DOCTORAT BIOLOGIE BRETAGNE SANTE LOIRE

UNIVERSITÉ DE NANTES

THESE DE DOCTORAT DE

L'UNIVERSITE DE NANTES

ECOLE DOCTORALE N° 605
Biologie Santé
Spécialité : Biologie cellulaire et moléculaire

Par

Giulia FRANGI

Study of the role of PiT2 at the interface between bone and adipose tissues

Thèse présentée et soutenue à Nantes, le 15/12/2020
Unité de recherche : INSERM, UMR1229, RMeS Regenerative Medicine and Skeleton

Rapporteurs avant soutenance :

Marie-Hélène Lafage-Proust	Professeur des Universités/Praticien Hospitalier - Université Jean Monnet, INSERM U1059 and University Hospital, Saint-Etienne, France.
Bram Van Der Eerden	Assistant Professor - Erasmus MC - Rotterdam, Netherlands.

Composition du Jury :

Président du jury :	Christophe Chauveau	Professeur des Universités - Université du Littoral Côte d'Opale (ULCO) - MABLab UR 4490 - Boulogne sur Mer, France.
Examinateurs :	Marie-Hélène Lafage-Proust	Professeur des Universités/Praticien Hospitalier - Université Jean Monnet, INSERM U1059 and University Hospital, Saint-Etienne, France.
	Bram Van Der Eerden	Assistant Professor - Erasmus MC - Rotterdam, Netherlands.
	David Magne	Professeur des Universités - ICBMS UMR 5246 CNRS, Lyon, France.
	Lene Pedersen	Associate professor - Aarhus University - Department of Molecular Biology and Genetics, Aarhus, Denmark.
Dir. de thèse :	Sarah Beck-Cormier	Chargée de recherche - Université de Nantes, Laboratoire RMeS INSERM UMRS 1229, Nantes, France.
Co-dir. de thèse :	Laurent Beck	Directeur de Recherche - Université de Nantes, Laboratoire RMeS INSERM UMRS 1229, Nantes, France.
Co-encadrant :	Xavier Prieur	Maître de conférences - Université de Nantes, Institut du Thorax, UMR-S 1087, Nantes, France.

A me stessa, per aver avuto il coraggio di partire
e la determinazione di arrivare fino alla fine.

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Jérôme Guicheux and Dr. Catherine Le Visage for having welcomed me three years ago at the laboratory RMeS INSERM UMRS 1229.

I would particularly like to thank Dr. Sarah Beck-Cormier, my thesis supervisor. Thank you for having entrusted me with this project in the very beginning, for being there every step of the way and, especially, for all the passion and love for research you demonstrate every day. You helped me grow in the person I am today and I am sincerely grateful for your guidance and your encouragement all along this three years.

I would also like to particularly thank my co-supervisors Dr. Laurent Beck and Dr. Xavier Prieur. Thank you both for your brilliant advice and for always finding the time to be there. It has been an honour to have you involved in my thesis work.

I would like to express my most sincere thanks to Pr. Marie-Hélène Lafage-Proust and Dr Bram van der Eerden for doing me the honour of accepting to judge this work as “rapporteurs”, and Dr. Lene Pedersen, Pr. Christophe Chauveau and Pr. David Magne for agreeing to judge this work as “examinateurs”.

I wish to express my gratitude to Dr. Celine Colnot and Pr. Christophe Chauveau for having accepted to be part of my “comité de suivi individuel” and for the helpful and enriching discussions we shared.

I would like to thank Dr. Greet Kerckhofs for having welcomed me in her laboratory at the University of Leuven for our collaboration and for having given me the possibility to learn everything I needed to know regarding microCT analyses. Your work is simply amazing.

I wish to thank all the staff members at the UTE-IRS-UN Animal Facility, in particular Julien, Marjolene and Malek. It has been a pleasure working with you. Mostly, my deepest gratitude goes to you my little mice, without all of you none of this work would have been possible.

A very special thanks goes to Philippe and Magalie, from the MicroPiCell imaging facility, and Nicolas and Nadège from the Cytocell facility, for your precious help and great expertise.

I would also like to thank all the members of the RMeS family, nothing would have been the same without each and every one of you. Thank you all for the unforgettable moments we have shared inside and outside of the lab.

In particular I would like to thank the family that I never imagined I would be lucky enough to find here in Nantes. What a crazy journey a PhD is and it gets a lot easier if you are surrounded by the right people.

Thank you, Anaïs, for being the wonderful person that you are. Your positivity and good humour have helped me through some difficult times. You light up a room the moment you enter it and you did the same with me. I am so grateful for our friendship. It is not easy to find someone you feel free to be yourself with and this is how I feel when I am around you. You make me feel at home, even though I am far away from home. I would also like to thank your Pierre, I can't imagine how my bathroom would have looked like without you! ;) You guys are amazing and I wish you all the best in this world, because you deserve it and even more.

Thank you, Solène, for your support, your kindness, your patience. You have been a rock to me this past year and I will never thank you enough for that. You are a great worker and a great mum, your family is wonderful and I am so lucky I got the opportunity to spend some time with all of you (otherwise who would have brought me to the airport at 3 in the morning to let me go home for the holidays? :P).

Thank you, Maude, from the bottom of my heart. You are the first person I bonded with when I arrived at the lab that June 2017 and we've been friends ever since. I knew you were my soulmate since the first time you told me you loved Doctor Who as much as I do (I'll give you Ten, the Twelfth is my doctor :P). Never forget how special you are, you can conquer the world if you want to.

My dear Leslie, where do I begin? You are an amazing human being, so bright and sunny, you warm up the heart of those around you. Thank you for your support and your friendship, they are invaluable to me. Thank you for that day, in the histology room, when you came to see me because you knew I was not okay and you told me that I was not alone. You really changed everything for me that day. Thank you for your everlasting support and understanding and for being there when I needed you the most.

I particularly wish to thank you, Pierre. Even though things went differently than we imagined, it has been a privilege for me to share my first two years of PhD with you. You supported me, listened to me, motivated and believed in me. You made me a better person. I will always be grateful for the time we spent together and for all the things we have done. I know you will achieve great things, both professionally and in your personal life, you have it in you. Just never give up on your dreams.

Thank you Nina, I barely knew you and you were already helping me for anything I needed as soon as I arrived. You are strong, confident and so brilliant. You inspired me every day. Whatever the future holds for you, I know you will make it your own and get the best out of it. I wish you and Julien all the most beautiful experiences in the world (a world that is a lot greener thanks to you ;)).

Thank you, Alexandra, for that night at the art museum. I still don't know how you knew, but it was exactly what I needed.

Thank you, Mélina, for your kindness and motivation, for speaking English with me when needed and for our adventures at the fitness club. I am already missing our body pump sessions!

Thank you, Benoit, for having shared with me the ups and downs of the last months of our PhD (even though you abandoned me near the end :P). I regret our friendship started so late, nonetheless I am very happy for it. Enjoy every moment of these last steps, it will be over sooner than you think.

Thank you Pierre G, for your support and understanding. You never judged and always listened, sorry I cried sometimes, you know I get emotional. I am glad we could always speak frankly to each other. I wish you the best in your new adventure.

A special thanks goes to Joëlle, Boris, Sophie and Julie, for all your advice and your incredible help. Especially, thank you Joëlle for the hours we spent together during the analyses of my scans and for your precious expertise, I could not have had better company.

And now, to my family and friends from home, I cannot properly thank you in a language we do not share. So, here it goes, in italian:

Questi tre anni non sono stati semplici, tra alti e bassi ci sono stati svariati momenti in cui avrei voluto essere a mezz'ora di macchina da casa invece che ad un'ora e mezza di aereo e poco più di mille chilometri di distanza. Certo, non posso lamentarmi, c'è chi abita talmente lontano dalla propria famiglia da non poterla vedere quasi mai e, diciamocelo, neanche due ore di aereo sono niente. Eppure la distanza si sente, quando non puoi rientrare per un compleanno o il matrimonio dei tuoi più cari amici o quando passano più di sei mesi tra un abbraccio e l'altro. Per non parlare di quando il mondo è colpito da una pandemia virale e gli abbracci, che tanto ti mancano, sono pure caldamente sconsigliati. Bello questo 2020!

Eppure è in questi momenti che le cose più semplici diventano le più importanti. Tua madre che ti invia le mascherine per posta, non sia mai che resti senza! Insieme al pacco c'è una busta e dentro la busta c'è un biglietto, piegato meticolosamente, con dei fiori di campagna seccati ed incollati con cura. Il biglietto dice: "questi fiorellini di campo li ho colti per te mentre andavo in paese, ho detto loro di starti vicino e farti tante coccole. Un abbraccio forte." e non puoi far altro che chiederti perché hai deciso di andartene così lontano quando il tuo cuore è sempre rimasto a casa. Grazie mamma, per tutto il tuo amore e il tuo supporto. Sei il mio punto fermo, la mia casa.

Insieme alle mascherine ci sono anche dei peperoncini del nostro orto, perché non possono mai mancare. Anche loro sanno di casa. Questi sono da parte di mio padre. Non abbiamo sempre avuto un grande rapporto noi due, vero papà? Questo perché ci somigliamo tanto e i nostri caratteri cozzano l'uno contro l'altro. Crescendo non è stato facile trovare un modo per venirci incontro senza allontanarci di conseguenza con uno scoppio. Credo, malgrado tutto, che ci siamo riusciti. Papà grazie a te ho trovato il coraggio di credere in quello che desidero e, sebbene questo dottorato mi abbia fatto capire cosa non voglio fare al posto di confermare le mie ambizioni, lo ritengo un grande passo per me verso la realizzazione di me stessa e del raggiungimento del mio fine ultimo, ovvero essere felice. Grazie papà per essere l'uomo che sei e avermi fatto volare, fin da bambina, tenendomi tra le tue braccia.

A mia nonna Noemi va un pensiero speciale. La tua vita non è stata semplice ma non ti sei mai arresa. Uno spirito forte e deciso, so che stai pregando per me anche da lassù o ovunque tu sia ora. Sei e sarai sempre nel mio cuore. Mi manchi ogni giorno.

All'altra mia famiglia, quella che negli anni ho potuto scegliere e che ha scelto me, un grazie di cuore. Siamo sparpagliati a ogni angolo del mondo ma non importa quanto tempo passa, quando ci rivediamo è come se ci fossimo lasciati solo qualche giorno prima.

