the maintenance of bone homeostasis. Indeed, our findings are the first to link sex steroid deficiency and the resulting bone loss to suppression of CBS/CSE expression in whole BM and m-SCs and down-regulation of H₂S serum levels. The difference
in serum H\textsubscript{2}S levels between sham operated and ovx mice was similar to that found between CSE\textsuperscript{−/−} mice (G. Yang et al. 2008) or CBS\textsuperscript{+/−} mice and their respective WT controls (Y. Liu et al. 2014), suggesting the relevance of sex steroids as regulators of H\textsubscript{2}S biosynthesis. Moreover \textit{in vitro} data of estrogen administration on h-MSCs evidenced up-regulation of CBS and CTH transcription, further confirming this statement. In keeping with our findings altered H\textsubscript{2}S levels (Brancaleone et al. 2008) (Perna et al.) (Matthew Whiteman et al. 2004) and CBS and CSE expression levels (Huang and Moore 2015) (L. Zhang et al. 2015) (Hwang et al.) have been found in many pathologies. Furthermore, recently depletion in H\textsubscript{2}S levels has been found to cause osteoporosis in homocystinuric patients (Y. Liu et al. 2014). \textbf{It is tempting to propose H\textsubscript{2}S depleted levels as one of the unifying mechanisms which account for the bone loss occurring in different pathologies.}

In an attempt to further establish whether H\textsubscript{2}S is essential for the proper function of bone tissue and in particular for preserving bone mass, we achieved replenishment of systemic H\textsubscript{2}S levels in ovx mice through administration of GYY, as assessed by gas-chromatographic analyses. Notably, in sham mice GYY increased H\textsubscript{2}S levels above the baseline. \textbf{Our data on one hand demonstrated that H\textsubscript{2}S levels modulates bone mass and on the other hand provided a proof of principle that a sulfur replacement therapy is a conceivable novel therapeutic option for preserving and restoring bone mass after menopause.} In particular we found that in femur, GYY prevented bone loss induced by ovx when it was administered before the onset of the disease, and reversed bone loss when it was administered after the four week necessary for the induction of bone loss by ovx, as evidenced by \textit{μ}CT analysis. Conversely, we found that GYY administration only partially prevented and reversed trabecular bone in spine of ovx mice. We hypothesize that these differential regulation accounts for the different nature of femur and spine. Moreover, based on our knowledge, our data are the first indicating a \textbf{GYY-mediated anabolic action in the femur of healthy mice.} Notably, GYY treated sham operated mice had the highest level of bone mass as well as the highest levels of free H\textsubscript{2}S, presumably because of the cumulative effects of estrogen on H\textsubscript{2}S synthesis and of GYY-mediated H\textsubscript{2}S release. The existence of a relationship between H\textsubscript{2}S levels and bone volume
suggest that it may be possible to increase bone volume above baseline and achieve a net bone anabolic effect by treating ovx mice with doses of GYY higher than the dose used in the current study.

Despite these advantages, whether \( \text{H}_2\text{S} \) modulates the balance between bone formation and resorption \textit{in vivo} was still unknown. In order to fill this gap on the knowledge we first performed histomorphometric analyses. This assay demonstrated a different behaviour in femur and spine in responding to ovx-induced changes in bone turnover. Only in spine we found the expected increase of static indices of bone resorption and bone formation by ovx; while dynamic indices of bone formation were not affected by ovx neither in spine nor in femur. We hypothesize that these unexpected results are due to the intrinsic low sensitivity and high variability of bone histomorphometry. Measurements of serum markers of bone resorption and formation further confirmed the changes occurring in bone turnover following ovx. Indeed, both CTX, an accurate marker of bone resorption, and P1NP, a sensitive marker of bone formation, were higher in ovx mice than in sham-operated mice. The increase was higher for CTX confirming that the compensatory bone formation is ineffective in balancing bone resorption during ovx. GYY treatment increased all spinal histomorphometric indices of bone formation in ovx mice and induced a further increase in P1NP serum levels. As a results, GYY treated ovx mice had the highest levels of P1NP. Conversely, we did not observe any down-regulation of static indices of bone resorption or CTX levels after GYY administration. Moreover we found that spinal histomorphometric static indices of bone formation and MAR were up-regulated by GYY in sham operated mice, further confirming an anabolic action of \( \text{H}_2\text{S} \) in control mice.