Un grazie immenso a te, Agnese, per avermi accolta nella tua famiglia ben 19 anni fa. Il catechismo ha fatto miracoli, ci ha fatte incontrare! Grazie per avermi sostenuta, grazie per tutto quello che abbiamo condiviso nel bene e nel male e grazie per non avermi mai lasciata sola, anche quando la distanza o le circostanze avrebbero potuto facilitarlo. Una volta mia mamma mi ha detto di essere grata che tu fossi mia

amica, perché sapeva che ci saresti sempre stata per me e questo la rincuorava. Non poteva avere più ragione! Spero di fare altrettanto per te e di essere tua amica, sempre.

Grazie a Giorgio, Davide, Sofia, Pez, Camilla, Susanna, Luca e Alessandro. Alcuni tra i miei più bei ricordi li condivido con voi. Mi mancate.

Grazie a Pez, tra i nostri mille impegni sei stato una costante della mia vita negli ultimi anni e sono enormemente grata della nostra amicizia. Sai sempre cosa dire e mi ascolti con una pazienza che in pochi avrebbero. Sei veramente una persona speciale e mi manchi moltissimo.

Un grazie immenso a Igor, la mia anima gemella. Sai che ti amo profondamente e che sei la mia altra metà. Sono così orgogliosa di te e di tutto quello che hai realizzato in questi anni. Sei una persona straordinaria e sono veramente felice di averti nella mia vita.

Grazie di cuore anche a Vittorio. Igor non avrebbe potuto trovare persona migliore con cui condividere la sua vita e non credo tu possa immaginare quanto sono contenta di averti come amico. Sei una persona meravigliosa, non arrenderti mai! Credo fortemente che tutto ciò che sogni e desideri si avvererà, perché è già dentro di te e non aspetta altro che di esprimersi.

Grazie Loris, per la costante che è stata la nostra amicizia in questi anni. Grazie per i tuoi consigli musicali, hai la capacità di condividere la canzone giusta al momento giusto e scaldarmi il cuore ogni volta. Sei brillante, una persona davvero speciale. Spero di condividere ancora tante cose insieme a te.

Grazie Francesca, per le nostre chiacchierate in macchina davanti casa, per i momenti condivisi e il tuo immenso supporto. Non ringrazierò mai abbastanza l'Islanda per averci fatte incontrare di nuovo.

Grazie a Lara, la mia compagna di avventure parigine. Dal master a oggi ti considero una tra le mie amicizie che stimo di più. Ammiro la tua dedizione e la tua forza, sei sempre stata una fonte di ispirazione per me. Spero continueremo a condividere le nostre vite, seppure lontane.

Finally, since success is more a state of mind than a goal to be achieved, I need to end these acknowledgments by saying that I consider this thesis a personal accomplishment. I learnt a lot about myself. It was hard and it was difficult, but it was also amazing. A journey I am happy to end, which nonetheless I am reluctant to leave behind me. I am not an easy person and I am aware that this has affected my project and the people who have been part of it. Thank you all for putting up with me these three years and helping me to get to the end. All the feelings I felt, all the adventures I lived and all the energy it took me to be here at the end, it was something I could have never imagined to experience. So, thanks to each and every single person that has contributed to this journey. And thank you, Giulia from three years ago, for having had the courage to leave and start this PhD and for always finding the strength not to give up, from the beginning and till the very last day.

SUMMARY

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BAT : brown adipose tissue

BM: bone marrow

BMAds: bone marrow adipocytes

BMAT: bone marrow adipose tissue

BMSCs : bone marrow mesenchymal stromal cells

CEBPα : the CCAAT-enhancer-binding protein α

CR : caloric restriction

ESCRT: endosomal sorting complex required for transport

EVs : extracellular vesicles

FGF23 : Fibroblast growth factor 23

HFD : high fat diet

IP7 : Inositol Pyrophosphate

Jam2 : junctional-adhesion-molecule-2

KO: knockout

MAP1B : microtubule-associated protein 1B

MGP : Matrix gla protein

MRI : magnetic resonance imaging

Myorg : Myogenesis Regulating Glycosidase

NGS: next-generation sequencing

Npt: Na-dependent phosphate transporter

OCN: osteocalcin

OLCs: osteolineage cells

OPN : Osteopontin

Osx-1 : Osterix-1

P4H: Prolyl 4-hydroxylase

PiT1 : Phosphate inorganic Transporter 1

PiT2 : Phosphate inorganic Transporter 2

pAT : peripheral adipose tissues

PDCD6IP : Programmed Cell Death 6 Interacting Protein

PDGFB : Platelet-derived growth factor subunit B

PDGFRB : Platelet-derived growth factor receptor beta

PDI: Protein disulfide-isomerase

PFBC : primary familial brain calcification

Pi : inorganic phosphate

PPARγ : -proliferator activated receptor γ

PTH : parathyroid hormone

Runx2 : Runt-related protein-2

scRNAseq: single cell RNA sequencing

Slc20a1 : Solute carrier 20 member a1

Slc20a2 : Solute carrier 20 member a2

TAG : triacylglycerols

TMD : transmembrane domains

USP7 : ubiquitin-specific peptidase 7

WAT : white adipose tissue

XPR1 : Xenotropic And Polytropic Retrovirus Receptor 1

FOREWORD

This PhD project entitled “Study of the role of PiT2 at the interface between bone and adipose tissues” was born from a “Pari Scientifique” grant (AdipOS), literally a “scientific bet”, sponsored by the “Région Pays de la Loire” back in 2017. This grant is allowed to projects aimed at exploring innovative and “high risk” scientific research.

It is in this context that my thesis work is placed. We hypothesized that the absence of PiT2 could have altered the function of adipose tissues, affecting bone quality and strength. This is why we focused on both the bone marrow adipose tissue and the peripheral fat depots in young and adult PiT2-deficient mice.

Within the first introductive part of this manuscript, we have detailed the PiT proteins, their known functions and expression pattern, both in skeletal and non skeletal tissues. We then focused on the relationship between the skeleton and the adipose tissues, focusing on peripheral fat depots and the bone marrow adipose tissue.

This introduction will be followed by the aims of this thesis and a summary of the results we obtained during this three years. We chose to present our work both in the form of the scientific article and also by adding the relevant experiments that were not included in the paper, mostly regarding the identification of potential protein partners interacting directly with the fourth intracellular loop of PiT2 and potentially involved in the observed *in vivo* phenotype of the PiT2-deficient mice.

The last part of this manuscript will include a conclusion and a general discussion, where we will try to contextualize our work, highlighting its contribution to the field and suggesting possible perspectives.

INTRODUCTION

I. The PiT proteins: PiT1 and PiT2

In the early nineties, PiT1 and PiT2 were identified as receptors for retroviruses. PiT1 is recognized by the Gibbon ape Leukemia virus (O'Hara et al., 1990), whereas PiT2 binds the rat amphotrophic leukemia virus (Miller et al., 1994 a; van Zeijl et al., 1994) and were therefore first named Glvr-1 (PiT1) and Ram-1 (PiT2). Nevertheless, the binding of a retrovirus on a membrane protein is no indication of its physiological function. Accordingly, they were later described as sodium (Na)-dependent phosphate transporters (Kavanaugh et al., 1994; Miller et al., 1994 b; Olah et al., 1994), that are now classified in the Slc20 family. In Xenopus oocytes exposed to different salts and nutrients, Kavanaugh and colleagues showed that PiT1 and PiT2 are electrogenic and that they mediate the uptake of inorganic phosphate (Pi) specifically in the presence of Na+ ions (Virkki et al., 2007).

I.1. Protein topology and structure-function relationships

PiT1 and PiT2 proteins have not been crystalized yet, and therefore their topology is only partially known (Figure 1). The current structure model for both proteins predicts 12 transmembrane domains (TMD) with a large intracellular loop (thereafter named "iLoop") between the 7^{th} and 8^{th} TMD, and unusual extracellular N- and C-termini (Virkki et al., 2007; Beck et al., 2009; Farrell et al., 2009). The presence of this iLoop was first predicted and confirmed by immunohistochemistry by Chien and colleagues (Chien et al., 1997) and later studied by other groups (Salaün et al., 2001; Virkki et al., 2007; Forster et al., 2012), which also described a large homology domain (referred to as PD1131) that is duplicated, reversed and present at both the NH_2 and COOH termini, suggesting that portions of the protein have been duplicated during evolution.

Various studies on the link between the structure of the PiT proteins and the related functions have been performed (see review from Forster et al, 2012 for more details). The first findings were obtained regarding their viral receptor function, for instance the putative virus-binding site of PiT1 has been located on its fourth extracellular loop (Johann et al., 1993; Dreyer et al., 2000), while the corresponding site on PiT2 has been found on the first extracellular loop (Feldman et al., 2004). Bottger and colleagues also showed how a mutant version of PiT2 lacking the iLoop was able to conserve its viral receptor function (Bøttger et al., 2004), while a more recent study also showed that the iLoop was also dispensable for the Pi transport capacity of PiT2 (Bøttger et al., 2011), suggesting that this large portion of the PiT2 protein is involved in yet unknown cellular functions. Finally, several residues identified in human PiT2 have been found crucial for the Pi transport function: in particular serine 113 and 593 (Salaun J Mol Biol 2004), glutamates 55 and 575 and aspartates 28 and 506, which are highly conserved in PiT1 (Bøttger et al., 2002,

2005; Beck et al., 2009). Ravera and colleagues also investigated the secondary topology features of PiT1 by cysteine mutagenesis, revealing six sites possibly involved in its Pi transport function (Ravera et al., 2013).

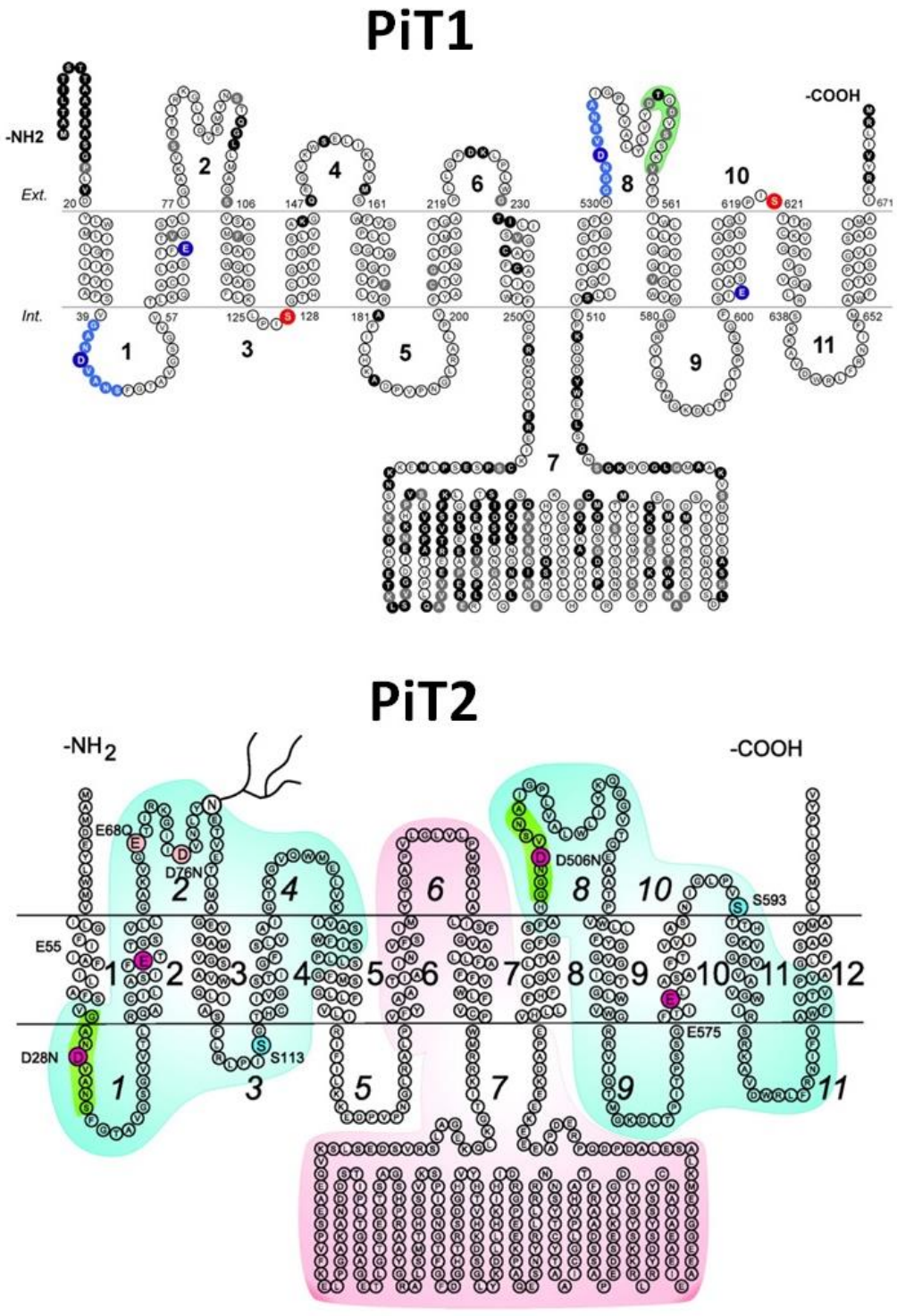


Figure 1. Topological model for human PiT1 and PiT2 (adapted from Beck et al, JBC, 2009 and Virkki et al, AJP 2007).
The 12 predicted TMDs were assigned according to Salaun and colleagues (Salaun et al., 2001). For PiT1, in red serine residues important for transport function, in blue conserved residues corresponding to those that have been shown to be essential for transport function of PiT2 (Bottger et al, 2005; Bottger and Pedersen, 2002). Light green shading indicates the region important for GALV binding. For PiT2, in light blue the PD1131 homology domains, in pink the region that can be removed without compromising retroviral receptor function of PiT2 (Bottger et al, 2004; Salaun et al, 2001).

I.1.1 Expression pattern

The mRNA expression of both PiT1 and PiT2 has been found in most tissues, both in humans and mice (figure 2). However, the level of expression of both PiT1 and PiT2 is not the same in all tissues. Indeed, those proteins present a large panel of expression together with very different levels, suggesting the possibility of non-redundant roles (Nishimura et al., 2008).

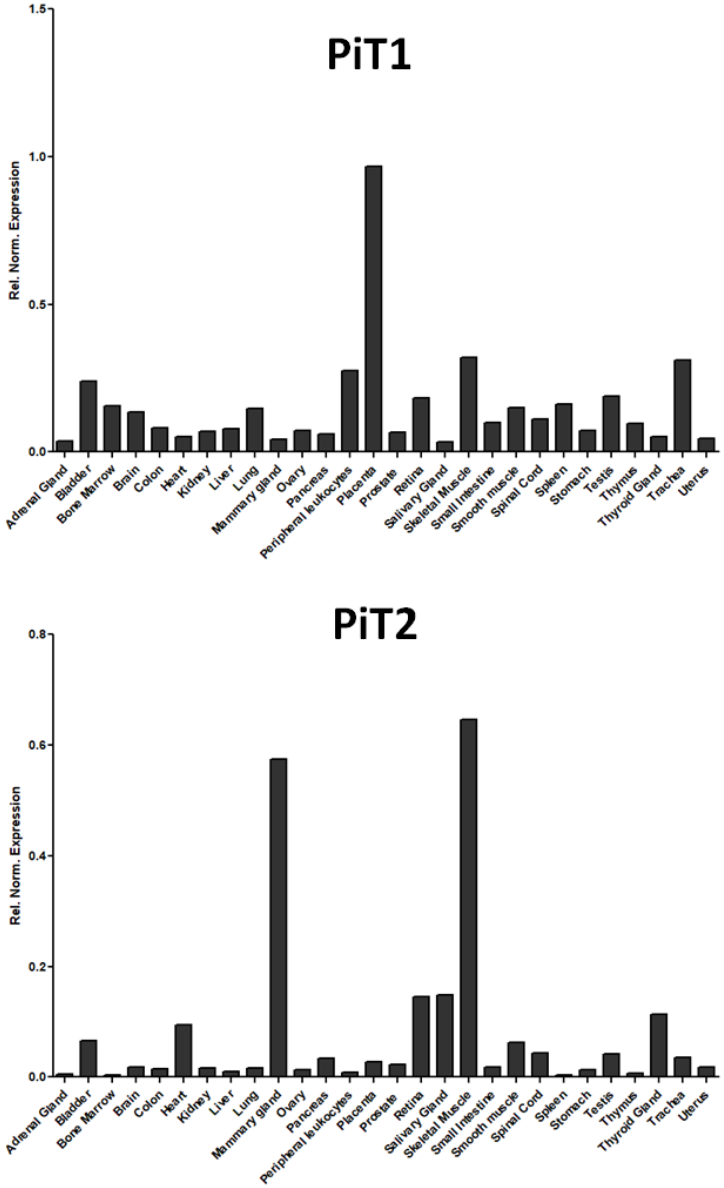


Figure 2. Expression of human PiT1 and PiT2 mRNAs in various tissues, adapted from Nishimura et al, 2008.
Experiments were performed in duplicate. The highest values among the various tissues are underlined. Data are expressed as the ratio of the target mRNA to the peptidylprolyl isomerase A (PPIA) mRNA.

At the protein level, while few reports did illustrate the tissue expression of PiT1 and PiT2, we are unable to confirm the specificity of these antibodies by using tissues from the corresponding KO mice.

Indeed, we tested a lot of antibodies from commercial and non commercial sources, without being able to confirm their specificity. Unfortunately, this lack of a specific antibody doesn't make things easy for the research, hampering its progress. Table 1 summarizes mRNA and protein expression pattern of PiT2 in the literature.

Table 1. PiT2 mRNA and protein expression pattern from the literature.

Organ	Species	RNA	Protein	Refs
Parathyroid	Rat	Negative		(1)
Thyroid	Rat, Human	Negative/Positive		(1); (2)
Spleen	Rat, Mouse Human	Negative (rat and mouse), low (human)		(3); (4); (2)
Thymus	Rat, Mouse Human	Positive/Negative/ low		(3) ; (4); (2)
Marrow	Rat, Human	Positive/low		(3); (2)
Liver	Rat ,Mouse Human, Pig	Positive		(3); (4); (5); (2) ; (6); (7)
Lung	Rat, Mouse Human, Pig	Positive		(3); (4); (2); (6); (7)
Heart	Rat, Mouse Human	Positive		(3); (4); (5); (1); (2); (6)
Muscle	Rat, Human Pig	Positive		(3); (2); (7) ; (8)
Kidney	Rat, Mouse Human, Pig Immortalized distal convoluted tubules (mouse)	Positive (apical; Villa-Bellosta et al, 2009 Positive		(3); (4); (5); (2); (9); (10); (11); (6); (7); (5)
Brain Brain (CP)	Rat, Mouse Human Rat, Shark	Positive	Positive (neuron, astrocytes, endothelial cells, ependyma, choroid plexus epithelium, SMC, purkinje cell layer, substantia nigra) Positive	(3); (4); (1); (2); (12); (13); (14); (15); (16); (6) (17)
Testis	Mouse, Human	Positive		(4); (2); (6)
Ovary	Mouse	Positive		(6)
Skin	Mouse	Positive		(4)
Hair follicle	Mouse	Positive		(6)
WAT	Mouse	Positive		(6)
Stomach	Mouse, Human Pig	Positive		(4); (2); (7)
BAT	Mouse	Positive		(25)
Intestine	Mouse, Rat	Positive (jejunum-		(4); (5); (18); (2);

	Human, Pig	colon)		(6), (9); (7)
Bone	Mouse, Pig	Positive		(5); (6); (7)
Odontoblast	Mouse	Negative		(19)
Pulp cells	Mouse	Positive		(19); (20)
Post-secretory ameloblast	Mouse	Positive		(19)
Stratum intermedium and stellate reticulum cells	Mouse	Positive		(19); (20)
Aorta	Mouse, Rat	Positive (media)	Positive	(21); (22)
Primary smooth muscle cells	Human	Negative		(23)
Placenta	Mouse	Positive	Positive (intracellular sublocalization in CK7+ cells (trophoblast marker), placental labirynth, pericytes)	(24)

(1) (Tatsumi et al., 1998)
(2) (Nishimura et al., 2008)
(3) (Kavanaugh et al., 1994)
(4) (Bai et al., 2000)
(5) (Tenenhouse et al., 1998)
(6) (Beck-Cormier et al., 2019)
(7) (Wubuli et al., 2019)
(8) (Chande et al., 2020)
(9) (Giral et al., 2009)
(10) (Villa-bellosta et al., 2009)
(11) (Kaneko et al., 2011)
(12) (Lagrue et al., 2010)
(13) (Inden et al., 2013)
(14) (Kimura et al., 2016)
(15) (Inden et al., 2016)
(16) (Wallingford et al., 2017)
(17) (Guerreiro et al., 2014)
(18) (Katai et al., 1999)
(19) (Zhao et al., 2006)
(20) (Merametdjian et al., 2018)
(21) (Villa-Bellosta et al., 2007)
(22) (Crouthamel et al., 2013)
(23) (Jono et al., 2000)
(24) (Wallingford et al., 2016)
(25) (Sun et al., 2020)

I.1.2 Regulation of expression

The expression of *Slc20a1* and *Slc20a2* genes, mostly at the mRNA level, has been shown to be regulated by different factors. Probably the most important factor regulating both *Slc20a1* and *Slc20a2* is phosphate (see table 3 focusing on PiT2 expression and its regulation by phosphate). Various data demonstrate that different phosphate contents within the diet modify the mRNA expression of both *Slc20a1* and *Slc20a2* genes in different models. Focusing on PiT2, in the kidney from different species, a low phosphate diet was consistently shown to increase its expression (Breusegem et al., 2009; Giral et al., 2009; Villa-bellosta et al., 2009, 2010; Wubuli et al., 2019), while a high phosphate diet was shown to influence PiT2 expression in a less consistent way, with some papers showing an increased expression and others no changes or even a decreased expression (Breusegem et al., 2009; Giral et al., 2009; Villa-bellosta et al., 2009, 2010; Wubuli et al., 2019). While the TFE3 transcription factor has been proposed to mediate the Pi-driven regulation of Slc34a1 (Kido et al., 1999), the involved mechanism of Pi-driven regulation of PiT1 and PiT2 remains unknown.