In our experimental model, ovx mice showed increased OCs number, Interestingly, elevated numbers of OCs were found in femur of CBS\(^{-/-}\) and CBS\(^{+/+}\) mice where circulating levels of \( \text{H}_2\text{S} \) were lower. Therefore we might speculate that blunted \( \text{H}_2\text{S} \) levels may hamper the compensatory inhibition on OCs differentiation and be part of the mechanism by which OCs number is increased.
in different pathologies with bone loss. However, the sulphur-replacement therapy did not significantly down-regulate OCs number and the serum index of bone resorption. It is unknown whether H2S treatments in CBS\textsuperscript{+/−} mice down-regulated OCs number or lowered the serum index of bone resorption (Y. Liu et al. 2014). Up to now, we were unable to explain the discrepancy between \textit{in vitro} and \textit{in vivo} data regarding osteoclastogenesis. A possible explanation is that higher micromolar concentrations are necessary to inhibit OCs differentiation while even lower micromolar concentration can induce osteogenic differentiation. Regimen of H2S administration higher than the ones used in the current study may elucidate whether H2S therapy may inhibit OCs differentiation and function \textit{in vivo}. Although we were unable to demonstrate inhibited bone resorption \textit{in vivo}, these data demonstrated that GYY acts in the physiological and pathological balance by increasing bone formation \textit{in vitro} and \textit{in vivo}.

Two important aspects have emerged by our \textit{in vitro} studies on the biological effects of H2S: its positive role in promoting cell viability and its osteoinductive properties. Thus, we decided to elucidate whether these aspects may account for the increase of bone formation \textit{in vivo}. Our experimental evidences demonstrated that GYY treatment: 1) inhibited apoptosis in h-MSCs; 2) increased the production of Wnt ligands in the BM; 3) further potentiates osteoblastogenesis induced by ovx as evidenced by increased number of CFU-ALP and mRNA expression of osteogenic markers and Wnt signaling in m-SCs. Canonical Wnt signaling is induced by Wnt ligands produced by bone and hematopoietic cells (Famili et al. 2015) and is known to induce OB proliferation (Kato et al. 2002), differentiation (Robert L Jilka 2007) (Bodine and Komm 2006), and promotes OB survival (Almeida et al. 2005) (Tobimatsu et al. 2006) (Bodine et al. 2005). During ovx Wnt signaling is activated in osteoblastic cells (J.-Y. Li et al. 2013), contributing to explain increased commitment and the differentiation of SCs into OBs (R L Jilka et al. 1998) during ovx. This compensatory increase in bone formation is known to be limited, among other factors, by m-OBs apoptosis (R L Jilka et al. 1998) (Di Gregorio et al. 2001). This partially explain why bone
formation does not increase as much as resorption after ovx (Kousteni et al. 2001) (Almeida et al. 2007). In this light, our data suggest that both a protective action on h-MSCs survival and induction of osteogenic differentiation of h-MSCs may account for the increased bone formation which contributes in reducing the gap with bone resorption in vivo by GYY treatments.

In summary, this work has shed new light into the mechanisms regulating bone homeostasis and the pathogenesis of osteoporosis. Beside this aspects, our data suggested a potential pharmacological relevance of H$_2$S in bone-wasting diseases and critical bone defects, as it modulated the two main processes involved in bone homeostasis: osteoclastogenesis (in vitro) and osteoblastogenesis (both in vitro and in vivo). Taken together the above findings led us to develop new therapeutic approaches with the aim of bridging the gap between basic research and clinics. Originally, H$_2$S-hybrid drugs have been developed in order to combine H$_2$S-inflammatory and anti-oxidant effects (Kodela et al. 2015) (Wallace, de Nucci, and Sulaieva 2015) to that of the original drug, mainly with the aim of preventing their side effects. However, recently, tanking to the wide range of function which H$_2$S plays in many organ targets they were developed in order to improve the effects of parent drug (Frantzias et al. 2012b) (Shukla et al. 2009) beyond anti-inflammatory and anti-oxidant effects.

With the aim of developing new pharmaceutical approaches for bone loss we took inspiration of the observation, postulated by others, that anabolic bone-inducing agents may be beneficial when prescribed with AL, one of the most currently used drugs in clinics (Patntirapong, Singhatanadgit, and Arphavasin 2014). Thus, we developed an original hybrid molecule of H$_2$S and AL, hereby named DM-22, with the aim of obtaining an H$_2$S-releasing drug displaying: a) anti-catabolic action similar to AL; b) pro-anabolic action similar to NaHS; and c) cytoprotective function due to H$_2$S moiety. First, we found DM-22 to be devoid of toxicity on h-OCs and inhibit with a less potency h-OCs differentiation compared to AL. Up to now, we did not investigate the mechanism underlying
this differential inhibition, but we could speculate that this difference may account to that DM-22 is not toxic for h-OCs. A growing attention towards the crosstalk between OBs-OCs is increased in the last years (Matsuo and Irie 2008) (Ikeda and Takeshita 2014) (Sims and Martin 2014), leading to the idea that AL inefficacy in preventing fragilities fractures might be due to the absence of bone turnover. In this light, we could speculate that DM-22 may leave a residual of h-OCs for maintaining bone turnover and preserving h-OCs to h-OBs communications.