Regarding the regulation of the expression of these two genes in bone cells, PiT1, but not PiT2, is also regulated by major osteogenic factors (PDGF, TGFß, IGF-I, FGF-2, BMP2) (Palmer et al., 2000; Suzuki et al., 2001, 2006; Villa-bellosta et al., 2009; Forster et al., 2011).

Table 2. PiT2 expression and its regulation by phosphate.

	Table 1.	**PiT2 expression**	**Organs (species)**	**References**
Low Pi diet	low Pi diet (3 months)	↑ (RNA)	Kidney /colon (pig)	(Wubuli et al., 2019)
	low Pi diet (0,1%, 14 days)	↑ (RNA and protein)	kidney (rat and mice)	(Breusegem et al., 2009)
	low Pi diet (0.1%, 4-5 days)	↑ (protein)	kidney (Rat)	(Villa-bellosta et al., 2009, 2010)
	low Pi diet (0.1%, 7 days)	↑ (RNA)	BBMV kidney (Rat)	(Giral et al., 2009)
	low Pi diet (0.03%, 8 days)	↔ (RNA)	kidney (mice)	(Tenenhouse et al., 1998; Hoag et al., 1999)
	low Pi diet (0,02%, 7 days)	↔ (and PiT1)	jejunum (rat)	(Katai et al., 1999)
High Pi diet	high Pi diet (3 months)	↑ (RNA)	intestine (pig)	(Wubuli et al., 2019)
	high Pi diet (1,2%, 14 days)	↓ (RNA and protein)	kidney (rat and mice)	(Breusegem et al, 2009)
Acute high Pi diet	acute high Pi diet (1,2%, 4h (after 7 days with low Pi)	↔ (RNA)	BBMV kidney (Rat)	(Giral et al, 2009)
	acute high Pi diet (1,2%, 4-5 days)	↓ or ↔ (protein)	kidney (Rat)	(Villa-bellosta et al., 2009, 2010)
In vitro experiments	1mM Pi	↑ (and PiT1)	fibroblast (rat 208F)	(Kavanaugh et al., 1994)
	Pi deprivation (24h)	↑ (RNA and protein) (no effect in HeLa and U937)	osteosarcoma cells (143B, HOS)	(Chien et al., 1997)

I.2. Functions of the PiT proteins

Since the discovery of the PiT proteins as Pi transporters in the early 90s, researchers have come a long way in the characterization of their physiological functions. The PiT proteins are mostly known as Pi transporters, part of the Slc20 Na-Pi co-transporters family. When PiT1 was mainly known as Glvr-1 (gibbon ape leukemia retrovirus receptor 1), Johann and colleagues suggested its role as a Pi transporter due to its

homology to a phosphate permease (PHO-4) from the fungi *Neurospora crassa* (Johann et al., 1992). Later on, after the discovery of PiT2 as the receptor for the amphotropic murine retrovirus (van Zeijl et al., 1994), the finding that both of them were Na^+-dependent Pi transporters was published (Kavanaugh et al., 1994; Miller et al., 1994 b; Olah et al., 1994).

Since these original descriptions, numerous other functions have been proposed for PiT1 and PiT2, which can be attributed to their ability to transport Pi (Pi-transport dependent functions), or that do not depend on this function (Pi-transport independent functions). Interestingly, the Pi transport-independent functions are now more abundant than the Pi transport-dependent ones (Table 3) and cover a large variety of physiological functions, defining the PiT proteins as multifunctional proteins.

In the next two paragraphs, we will detail these PiT functions, both in the context of extra-skeletal and skeletal tissues.

Table 3. Phosphate transport dependent and independent functions of PiT1 and PiT2.

Pi transport dependent	Pi transport independent	Unknown mechanism
	Retrovirus receptor (PiT1/PiT2)[1,2,3,4]	Bone and dentin mineralization (PiT2)[5]
Brain calcifications (PiT2)[14]	Proliferation/apoptosis (PiT1)[6,7]	Liver development and cancer (PiT1)[13]
	Chondrocyte survival (PiT1)[8]	Myocyte function and survival (PiT1/PiT2)[18]
	Neuronal growth (PiT2)[9]	
	Glucose/fat metabolism (PiT1)[10] Erythroid maturation (PiT1)[11,12]	
Vascular calcifications (PiT1/PiT2)? [16]	Vascular calcifications (PiT1/PiT2)? [15]	
	Pi sensing (PiT1/PiT2)[17,19]	

1) (O'Hara et al., 1990)
2) (Miller et al., 1994 a)
3) (Pedersen et al., 1995)
4) (Pedersen et al., 1997)
5) (Beck-Cormier et al., 2019)
6) (Beck et al., 2009)
7) (Salaün et al., 2010)
8) (Couasnay G et al., 2019)
9) (Ma et al., 2017)
10) (Forand et al., 2016)
11) (Forand et al., 2013)
12) (Liu et al., 2013)
13) (Beck et al., 2010)
14) (Wang et al., 2012)
15) (Chavkin, N. W et al., 2015)
16) (Crouthamel et al., 2013)
17) (Bon et al., 2018 a)
18) (Chande et al., 2020)
19) (Bon et al., 2018 b)

I.2.1. Functions of the PiT proteins in extra-skeletal tissues

Role of PiT1 in cell proliferation and liver development

In 2009, the first study demonstrating the role of PiT1 in the regulation of cellular proliferation was published. The authors showed that deletion of PiT1 in HeLa cells resulted in dramatically reduced cell proliferation both *in vitro* using siRNA and *in vivo* in nude mice (Beck et al., 2009). The loss of PiT1 in HeLa cells resulted in decreased Pi transport, suggesting that this reduction could explain the reduced proliferation. However, the reduced proliferation could not be rescued when Pi transport was corrected by over-expression of PiT2 or Npt2b (Slc34a2), while proliferation was rescued by over-expressing a Pi-transport deficient mutant of PiT1. These experiments were the first to identify Pi-transport independent functions of PiTs (apart from their use of receptors by retroviruses). Soon after, Salaün and colleagues also determined another transport-independent function for PiT1, demonstrating its association with programmed cell death. Indeed, they revealed how PiT1 depletion sensitizes cells to TNF-mediated apoptosis (Salaün et al., 2010).

Deletion of PiT1 in mice resulted in embryonic death at E12.5 (Beck et al., 2010). This was reported to be due to severe defects in liver development, coming both from reduced proliferation and massive apoptosis of hepatoblasts (Beck et al., 2010). This impaired liver development caused dramatic anaemia with an abnormal yolk sac vasculature, as also described by Giachelli's group (Festing et al., 2009), that most probably led to the death of the embryo. During embryogenesis, liver formation is a crucial checkpoint in fetal hematopoiesis. Indeed, it is the main organ for hematopoietic stem cells expansion and differentiation until late fetal life, at which time bone marrow hematopoiesis is established. Interestingly, erythroid progenitors still had the ability to migrate to the PiT1-deficient liver even though it was hypoplastic, arguing against a fetal liver homing defect. The reduced proliferation and massive apoptosis of liver cells, then, illustrated a role for PiT1 in proliferation and survival for hepatocytes during liver morphogenesis. Of note, PiT2 could not compensate for the loss of its paralog, revealing PiT1 as an essential gene for mouse liver development.

Role PiT1 in hematopoietic tissues

As already mentioned before, PiT1-deficient mice revealed a lethal phenotype caused by severe anemia due to defects in liver development (Beck et al, 2010). These results have led the same team to investigate the maturation of erythroid cells via specific deletion of PiT1 in the hematopoietic system using PiT1-floxed and Mx1-Cre mice (Forand et al., 2013). The Erythroid Krüppel-like Factor (EKLF/KLF1), a DNA-binding protein, plays a crucial role in the development of red blood cells by its implication in hemoglobinization, cell-cycle arrest, and terminal differentiation of erythrocytes *in vitro.* The authors showed that EKLF-induces an up-regulation of PIT1 during erythroid maturation *in vivo*, which can explain

the anemia observed in PiT1-deficient mice. *In vitro* down regulation of PiT1 in a human erythoid cell lineage was shown to impair the terminal differentiation of primary erythroid progenitors, further supporting a role of PiT1 in red blood cell maturation. Similar results were obtained by another team that showed that adult mice lacking PiT1 within hematopoietic tissues after birth develop a profound anemia characterized by mild macrocytosis, dyserythropoiesis, increased apoptosis, and a near complete block in terminal erythroid differentiation (Liu et al., 2013). Conditional deletion of PiT1 in hematopoietic tissues showed, in addition to the red blood cell defects, a serious lack of B cells, mainly due to an alteration of their development, accompanied by a mild neutropenia and thrombocytosis. In their paper Liu and colleagues also suggest that this function of PiT1 is independent of its role as a phosphate transporter, since no major differences were observed concerning the uptake of phosphate between mutated and control erythroblasts.

PiT1 and glucose metabolism

The discovery of PiT1 as a regulator of proliferation and apoptosis independently of its Pi transport function (probably allowing a proper liver development) had contributed to the demonstration that PiT1 is indeed a multifunctional protein. This was confirmed some years later with the finding of another function of PiT1 within the liver, in particular in the regulation of glucose metabolism and insulin signalling (Forand et al., 2016). Conditional PiT1 KO mice in the hepatocytes display improved glucose tolerance, while gluconeogenesis was not altered. In the meantime, those mice had a higher insulin sensitivity, which could be explained by the interaction of PiT1 with a protein partner named ubiquitin-specific peptidase 7 (USP7). Through its interaction with USP7, PiT1 was shown to consequently prevent the USP7-IRS1 interaction important for the signalling pathway connected to the insulin receptor (Figure 3). As a result, mice KO for PiT1 in hepatocytes display improved glucose tolerance, insulin sensitivity, and enhanced insulin signalling when compared to WT mice.

Using a transport-deficient PiT1 mutant, they also demonstrated that the effects of PiT1 deletion observed on insulin signalling were independent of the phosphate transport function of PiT1. Interestingly, Forand and colleagues also showed that the specific deletion of PiT1 within the hepatocytes protected against the weight gain induced by ageing, also improving glucose tolerance and insulin sensitivity in mice fed a normal chow diet. Moreover, when those mice were fed a high fat diet (HFD), the absence of PiT1 within the hepatocytes also protected against the HFD-induced obesity and insulin resistance.

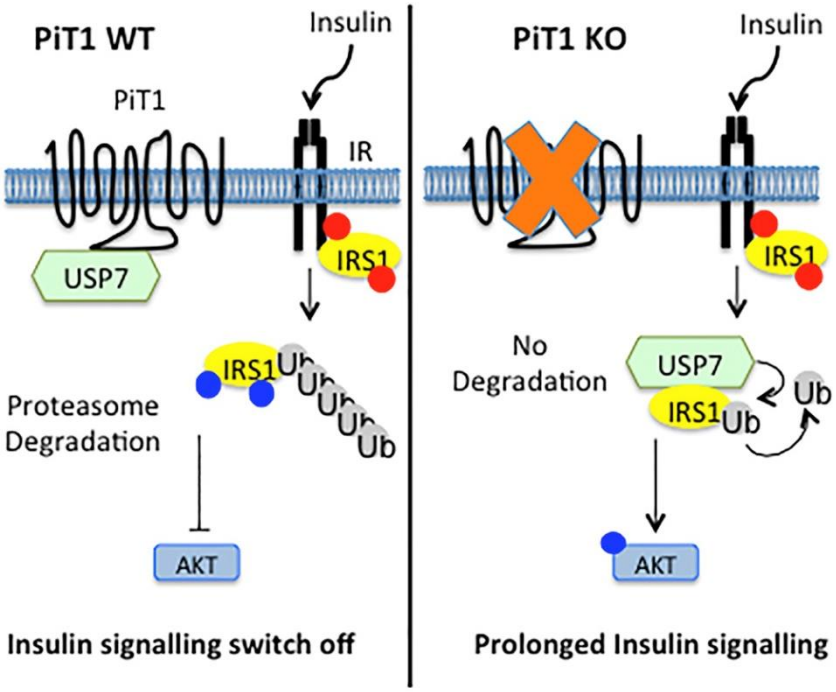


Figure 3. Schematic representation of the effect of the invalidation of PiT1 in hepatocytes on insulin signalling. Briefly, the binding of insulin to its receptor leads to the autophosphorylation of the receptor and its activation, which induces the recruitment and phosphorylation of IRS1, which mediates the biological action of insulin. Insulin signalling is tightly regulated by inhibitor proteins and negative feedback loops. IRS1 is a major adaptor protein in insulin signalling. Its ubiquitination and degradation are part of a negative feedback loop that attenuates insulin signalling. PiT1, through regulation of the USP7/IRS1 interaction, modulates this insulin negative feedback loop, sustaining insulin signaling. Graphical abstract from Forand et al., Cell Reports, 2016.

Roles of the PiTs in myocyte function and survival

Even if not directly involved in Pi homeostasis, voluntary muscles contain a large portion of the phosphorus present in soft tissues, mostly as ATP and phosphoryl creatinine (Peacock, 2020). As already mentioned before, patients with hypophosphatemic disorders display a low muscle function due to low blood phosphate. PiT1 and PiT2 are known to be expressed in muscle cells (Kavanaugh et al., 1996). Notably, early this year, Chande and colleagues showed the importance of both co-transporters in skeletal muscle. They showed how a double deletion of both PiT1 and PiT2 in skeletal muscle (*smPit1−/−; smPit2−/−* mice) leads to myofiber necrosis, skeletal muscle atrophy and early death in mice (Chande et al., 2020). This phenotype is due to energy stress that leads to reduced ATP production and abnormal mitochondrial morphogenesis and function. The molecular mechanisms are yet unknown and the authors propose genetic rescue experiments using adenoviral constructs expressing WT and transport-deficient forms of PiT1 and PiT2, to test whether Pi uptake is required to rescue the myopathy of these mutant mice.

PiT2 and neuronal growth

The various Pi transport-dependent and independent functions of PiT1 uncovered its multifunctional nature, and it is tempting to speculate that the same molecular mechanisms may exist for PiT2.

Accordingly, a recently published paper on neuronal growth suggests that PiT2 could also be a multifunctional protein, revealing a role that is not dependent on its capacity to transport phosphate (Ma et al., 2017). Ma and colleagues showed that neuronal cells transfected with a version of PiT2 lacking the 4th intracellular loop (a.k.a. PiT2 iLoop) display a decrease in neurite length. Interestingly, they identified a proteic partner of the iLoop called microtubule-associated protein 1B (MAP1B) through a yeast two-hybrid screening approach. MAP1B, a cytoskeletal protein, is important for the cytoskeletal changes that accompany neurite extension. They confirmed MAP1B interaction with PiT2 *in vitro* and *in vivo*, showing how it regulates the differentiation of neuronal cells through interaction with MAP1B. They also showed how mutating the MAP1B binding site on PiT2 affected neurite length, while PiT2 lacking its phosphate transport function did not affect it, suggesting that PiT2 modulates neuronal outgrowth independently of its role as a phosphate transporter. Finally, they switched on a drosophila model, where both the homolog for human PiT2, dPiT, and for microtubule-associated protein, Futsch, were identified. In drosophila, they observed that dPiT is essential for a normal development of the synapses and, moreover, that dPiT and Futsch interact within the brain and this interaction regulates synapses development (Ma et al., 2017). Overall, this study showed that PiT2 modulates neuronal outgrowth by interacting with MAP1B independently of its phosphate transport function. Like its paralog PiT1, it is probably just a matter of time before revealing additional molecular mechanisms of action of PiT2, whether or not depending on its Pi transport function and/or the interaction with potential protein partners.

PiT2 and brain calcifications

Primary familial brain calcification (PFBC) is a pathological condition generated by mutations in the *Slc20a2* gene. In particular, a deletion and five missense variants in the *Slc20a2* gene have been identified in 2012 as the first genetic cause for PFBCs (Wang et al, 2012). This condition is characterized by a decreased phosphate uptake in brain vessels and consequent local increase in Pi concentrations in the cerebrospinal fluid (CSF) and this has been suggested to contribute to Ca-Pi deposition in related tissues and blood vessels (Lemos et al., 2015; Jensen et al., 2016). PFBC is a rare genetic disorder caused by pathogenic sequence changes in at least six different genes identified so far: *Slc20a2*, *Xpr1*, *PDGFB*, *PDGFRB*, *Myorg* and *Jam2* (Westenberger et al., 2019; Cen et al., 2020). Pathogenic variants in *Slc20a2*, *Xpr1*, *PDGFB*, and *PDGFRB* are inherited in an autosomal-dominant manner, while mutations in *Myorg* and *Jam2* have been recently identified as inherited in an autosomal-recessive way (Westenberger et al, 2019; Cen et al, 2020). This pathology is characterized by the presence of symmetrical calcifications of the basal ganglia and other brain regions, like the subcortical white matter, the thalamus or the cerebellum. Functional data suggested that *Slc20a2* mutations, found in approximately 50% of PFBC families, are caused by haploinsufficiency (Wang et al., 2012; Hsu et al., 2013). However, recent observations showed a dominant-negative effect of three *Slc20a2* variants, suggesting an alternative mechanism for at least some

Slc20a2 pathogenic mutations (Larsen et al., 2017). To date, on a total number of 95 patients, 59 different mutations in the *Slc20a2* gene causing PFBC have been identified, mostly point mutations and frameshift changes (Source: The Movement Disorder Society Genetic mutation database (MDSGene); Westenberger et al, 2019). The PFBC phenotype is recapitulated in PiT2-deficient mice, where ectopic brain calcifications have been observed both thanks to microtomography and histological analyses (Jensen et al., 2018; Beck-Cormier et al., 2019). It is not clear yet if *Slc20a2* heterozygous mice also display a PFBC phenotype, with ectopic calcification within the brain. The only two publications regarding this topic show contradictory results (Wallingford et al., 2017; Jensen et al., 2018).

I.2.2. Functions of the PiT proteins in skeletal tissues

PiT1 and PiT2 as phosphate sensors

Phosphate is essential for our organism and a reduction (hypophosphatemia) or an excess (hyperphosphatemia) of blood phosphate result in pathological conditions leading to severe biological repercussions like rhabdomyolysis, muscle weakness or soft tissue calcification (Peacock, 2020). Thus, maintaining a constant concentration of serum Pi is fundamental and is achieved through complex mechanisms underlying Pi homeostasis. The regulation of Pi homeostasis is tightly controlled by various factors managing Pi absorption by the intestine, its excretion by the kidney, and its storage and mobilization from the skeleton. The major regulators of phosphatemia are the parathyroid hormone (PTH), vitamin D and FGF23 (Bergwitz et al., 2010; Peacock, 2020), which have been the subject of intense research since the last 50 years. What is much less known, but is essential to the regulation of Pi homeostasis, is how the cell or the organism can detect changes in extracellular Pi concentration to trigger the adaptive response? Importantly, during the last 15 years, the phosphate ion has been described as a signalling molecule capable of switching on intracellular signalling pathways (such as ERK1/2 phosphorylation) and regulate the expression of target genes (MGP, OPN or FGF23) (Khoshniat et al., 2011; Chande et al., 2018). In particular regarding bone cells, phosphate can regulate differentiation and production of cartilage matrix by chondrocytes and it can also regulate bone remodelling by affecting the differentiation and function of osteoblasts, osteocytes and osteoclasts. To do so, extracellular phosphate is capable of stimulating or inhibiting gene expressions through the activation of ERK1/2, for example it induces the expression of OPN in osteoblasts to support the formation of bone matrix (Beck et al., 2000; Beck, 2003; Chande et al., 2018). The molecular mechanism of Pi as a signalling molecule requires that the cells are capable of detecting variations in the concentration of extracellular Pi, a mechanism that is referred to as Pi sensing. This mechanism is already well described in bacteria, yeasts and plants, while less is known regarding mammals. As well described by Kritmetapak and Kumar in their review (Kritmetapak et

al., 2019), in bacteria and yeasts changes in the extracellular concentrations of phosphate induce signalling pathways that regulate the expression of phosphate transporters and the transcriptional regulation of target genes. In particular, in Escherichia coli the concentration of extracellular phosphate is sensed by a specific phosphate transporter (Pst). When the amount of extracellular phosphate is low, Pst facilitates the phosphorylation of another membrane protein called PhoR. PhoR then is capable of phosphorylating PhoB, a cytoplasmic protein, that is then functioning as a transcription factor regulating the transcription of target genes involved in Pi conservation. In the same way, in Saccharomyces cerevisiae when extracellular phosphate is low, the synthesis of IP7 is augmented leading to the activation of the transcription factor Pho4. This induces the transcription of genes encoding for phosphate transporters like Pho84. Interestingly, in both those unicellular organisms by default the mechanism of adaptation to phosphate deprivation is activated, because the amount of phosphate in the external environment are usually very low, and when the extracellular phosphate is at saturating levels the system is switched off.