Second, our data showed an unexpected negative aspect of AL treatment. Indeed, we found that high concentrations of AL induced h-MSCs acute toxicity at higher concentrations and inhibited h-MSCs proliferation and differentiation. To date the role of AL on h-MSCs and h-OBs is still a highly debated topic. An abundance of experimental evidence suggests that AL induced osteogenic gene expression in MSCs of different species and sources (Duque and Rivas 2007) (H. K. Kim et al. 2009) (C.-Z. Wang et al. 2010). Besides the above, it was reported that high concentrations of AL altered cell viability and inhibited osteogenic differentiation of MSCs (Patntirapong, Singhanadgit, and Arphavasin 2014). Currently, there is no explanation to account for these opposing reports; however authors suggested that this controversy may account to cell types, stages of cell differentiation and AL concentrations (Patntirapong, Singhanadgit, and Arphavasin 2014). AL-dependent inhibition of mineralization is regarded to be partly due to the drug effect on the viability (Patntirapong et. Al., 2014), however we found inhibition even in absence of toxicity. Interestingly, we found that the same concentrations of DM-22 exhibited a lower inhibition of proliferation, did not induce h-MSCs cytotoxicity and increased h-MSCs differentiation even at the lowest concentration compared to the parental drug. It is tempting to propose that the H2S moiety of DM-22 could have prevented AL-dependent acute toxicity and induced osteogenic differentiation of h-MSCs.

Interestingly, the ability of DM-22 to inhibit h-OCs differentiation and function and inducing h-MSCs differentiation while not impairing h-OCs and h-MSCs viability, resembles NaHS-mediated modulation of osteoclastogenesis and osteoblastogenesis. In summary, our in vitro data revealed
that the incorporation of H$_2$S into AL molecule might provide effective treatments for osteoporosis.

Besides the development of new drugs targeted for bone loss pathologies, the biological insights obtained from this study might be translated in the field of bone tissue engineering. To this end, the anabolic action and the anti-catabolic action of H$_2$S could be used in orthopaedic application to increase osteoinductivity of scaffolds currently employed in bone regenerative medicine. Preliminary experiments demonstrated that SF_GYY, is permissive for cells colonization and supported the osteogenic differentiation in a dynamic system of culture in perfusion in bioreactor. Further experiments for improving GYY adhesion and its release are in progress.
7. CONCLUSIONS

Our findings firstly highlighted a crucial role of H$_2$S role in bone tissue. In particular, we evidenced that: 1) CBS and CSE are expressed in human bone tissues and human bone derived cells; 2) CBS and CSE expression is a distinctive feature of h-MSCs transition toward mature h-OBs; 3) loss-of-function of CBS and CSE resulted in impaired osteogenic differentiation in vitro; 4) CBS, CSE and H$_2$S levels played a critical role in the maintenance of bone homeostasis in vivo; in particular we found that H$_2$S replacement therapy in ovx mice, where CBS, CSE and H$_2$S levels are blunted, prevented ovx-induced bone loss.

Secondly, our data validated the use of H$_2$S-donors as novel potential candidates for the treatment of bone pathologies since: 1) exogenous administration of H$_2$S modulated bone remodeling both in vitro as well as in vivo; 2) H$_2$S replacement therapy reversed ovx-induced bone loss in vivo.

Based on these evidences, we developed a) an H$_2$S-hybrid with AL to improve the therapy of bone loss; b) an H$_2$S-releasing SF scaffold to improve bone regenerative medicine. We demonstrated in vitro that DM-22 has improved biological properties compared to the parent drug in terms of h-OCs and h-MSCs cellular viability, h-MSCs cellular proliferation and h-MSCs osteogenic differentiation and may preserve a residual h-OCs function fundamental for h-OCs to h-OBs communications. Finally, we provided preliminary evidence that SF_GYY, the H$_2$S-releasing SF scaffold, is permissive for cells colonization and supported the osteogenic differentiation.
8. FUTURE DIRECTIONS

Our data opened new lines of investigation in the field...:

... of OBs-OCs communications. One intriguing aspect of H₂S regulation that will be very interestingly to investigate is whether OCs and OBs may communicate and direct the transition between the different phases of bone remodeling through H₂S or H₂S downstream targets.

... of gaining knowledge on post menopausal osteoporosis. It will be of interest to evaluate CBS-CSE-H₂S levels in estrogen replacement therapy.

... of the search of valuable markers able to predict bone formation ability. We should direct our efforts into first verify whether the population identified by increased CBS and CTH expression have improved bone formation ability in vivo. Further investigations of downstream target of H₂S activation during osteogenesis may hopefully reveal surfaces markers able to make a perspective isolation of cells with improved capacity of bone formation in vivo.

... of the development of therapies for bone loss. First we need to test DM-22 compared to AL and GYY in the mice model of postmenopausal osteoporosis. Besides μCT, histomorphometric and serum marker measurements will be of interest assaying the potential prevention on gastric cytotoxicity. Indeed the cytotoxic induction on gastric cells is one of reason of the side effects due to AL therapy (Segal, Tamir, and Ish-Shalom 2003) (Strampel, Emkey, and Civitelli 2007) which lowered the adherence to therapy. It is conceivable that is H₂S is devoid of toxicity even in gastric cells, DM-22 is likely to be more tolerated than AL and we can speculate that DM-22 could ameliorate the compliance of AL therapy. Third as the primary aim of pharmacological therapy is to reduce the risk of osteoporotic fractures, one of the main questions to address in the main future is whether H₂S therapy may be beneficial into reducing the risk of fractures. Furthermore we may test whether regimen of GYY higher to that used in the current study may achieve a net bone anabolic effect and may reduce the number of osteoclasts in ovx mice.
... in the diagnosis / monitoring of therapies. An important field of investigation should be in verifying whether CBS, CSE and H₂S levels may be used as diagnostic, prognostic factors for identifying individuals prone to osteoporosis and fracture risk and for monitoring efficacy of therapies. Further investigations are needed to determine whether CBS, CSE and H₂S levels down-regulation may be involved other pathologies characterized by bone loss.

... of the development of scaffolds for bone regenerative medicine. Of great importance is to clearly define the amount and the time course of H₂S release from the scaffolds. Moreover we are planning to make a better tuning, controllable and distributed in time, of H₂S release from scaffolds. A strategy we will investigate will be creating a system that can protect donors from water. Next step will be directed in test the osteoinductivity of those scaffolds in vitro and in vivo.
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10. OTHER INFORMATION

LIST OF PEER REVIEWED PUBLICATIONS DERIVED FROM THE PHD PROJECT


LIST OF OTHER PEER REVIEWED PUBLICATIONS


LIST OF Oral Presentation


LIST OF Poster Presentation


LIST OF OTHER ABSTRACTS


5. Paolella F, Gabusi E, Manferdini C, **Gambari L**, Schiavinato A, Grigolo B, Lisignoli G; Effect of growth hormone and hyaluronan amide derivative on human osteoarthritic chondrocytes; OARS1 world conference, Seattle, 30/04-03/05/2015.


PATENT

"Numero di deposito ITALIA MI2014A001919 del 7 novembre 2014”

AWARD

“New Investigator Award” at the “International Congress on Children’s Bone Health” (ICCBH, 10).
This study was supported by grants from FORST, the National Institutes of Health (DK91780, DK007298 and RR028009) and by grant 'Ricerca Finalizzata' from the Italian Ministry of Health (RF PE-2011-02348395).

This study would not be achieved without the important collaborations with Prof. Pacifici at Emory University, Department of Medicine (Atlanta, USA); Prof. Calderone and Dr. Rapposelli at University of Pisa; Prof. Motta at University of Trento.

Dr Gambari was supported by a fellowship granted by Spinner 2013 and co-founded by University of Bologna. Dr Gambari and Dr Grassi designed this Study. Dr Gambari carried out the experiments in vitro and performed some ex vivo experiments on animals. Dr Grassi and Dr Tyagi carried out the experiments in vivo. Prof. Calderone and Dr Rapposelli developed the DM-22 molecule. Prof. Motta and Dr. Raggio developed and performed the structural and chemical analyses on SF scaffolds. Data collection, Data Analysis was done by Dr Gambari, who interpreted the data. The doctoral thesis was drafted by Dr Gambari and revised by Dr Grassi, Dr Lisignoli and Prof. Mariani. A special acknowledgment goes to Dr Desando and Prof. Gambari who read my Doctoral thesis with great care and love.

We are researchers; we study and work for the most of our time with English language, and this is the main reason why I wrote this Doctoral Thesis in English. But this is the time for me to acknowledge, and as my language of hearth is Italian, this is the reason why I want to continue ... questi ringraziamenti nella mia amata lingua. Con questi tre anni di Dottorato, preceduti dai due anni di borsa di studio, si chiude un ciclo. Il mio primo scherzoso ringraziamento deve essere, quindi, rivolto a te, mio piccolo e puzzolente “solfone”, il mio amato Solfuro di Idrogeno; eh si, perché senza di te, io non sarei arrivata qua. Com’è stato detto da altri siamo stati molto fortunati nell’imbatterci in te! Da quell’ormai lontano novembre 2010, la mia vita è cambiata, non posso più esimermi dal bere quell’acqua dal sapore di uovo marcio quando ne trovo una fonte o ricercare qualche sorgente termale dove farmi un bel bagnetto. Perché ho voluto dedicare la mia vita alla ricerca e, almeno per ora, al centro della mia ricerca ci sei tu!