Unlike yeasts and bacteria, plants are multi-cellular organisms, which implies a regulation of Pi by intra- but also inter-cellular regulation pathways of different tissues and thus a communication system. In this case, the answer to a low phosphate state needs to integrate not only the information at the roots level but also regarding branches and leaves. In plants, the acquisition of Pi and its regulation depends on various parameters, which won't be detailed in this introduction (for more information see the review from (Chiou et al., 2011)). It is indeed a complex network, from the local root sensing to the systemic signalling and regulation.

In mammals several proteins have been identified as potential phosphate sensors (Beck et al., 2020), for the sake of this introduction, I will focus on the PiT proteins. Because PiT1 and PiT2 are able to bind Pi with high affinity, our group recently investigated the role of these proteins in mediating Pi signalling in murine skeletal cells (Bon et al., 2018 a). They found that deletion of PiT1 or PiT2 attenuated the Pi-dependent ERK1/2-mediated phosphorylation and subsequent gene up-regulation of the mineralization inhibitors MGP and OPN. Importantly, the ERK1/2 phosphorylation could be rescued by overexpressing Pi transport-deficient mutant forms of PiT1 and PiT2. Using BRET (Bioluminescence Resonance Energy Transfer), our group also found that PiT1 and PiT2 form both homodimers and heterodimers. Interestingly, only the formation of heterodimers was regulated by variations of extracellular Pi concentration suggesting that Pi-regulated PiT1-PiT2 heterodimerization could mediates Pi sensing. Experiments using transport-deficient mutants of PiT1 and PiT2, and experimental conditions without Na+ (necessary for Pi transport) further suggested that Pi sensing was dependent upon Pi binding on the PiT1-PiT2 heterodimer, without the need to be transported within the cell. The physiological consequence of a role of PiT1-PiT2 in Pi sensing was illustrated in the control of FGF23 secretion upon Pi challenges, a work in which I have been involved during my first year of PhD (Bon et al., 2018 b) See the results chapter and Figure 4).

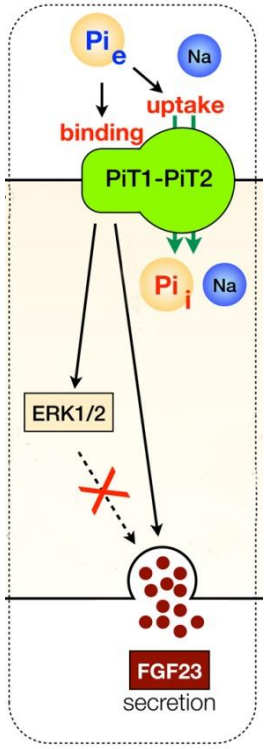


Figure 4. Schematic view of PiT1 and PiT2 as phosphate sensors. PiT1 and PiT2 are forming membrane-bounded heterodimers allowing for extracellular Pi binding, triggering the ERK1/2 MAPK pathway and FGF23 secretion in vivo. However, the use of a MEK inhibitor did not block Pi-dependent PiT-mediated secretion of FGF23 indicating that other signaling pathways are involved. (Adapted from Beck et al, 2020).

PiT2, mineralization and bone quality

Bone mineralization is a strongly regulated process, which promotes the deposition of hydroxyapatite in specific areas of the extracellular matrix (ECM). Even though the molecular mechanisms regarding hydroxyapatite crystal formation remains controversial, matrix vesicles sprouting from mineralizing cells (osteoblasts or chondrocytes) are thought to be the early site of formation of hydroxyapatite crystals, which will be deposited on collagen fibrils after rupture (Millán, 2013). This mechanism requires yet unknown phosphate transporters located at the membrane of matrix vesicles. Since phosphate transport in mineralizing cells is assumed (even if not proven) to be primarily handled by PiT1 and PiT2, they were thought to have a fundamental role in supplying phosphate for the mineralization process. In particular, early publications have first studied the putative role of PiT1 as the phosphate transporter fundamental for the bone mineralization process, since many *in vitro* studies illustrated its regulation in skeletal tissues. For example, Palmer and colleagues reported PiT1 expression in hypertrophic chondrocytes, suggesting a role for the Na-Pi cotransporter in the progression of the mineralization front during endochondral ossification (Palmer et al., 1999). PiT1 was also shown to be essential for the differentiation of osteoblastic MC3T3-E1 cells in culture over time and for bone matrix calcification (Nielsen et al., 2001; Suzuki et al., 2006; Yoshiko et al., 2007). However, later *in vivo* published data failed to validate the early *in vitro* studies, suggesting that PiT1 is actually not responsible, or not sufficient for the mineralization process.

First, our group showed that PiT1 hypomorphic adult mice have normal bone mineralization (Bourgine et al., 2013). PiT1 expression in hypomorphic mice was reduced by 85% and while they exhibited

growth retardation, no significant mineralization differences were detected between femurs of WT and hypomorphic mice from 15 to 300 days after birth.

Two other studies also showed using distinct animal models (*Col2a1-cre;Pit1*$^{flox/flox}$; or *Agc1*$^{CreERT2/+}$*;PiT1*$^{lox/lox}$ mice) how a chondrocyte-specific deletion of PiT1 reveals only subtle or no bone mineralization abnormalities (Yadav et al., 2016; Couasnay et al., 2019). Yadav and colleagues performed a conditional ablation of PiT1 in chondrocytes by using the Col2a1-Cre strain, showing a minor or even negligible role for PiT1 in mineralization, while the work of our group showed how PiT1 is required for the growth plate maturation process, but not for the mineralization potential of the growth plate (Couasnay et al., 2019). In particular, PiT1 ablation in the chondrocytes shortly after birth generates an uncompensated ER stress leading to the intracellular retention of the pro-survival factor VEGFA and a rapid and massive cell death in the center of the growth plate (Couasnay et al., 2019). Interestingly, this newly described function of PiT1 is Pi-transport independent and mediated by its interaction through its iLoop with the protein disulfide isomerase (PDI), an ER chaperone strongly induced in ER stress conditions.

Last but not least, two other independent publications showed how PiT1 overexpression in rats and mice did not affect bone mineralization (Suzuki et al., 2010; Chande et al., 2019). Suzuki and colleagues overexpressed PiT1 in a transgenic rat model, showing no major bone deformity during their skeletal development. Chande and colleagues on the other hand generated transgenic mice overexpressing PiT1 under the control of the CAG (cytomegalovirus/chicken beta actin/rabbit beta-globin gene) promoter and showed no change in bone mineral density upon microCT analysis.

Since the role of PiT1 in skeletal mineralization has been challenged by *in vivo* approaches, which set aside the previous assumption of its critical role in this process, the focus has consequently shifted to PiT2. Moreover, the identification of heterozygous pathogenic variants in the *Slc20a2* gene that cause primary familial brain calcification (PFBC) indicated a role for PiT2 in the regulation of tissue mineralization. To answer this question, our group has investigated the dental and skeletal phenotype of the PiT2KO mice.

Dental phenotype of PIT2KO mice

PiT2-deficient mice showed a disrupted dentin mineralization with the presence of calcospherites at the mineralization front leading to severe dental fragility, evidenced by frequent incisor fractures observed from P28 (Fig.5A and D). Interestingly, among other Pi transporters, our group showed that, mostly during the postnatal stages, PiT2 is expressed 2- to 10-fold higher than the other Pi transporters and mainly within the stratum intermedium and the subodontoblastic cell layer (Fig. 5B and C, (Merametdjian et al., 2018)), which does not support a major role of this transporter in the initiation of dental mineralization. The results obtained within the tooth regarding both PiT1 and PiT2 suggested that they may rather participate, directly or indirectly, to the regulation and maintenance of tooth mineralization throughout life span, rather than in the initiation of dental mineralization. Since mineralization of the tooth

is possible without PiT1 or PiT2, this opens up the chance to a role other than mineralization itself for PiT1 and PiT2 in the dental context. In particular, the possibility of their involvement in Pi sensing and signaling pathways in neighboring cells has been proposed, also on the basis of the low transporting capacity of these transporters compared to the high quantities of phosphate needed for mineralization, which suggest a role for PiT1 and PiT2 other than related to the transport of phosphate.