Facciamoci ora più seri… E’ evidente che io sia giunta alla fine di un percorso molto importante che ha segnato molti cambiamenti nella mia vita lavorativa. Il Dottorato a detta di molti dovrebbe essere quel periodo di tre anni in cui il Dottorando si mette alla prova su un progetto, matura e cerca di diventare a tutti gli effetti quello che ha sempre desiderato di essere, o almeno così vale per me, un ricercatore. Questi sono stati anni per me di dedizione, di duro lavoro… Non mi sono mai risparmiata per perseguire quest’obiettivo. Non posso certo dire di essere del tutto soddisfatta per il lavoro svolto… Sono una perfezionista. Spero che un giorno i fatti parlino per l’impegno e la passione profusi.

I miei primi fortemente sentiti ringraziamenti vanno a tutto il gruppo di ricerca in cui sono stata accolta. Penso proprio di essere capitata al posto giusto al momento giusto. Per questo devo ringraziare anche Roberta Piva che in diversi momenti della mia carriera mi ha indirizzato verso il gruppo della Dott.ssa Lisignoli. Ti dovrei chiamare Prof. Piva ma per me sarai sempre la Roberta
che ha accompagnato alcuni dei miei più bei ricordi d’infanzia… Quando Papi mi portava in Lab. e tu mi accoglievi a braccia aperte, ricordo ancora quando mi hai portato in bicicletta sul fiume… Con ancora più affetto ricordo la tua presenza in montagna quando ancora eravamo tutti felici!!

Grazie anche per avermi accolto per lo stage nel tuo gruppo di ricerca nel quale ho incontrato persone favolose e grazie alle quali ho imparato molte cose: Elena, Elisabetta, Letizia, Andrea…

Grazie per tanti bei momenti che porto sempre nel cuore. Sarà perché da voi mi sento a casa, ma ho passato un periodo veramente felice e sereno da voi!

Torniamo ora al nostro Lab. In primo luogo voglio ringraziare Francesco Grassi, PI di tutti i progetti che abbiamo seguito insieme. Grazie per aver visto qualcosa in me, insieme a Gina, in quel nel lontano 2010. Grazie per avermi dimostrato nel tempo la tua stima. Grazie soprattutto per avermi aperto le porte per un Dottorato nel nostro laboratorio. Grazie per avermi dato il tuo supporto quando il primo tentativo di ottenere il Dottorato è sfumato nel nulla. Grazie per aver insistito ed aver speso il tuo tempo per scrivere il progetto SMARTBONE grazie al quale mi si sono aperte le porte del Dottorato. Grazie per aver rinunciato a gran parte del tuo compleanno per riuscire a metterti in contatto con il Prof. Facchini, il Prof. Cocco e il Prof. Vitale, perché tutta la documentazione fosse valida. Grazie perché so che lavorare con una testona come me non è sempre semplice. Grazie per avermi dato nel tempo la possibilità di essere indipendente nell’esecuzione degli esperimenti. Grazie per avermi dato la possibilità di far crescere la nostra ricerca, alimentata dal fuoco che mi anima. Grazie per avermi permesso di presentare poster e presentazioni orali a congressi. Insomma come avrai capito, grazie.

Un pensiero triste va a chi non può essere qui oggi. Grazie Prof. Andrea Facchini, per avermi accolto nel laboratorio, per aver appoggiato Francesco nel farmi ottenere il Dottorato. Grazie per i consigli che mi hai dato sino a quando ha potuto. Grazie per le belle parole che mi ha dimostrato quando i piani non sono andati come entrambi ci aspettavamo. Lei rimane per me il mio vero tutor. Ogni volta che passo davanti allo studio il mio pensiero va sempre al suo ricordo. La sua guida sempre presente e silenziosa non ci abbandonerà mai.

Grazie infinite anche a Gina Lisignoli per avermi accolto sin dal lontano 2010. Grazie per i consigli che mi ha dato in questi anni. Grazie per la serietà e la diligenza con cui ha dato il suo contributo per la realizzazione di questo Dottorato. Grazie per il periodo in cui mi ha coordinato più da vicino. Per me è sempre molto stimolante avere un confronto scientifico con Lei. Le sono veramente molto grata.

Grazie alla Prof. Mariani che ha egregiamente sostituito il Prof. Facchini come mia co-tutor. Grazie infinite per la disponibilità e la serietà con cui ha preso questo impegno. I suoi consigli sono sempre stati per me molto graditi e molto utili. Grazie anche per il suo sostegno ed il suo incoraggiamento di quest’ultimo periodo.

Ringrazio infinitamente anche il Coordinatore di Dottorato Lucio Ildebrando Cocco per avermi accolto come tutor. La ringrazio per avermi così concesso di finire il Dottorato. La ringrazio inoltre per aver organizzato sessioni di seminari anche con premi nobel in cui ho potuto respirare l’aria di scienza. E’ stato bello sentir dire a premi Nobel di perseverare e “DO WHAT OTHER PEOPLE THINK IS IMPOSSIBLE”. Perché tutti quelli che hanno deciso di intraprendere questo lavoro sanno che ogni giorno è un’incognita, percorriamo un sentiero che potrebbe essere quello giusto o
no, che potremmo seguire per poco o lungo tempo; che è molto facile sbagliare la strada, che la vetta viene raggiunta dopo innumerevoli sforzi e fatiche… Ma che soddisfazione quando la si raggiunge!!; quindi ora elencherò la top-ten (non in ordine di rilevanza) dei dieci momenti lavorativi più belli di questi anni di Dottorato: 1) Emozione di presentare i propri dati all’ECTS doctoral program con tutti i complimenti che ne sono venuti. 2) Il progetto SMARTBONE arriva primo tra i progetti proposti!! Questo per me vuol dire una sola parola: finalmente Dottorato!!!! 3) Il lavoro di OCs viene accettato!! Dopo tanta tanta tanta fatica… 4) Vinciamo la Ricerca Finalizzata!! Il nostro lavoro dà i suoi frutti!! 5) Ho vinto la (mia prima e ultima?) short oral presentation e il New investigator Award!! Impensabile!!! 6) La mia presentazione di laboratorio su CBS e la mia presentazione al kick off meeting hanno riscosso successo! 7) L’articolo su JBMR viene accettato!! Evvai! 8) Il feedback positivo è vero!! Non è solo un sogno! 9) DM-22 induce mineralizzazione, il brevetto si può fare!! 10) Uhm… poiché non mi vengono in mente altri momenti storici dirò che il più bello deve ancora venire…

Tra le mie care amiche del Lab ringrazio prima fra tutte Francesca… Ora sarà un po’ banale e ripeterò i tuoi di ringraziamenti… Se c’è una cosa per cui sono davvero infinitamente grata al Rizzoli è perché ci ha permesso di diventare in questi anni grandi amiche. Senza di te non ce l’avrei fatta e non ce la farei. Sei la mia ancora di salvataggio che mi ricorda quando vengo dirottata nella direzione sbagliata. Grazie infinite per il tuo elevato grado di sopportazione. Grazie per le risa, gli abbracci. Grazie perché cerchi sempre di proteggermi…

Ringrazio infinitamente anche Giovanna, grazie per tutte le serate in cui torniamo a casa insieme, grazie per tutti gli sfoghi, per me è un momento ormai irrinunciabile e catartico. Grazie per le graniti al baretto nei miei momenti di più grande disperazione. Grazie per i pomeriggi passati all’acquagym… Grazie infinitamente anche per quest’ultimo periodo di follia, mi hai aiutata moltissimo. Grazie insomma per esserci sempre…

Grazie infinite anche a Elena e Cri, che insieme a Francesca, sono le mie GL girls. Ragazze non potevo sperare di capitare in un gruppo migliore. Ognuna di voi è veramente speciale. Anche voi devo ringraziare per la sopportazione, sono una grande testona! So di essere insopportabile alle volte! Grazie per le risate che ogni giorno mi regalate! Perché il lavoro al Rizzoli sarebbe un po’ più grigio senza tutte voi.

Grazie Manu… mi sento tanto tanto vicina a te, per tanti motivi che sappiamo bene solo io e te. sono davvero felice che tu sia tornata perché mi sei mancata troppo… grazie mille per il tuo appoggio, per il tuo incoraggiamento, per la tua amicizia, irrinunciabile…

Grazie a Mariassunta… Grazie a Ylenia… Grazie soprattutto a Marli… Insegnare è sempre un motivo di crescita e spero con tutto il cuore di esserti stata utile. Cara Sung, ti rimpiango molto, da quando non ci sei il lab. è molto più triste, mi mancano tantissimo i tuoi sorrisi, la tua gioia di vita!! Marli, spero che il tempo consolidi questa amicizia che sta nascendo, penso che tu sia proprio una persona speciale e penso che non potesse capitarmi di meglio come ragazza da seguire…

Grazie a tutto il laboratorio… Tutti voi in diversi momenti mi avete regalato un sorriso, una frase che mi è stata di gran supporto e aiuto! Grazie anche a chi mi ha messo i bastoni tra le ruote… A chi
non mi apprezza… Perché alla fine si è chiusa una porta ma si è aperto un portone! E poi non si può piacere a tutti e va bene così…

Un grazie in particolare a Isa, Vale, Carol, Mauro… Grazie per rendere speciale e gradevole quella mezzora-oretta che dedichiamo al pranzo!! Grazie infinite anche per il vostro supporto ed incoraggiamento di quest’ultimo periodo!