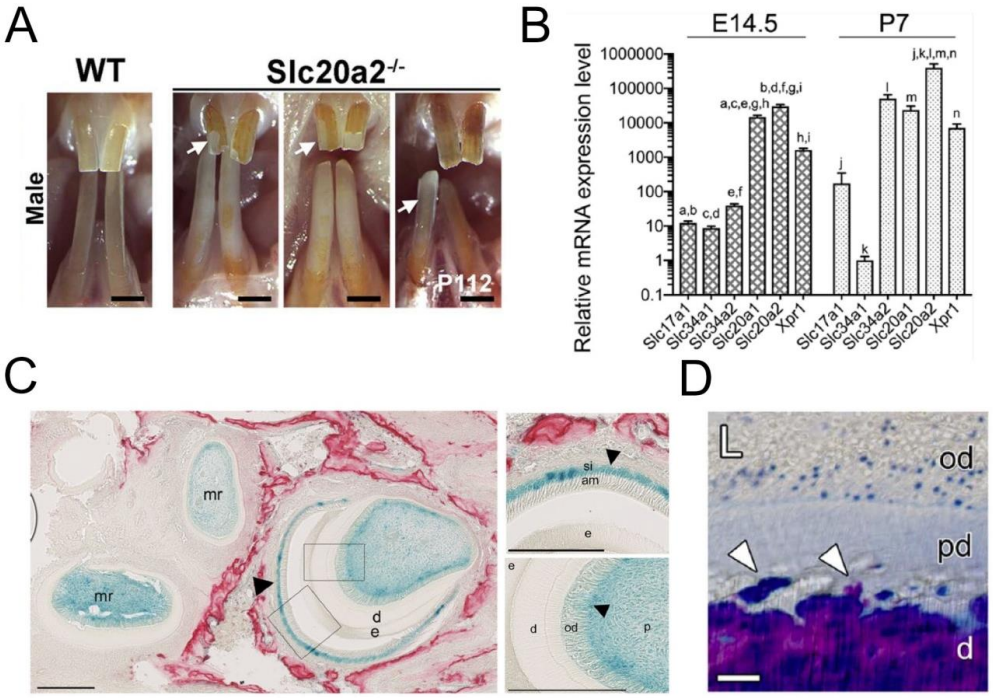


Figure 5. Dental phenotype of PiT2-deficient mice.
A) Images of incisors from P112 PiT2 WT and KO male mice. B) Comparison of the RNA expression levels of Pi transporters at E14.5 and P7 in molar germs. The expression of Pi transporters was calculated relative to the expression of Slc34a1 at P7, set as 1. Note that Slc34a3 was not detected. Data are expressed as mean — SEM. One-way analysis of variance, followed by Tukey's post hoc multiple comparison test, was used to compare expression levels within each developmental stage group (E14.5 or P7). C) X-gal stainings of 1-mo-old paraffin-embedded Slc20a2 KO incisors. The strongest staining was observed in the stratum intermedium and subodontoblastic layer (black arrowhead); a more diffuse signal in the pulp was observed; and no signal could be seen in the cementoblasts. Bar = 250 µm. D) Presence of calcospherites (arrowheads) at the predentin layer of mutant mice. Bar = 25 µm. am, ameloblasts; d, dentin; e, enamel; mr, molar root; od, odontoblasts; p, pulp; si, stratum intermedium; pd, predentin. (From Beck-Cormier et al, 2019 and Merametdjian et al JDR 2018).

Bone phenotype of PiT2KO mice

In 2017, Yamada and colleagues suggested for the first time the necessity of PiT2 expression for normal bone growth and development *in vivo,* showing abnormal bone development and decreased bone mineral density in PiT2-deficient mice (Yamada et al., 2017). At the same time, our group performed a detailed characterization of the skeletal phenotype of PiT2-deficient mice (Fig 6, (Beck-Cormier et al., 2019)). PiT2-deficient mice have shorter long bones, reduced cortical thickness (and modest reduction of trabecular thickness) visible from 3 weeks of age, reduced bone mineral density (BMD) and bone mineral content (BMC) and severe reduction of bone biomechanical properties (Fig.6A-C). Measures of bone quality in PiT2-deficient mice were abnormal, and all below WT predicted values (Fig.6D). Interestingly, *in vivo* and in vitro experiments showed that maturation, activity and mineralization of PiT2-deficient skeletal cells

(osteoblasts, osteoclasts and chondrocytes) are similar to controls. Altogether, these results showed that *PiT2KO* mice have an important functional impairment of bone quality with a severely abnormal skeletal phenotype characterized by a disproportionate reduction in bone strength that, however, cannot be accounted for by the modest reductions in cortical thickness and BMC. Bone quality is a complex concept, which involves structural and material parameters such as bone geometry, bone microarchitecture, the orientation and interrelationship of matrix proteins and collagen fibrils and hydroxyapatite crystal size and packing. It has only recently been appreciated as a major contributor to fracture risk (Felsenberg et al., 2005; Papaioannou et al., 2015). Therefore, it would be interesting to analyze these parameters in PiT2-deficient mice.

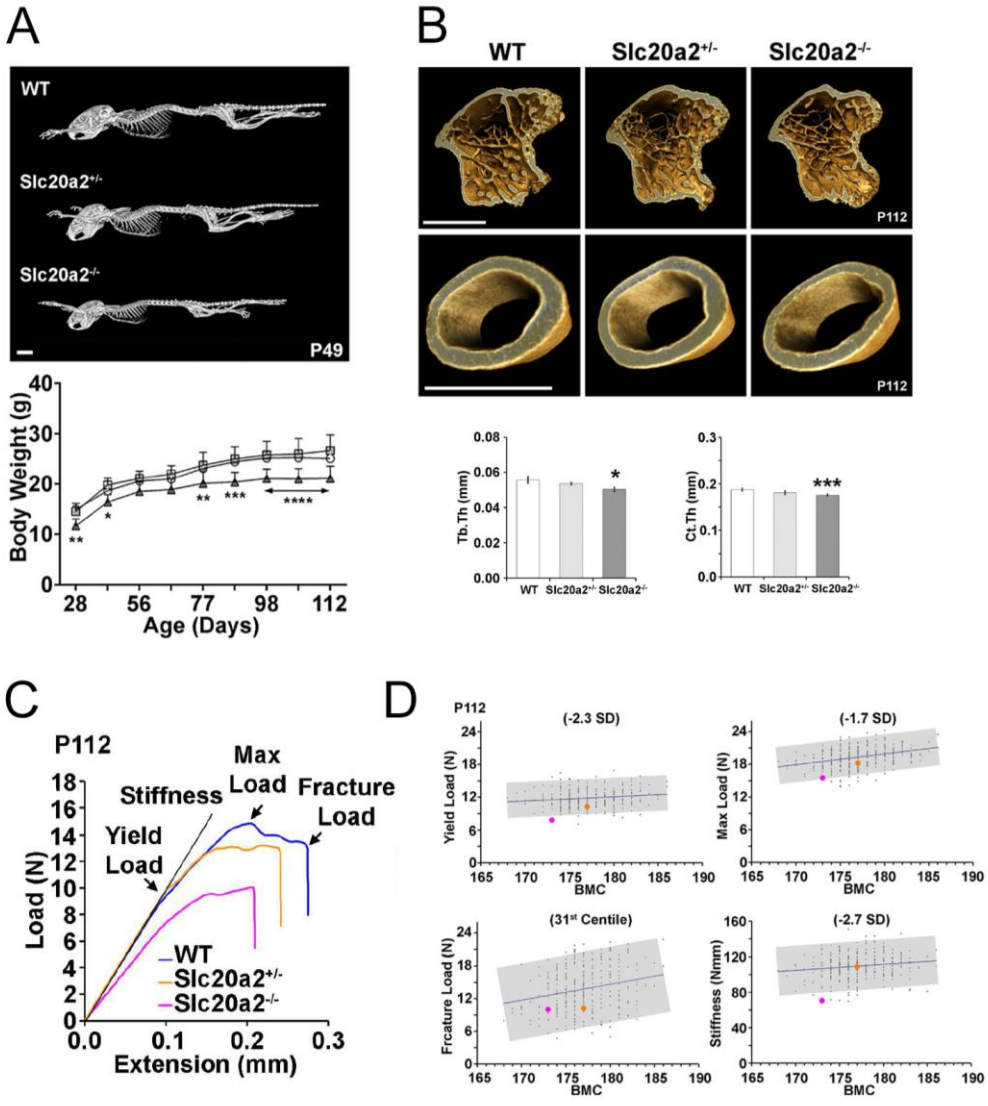


Figure 6.Skeletal phenotype of PiT2-deficient mice.
A) µCT images and body weight of P49 female PiT2 WT, Het and KO mice. Scale bar: 5 mm. B) µCT images of the proximal tibial metaphysis and mid-diaphyseal tibial cortical bone of P112 PiT2 WT, Het and KO female mice. C) Representative load displacement curves from three-point bend testing of humeri from P112 PiT2 WT, Het and KO female mice showing yield load, maximum load, fracture load, and the gradient of the linear elastic phase (stiffness). D Bone quality analysis. Graphs demonstrating the physiological relationship between relative bone mineral content (median gray level determined by quantitative X-ray microradiography) and yield load, maximum load, fracture load, and stiffness in femurs from P112 PiT2 Het and KO (pink dot) female mice. (From beck-Cormier et al JBMR 2019)

II. Communication between the skeleton and the adipose tissues

As you will read throughout the next chapter of this introduction, it is well described in the literature that a strong relationship exists between bone and the adipose tissues. This is what led us to develop an interest in the association between bone and adipose tissues in the context of PiT2KO mice. Hence, I will now proceed to the introduction of skeleton, adipose tissues and their relationship.

II.1. The skeleton

The human skeleton is composed by 206 bones and it is the internal framework of the body, which shapes it and has also many other important functions: support, movement, protection of internal organs, production of blood cells, storage of ions and endocrine regulation. The bone matrix is made mostly of type I collagen and hydroxyapatite, which are secreted by bone forming cells called osteoblasts. There are two other main types of cells that allow bone homeostasis: the osteocytes, mature integrated osteoblasts that are resident in the cortex, secrete FGF23 and are the sensors of mechanical strength, and the osteoclasts, the bone resorbing cells. Together osteoblasts, osteocytes and osteoclasts allow our bones to be in constant remodeling through life and after injuries (Clarke, 2008). The bone structure is characterized by four main parts: (i) the cortex, that gives bone its shape; (ii) the bone marrow, where hematopoietic stem cells and bone marrow stromal stem cells are produced, (iii) the articular cartilage, that covers the epiphyses of the bone (joints) and (iv) the periosteum, a thin membrane covering the cortex and allowing the bone to grow in thickness. During skeletal development, bone can form via endochondral ossification or intramembranous ossification depending on the anatomical site (Fig.7).