Ora non voletemene ma arrivo ai ringraziamenti più speciali… quelli alla famiglia…

Negli ultimi anni, pensando alla professione di ricercatore e avvicinandomi al mondo dei congressi, mi è frullata sempre più per la testa la parola “mentore”. Si dice che ognuno di noi abbia avuto almeno un mentore nella propria vita. Credo che non me ne possa volere nessuno per questo, ma il mio vero e unico mentore è il MIO PAPA’. Perché per me il mentore è qualcuno da cui hai appreso tanto e di cui vorresti ripercorrere le orme. Definire il mio amore per te è molto difficile, impossibile da rendere a parole. Io a te devo tutto. Lo sai… Ho sempre avuto una venerazione per te… e non importa quanto mi provochi scherzosamente o quanto tu possa commettere (eventualmente) errori tu per me sei e sarai sempre perfetto!! Ti voglio un bene dell’anima… Grazie anche perché ci sei sempre quando ho bisogno di un consiglio o aiuto anche sul piano lavorativo.

Grazie Mami, per anni so che ti sei sentita come con la medaglia d’argento, e di questo mi dispiace moltissimo; ma credimi, amo infinitamente lui quanto te. Siete i miei modelli di vita. A te devo tanti lati speciali del mio carattere… la dolcezza, la testardaggine, la precisione, la cura verso gli altri… e tanto altro…

Vi amo infinitamente, non avrei potuto volere genitori migliori. Siete la mia forza… Siete la mia certezza… Siete la mia guida… Siete il mio equilibrio… Siete tutto per me… Siete e sarete sempre la mia ragione di vita…

Grazie ai miei ANGELI. Grazie al mio piccolo Tommy, al mio fratellino a cui dedico ogni respiro. Quando ti abbiamo perso il mio cuore si è spezzato e non tornerà mai del tutto a battere. Ho perso la luce che rischiara le mie giornate. Mi manchi da morire ogni giorno… Grazie ai miei nonni (Mauro e Marisa; Renato e Liliana) che hanno cresciuto con amore i miei genitori e che hanno condiviso con me momenti felicissimi che porto ogni giorno nel cuore…

Grazie anche a Nonna Laura che non ho potuto conoscere personalmente ma che ho sentito molto vicina…

Grazie a Maria Luisa che ci hai lasciato da così poco, lasciando un nuovo vuoto nel nostro cuore… Grazie a Freezer, l’amore della mia vita, l’altro mio fratello che non è più qui. Sei unico e indimenticato… Grazie a Fred che ho amato infinitamente e che mi ha salvato la vita… Grazie a Cippo, Cippa, Pappi, Gallà, Artù, Merlin, Maya, William e Ginervra. Vi ho voluto tanto bene!!! Vi voglio tanto bene!!!

Grazie all’AMORE MIO, il MIO FRANCESCO… non mi stancherò mai di dirti che sei la luce che illumina il mio buio, sei il sole che riscalda il mio inverno… Ti amo immensamente… Grazie per essere “caduto dal cielo” ed avermi trovata. Hai cambiato la mia vita regalandomi “nuovamente” la felicità, la speranza… Senza di te ormai sarei persa… Grazie per la tua infinita pazienza in generale
e soprattutto per la cura e l’amore con cui mi hai supportata in questo ultimo periodo. Per avermi servita e riverita mentre studiavo ininterrottamente… Grazie perché in questa lunga notte prima della consegna stai dormendo a fianco a me con la luce accesa per farmi studiare… Ti amo…

Grazie infinite alla mia famiglia acquisita. Sono stata estremamente fortunata ad incontrarvi. Grazie ad Anna, Andrea, Nonna Gina, Lorenzo, Elena, Giuliano, Patrizia, Martina, Zia Giovanna, Lula, Susi e Mosè. Grazie per avermi accolto da sempre con tanto affetto. Grazie per rendere ogni momento passato insieme sereno e speciale. Grazie anche per aver reso Francesco la persona speciale che è, ve ne sarò sempre infinitamente grata. Si dice che dietro ogni grande uomo c’è sempre una grande donna, dietro questo grande uomo c’è una grande famiglia! Grazie infinite… Vi voglio bene!

Grazie zietto (Davide), grazie per le risate, grazie per la dolcezza con cui mi abbracci e mi coccoli, come se fossi ancora (ed ancora di fatto lo sono) quella piccola bambina spaurita che da piccola sollevavi fino quasi a raggiungere il soffitto! Momenti irrinunciabili che mi riempiono il cuore. Grazie per esserci SEMPRE stato. Grazie per quello che hai fatto per me, per la nonna e che ora fai per la zia Marta. Te lo dico a maggior ragione perché pensi di non esserlo: sei veramente una persona d’oro! Ti voglio bene!

Grazie zietto (Cesare), grazie per essere stato sempre un mio grande sostenitore. Grazie infinite per avermi incoraggiato sempre ad essere più sicura di me stessa. Grazie per le chiacchierate. Grazie per avermi fatta sempre sentire al centro del tuo mondo. Da quando ti sei trasferito qui da noi, ho un motivo di felicità in più! Ti voglio bene!

Grazie zietti (Stefano e Daniela), grazie per essere la mia seconda famiglia. Grazie per aver reso sempre indimenticabili i momenti (sempre troppo pochi) passati insieme. Quando tornate a casa dalle vacanze estive si crea sempre un vuoto… La casa è vuota e più triste senza di voi. Grazie per esserci sempre stati… Per avermi amata come una figlia… Vi voglio bene!

Grazie zietto (Paolo), grazie per essere entrato in punta di piedi, insieme alla zia, nella mia vita. Purtroppo la lontananza non ci ha permesso di condividere molti momenti. Tutto è cambiato, però, da quella lettera che mi mandasti per la laurea… per me è stata come un dono dal cielo… in te vedo i miei nonni che non posso più vedere. Nei tuoi racconti vedo gemme preziose di un passato che altrimenti non potrei conoscere. Grazie per avermi dato la tua stima… Grazie per i regali che mi avete fatto sempre perfetti, così ricercati e speciali… Primo fra tutti perché condiviso con Tommy, l’orso gigante di peluches…

Grazie Zio Massimo (Roma) perché ti ho conosciuto un po’ tardi ma mi sei entrato subito nel cuore…

Grazie zietto (Luca), grazie per i pomeriggi in cui vieni a trovare questi “orsi” di familiari che vi ritrovate. Grazie di cuore, perché sono sempre momenti speciali. Grazie perché fin da piccola ho sempre sentito un legame speciale con te poiché mio Padrino. Grazie zii (Annarita, Massimo, Monica) per l’affetto che mi dimostri ogni volta che ci vediamo.

Grazie “sorella” mia (Ilaria). Il nostro legame è indistruttibile e indissolubile… sei stata e sarai sempre fondamentale per me… il mio bastone della vecchiaia!

Grazie cugini… A Giovanni, Carlotta a Caterina va il mio sincero ringraziamento per un dolce passato in cui siamo stati molto vicini. A Maddalena, Marina, Michele grazie per i momenti felici condivisi. A Marco va il mio sincero ringraziamento perché seppur nella distanza ho sentito una
particolare vicinanza con te, soprattutto dopo la perdita della Nonna. Ai miei cugini acquisiti Elena, Luca, Luigi ed alla nuova linfa vitale della famiglia, Cristian, Manuel, Tommaso, Massimo, Sofia, Daniele, Giovanni un grande bacio.

Grazie alle mie più care amiche: Ale, Eli, Ila. Ognuna di voi è a suo modo fondamentale per me. Forse anche voi mi siete state un po’ mandate dal cielo perché sono certa che ognuna di voi abbia avuto un ruolo insostituibile nella mia vita. Grazie perché sono cresciuta e continuerò a crescere con voi… Purtroppo non riusciamo a vederci spesso ma vi ho sempre accanto a me. Grazie ai miei nipotini: Gioele, Daniele ed al piccolo Leonardo che sta ancora nella pancia della mamma… Grazie per la gioia che mi avete dato e che mi darete! Un ringraziamento speciale va alle vostre famiglie che mi hanno sempre accolta a braccia aperte. Un particolare ringraziamento va a Katia, Marco, Nicola e Christian che mi hanno sempre fatto sentire a casa, vi porto sempre nel cuore. Grazie di cuore anche a Vanes ed Albi per il vostro affetto.

Grazie ai miei amici Andrea, Matte, Ando, Velli, Monica, Daniele. Grazie per i bei momenti passati insieme e per il vostro affetto.

Grazie a Marco, Paolo, Nata, Enrico, Maurizio, Elena, Valentina, Simona, Fabio, Riccardo, per i momenti passati insieme.

Grazie ai vecchi amici che porto nel cuore… Tommy, Manu, Ele, Anto, Costy, Lucia, Fede, Erica, Alice, Totta, Silvia, Margherita… Grazie a Rosi, Francesco, Lorena… e sicuramente mi starò scordando qualcuno…Con voi ho passato momenti veramente speciali ed indimenticabili…

Mi sarò sicuramente dimenticata qualcuno o avrei potuto esprimermi meglio… Quindi scusatemi per questo…

Ed infine un ringraziamento a me… testona, permalosa, integerrima, dolce, sensibile, protettiva… perché nonostante mi critico tanto… penso di meritarmi un po’ di felicità…