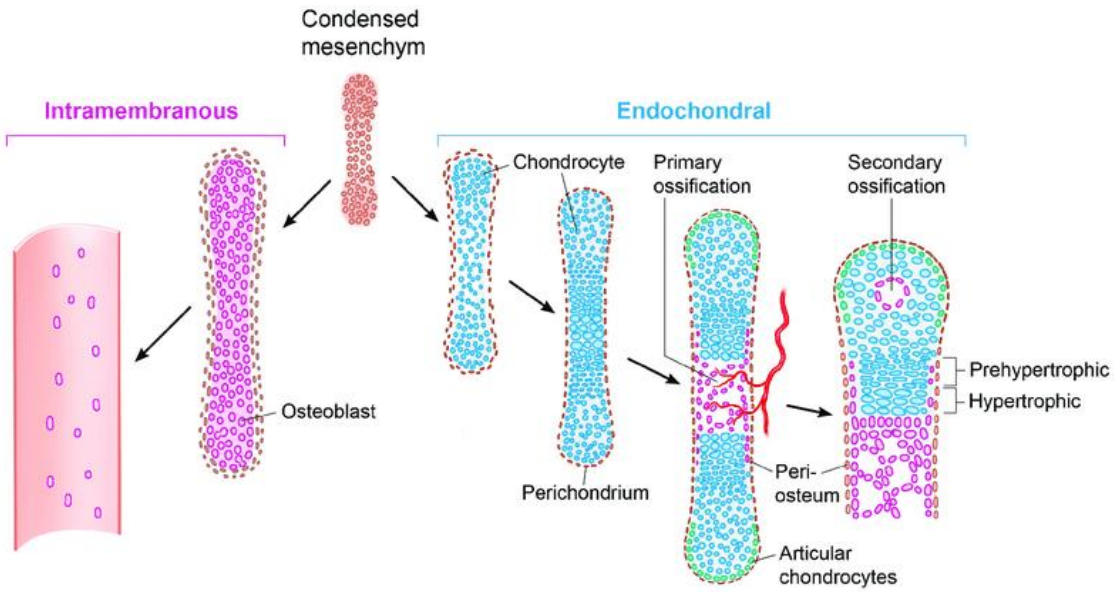


Figure 7. Intramembranous and endochondral ossification, the two essential processes during fetal development by which bone tissue is created. In intramembranous ossification, bone develops directly from mesenchymal connective tissue. In endochondral ossification, bone develops by replacing hyaline cartilage. Adapted from (Houschyar et al., 2019).

Endochondral ossification mostly occurs in the axial skeleton, limbs and part of the clavicle. Intramembranous ossification occurs in flat bones of the skull and part of the clavicle. These ossification processes involve the transformation of a pre-existing mesenchymal tissue into bone tissue. During endochondral ossification, bone formation occurs in a two-step mechanism. First, undifferentiated mesenchymal cells differentiate into chondrocytes that will form a matrix template surrounded by the perichondrium. Second, vascular invasion of the cartilage template allows the migration of osteoblast precursors and establishment of the growth plates and primary ossification center. During this process, cartilage is removed and replaced by bone and bone marrow. During intramembranous ossification, mesenchymal cells differentiate directly into osteoblasts to form bone without an intermediate cartilage template (Karsenty et al., 2009; Berendsen et al., 2015).

Bone is a highly vascularized tissue and bone vessels are fundamental for bone development, regeneration and remodeling (Temple et al., 2014). A correct angiogenesis during bone formation, both in endochondral and intramembranous ossification processes, is pivotal to the proper development of bones (Berendsen et al., 2015). Likewise, during bone regeneration, newly formed vessels are fundamental for bringing oxygen and nutrients to the forming bone, in addition to inflammatory cells and bone precursors (Filipowska et al., 2017). Lastly, during bone remodelling vasculature is essential to the bone resorption/formation coupling, bringing osteoclast precursors and osteoprogenitors to the site where the remodeling process will take place (Lafage-Proust et al., 2015).

Even if this thesis work was focused on deciphering the role of fat on bone formation and development and not the one of vessels and their related factors, it is important to know that also this tissue is fundamental for a proper skeletal development and maintenance.

II.2. The adipose tissues

The adipose tissue plays a central role in the maintenance of energy homeostasis. The major constituents of the adipose tissue are the adipocytes, cells characterized by the ability to store lipids in periods of energy excess (mainly in the form of triacylglycerols) and to mobilize them during energy deprivation. A mature adipocyte is usually characterized by a large lipid droplet (LD) surrounded by a thin layer of cytoplasm with the nucleus displaced at the margin of the cell (Ali et al., 2013). In humans, the adipose tissue consists mainly of adipocytes (35-70%) and a stromal fraction characterized by endothelial cells, immune cells, pre-adipocytes, and adipose progenitor/stem cells (stromal vascular fraction) (Frühbeck, 2008). As well described in the literature (Rosen et al., 2014), in mammals two principal types of adipose tissue exist: the white adipose tissue (WAT) and the brown adipose tissue (BAT). More recent studies have highlighted the existence of a third type of adipose tissue known as beige or brite (brown and white), possessing both WAT and BAT characteristics (Harms et al., 2013). Last but not least, a fourth

adipose depot is present in mammals, mostly within the bone marrow of long bones, which is the bone marrow adipose tissue (BMAT) (Paula et al., 2020) (Figure 8).

Each one of these four adipose depots possesses distinct characteristics and functions, which define their unique nature. For instance, we can separate WAT, BAT and beige adipose tissues from BMAT thanks to their location and generally refer to them as peripheral adipose tissues.

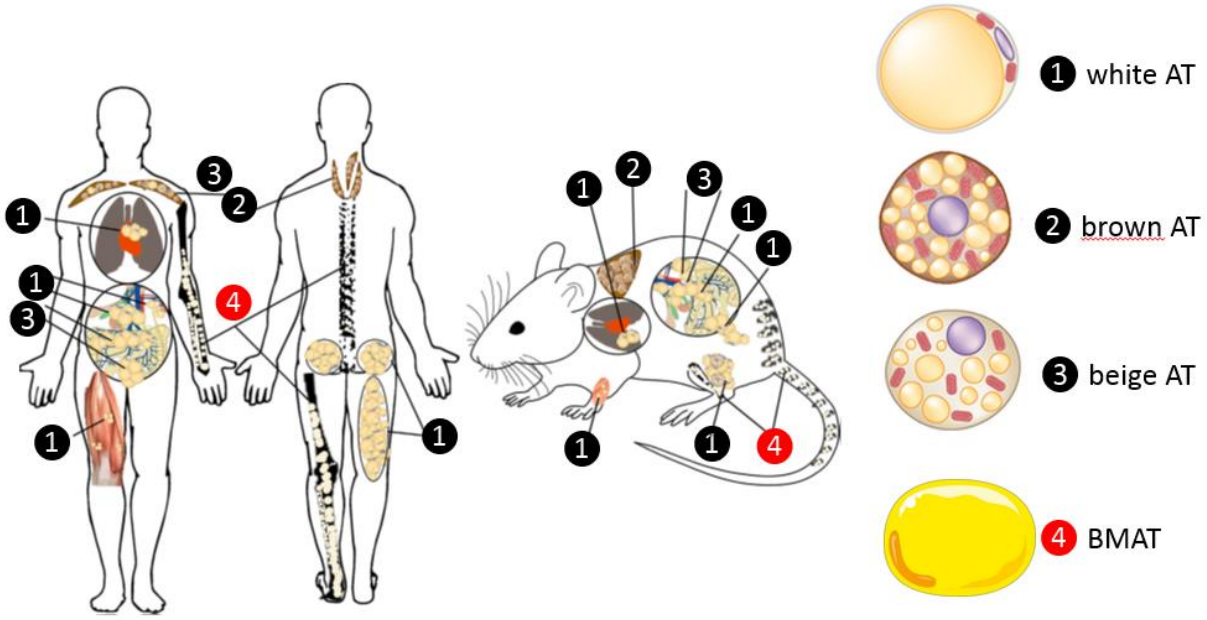


Figure 8. Human and rodent adipose tissues.
In human adults and rodents the main white adipose tissue depots are subdivided into subcutaneous and visceral adipose tissue. Brown adipose tissue is located deep within the neck (humans) and in the interscapular region (rodent), with both of these depots containing classical brown adipocytes. Humans also contain some brown adipose tissue superclavicularly, although this may also contain white and beige adipocytes. BMAT shows a similar distribution in rodents and humans, predominating in the arms and legs. Adapted from Suckachi et al, Curr Op Pharm, 2016.

II.2.1. WAT

Considered the most common adipose tissue in mammals, WAT is composed by big, unilocular white adipocytes. The two most important WAT deposits in our body are the visceral depots (vWAT), which include the omental, mesenteric, retroperitoneal, gonadal, and pericardial WAT and the subcutaneous depots (sWAT), mainly located under the skin within the abdominal and gluteofemoral regions (Wajchenberg, 2000; Kwok et al., 2016). Interestingly, expansion of the visceral fat has been frequently associated with pathologies like obesity, also linked with cardiovascular diseases and cancer (Chait et al., 2020). At the opposite, the important lipid storage capacity in the subcutaneous fat has been shown to protect the individuals against the metabolic complication associated with overweight (Tran et al., 2008). The WAT has various functions in mammals, mainly associated with the tissues that are physically close to it. WAT supplies adjacent tissues with nutrients, it has shock-absorbent roles when close to the skeleton

(for example the multiple fat cushions present within the feet for pressure dissipation), it also has antimicrobial functions within the skin and the instestine (Zwick et al., 2018). However, probably the most important function of WAT is the maintenance of the energy homeostasis, which happens through its ability to store energy in the form of lipids and release it to fuel the body when needed. In the post-prandial state, triglycerides (TGs) are transported towards the adipose tissue as TG-rich lipoproteins, chylomicron and Very Low Density Lipoproteins (VLDL). The uptake of dietary TGs in WAT is mediated by the lipoprotein lipase (LPL), an enzyme secreted from adipocytes and relocated on the lumen of WAT capillaries. Here, it intercepts and processes TGs to release FFAs, that will then be internalized and directed towards lipogenesis, the step which involves the processing of FFAs for storage (Goldberg, 1996; Sethi et al., 2007). In the fasting state, the LD TGs are hydrolysed through a regulated mechanism called lipolysis that releases free fatty acids (FFAs) (Sethi et al., 2007). Lipolysis is catalysed by two main lipases, the hormone sensitive lipase (HSL) and the Adipose Triglyceride Lipase (ATGL), activated during the fasting state and inhibited during the post prandial state. HSL is a lipase of 81 kDa and its activity is highly regulated by hormones like catecholamines, secreted by the adrenal medulla or directly by the sympathetic innervation of the adipose tissue, the growth hormone (GH) or the atrial natriuretic peptide (ANP), while insulin is an inhibitor of its activity (Bolsoni-Lopes et al., 2015). ATGL converts TAG to DAG, being highly specific for TAG hydrolysis, and it is responsible for large part of the lipolytic activity. Together, ATGL and HSL are responsible for about 95% of the hydrolysis of TAG (Bolsoni-Lopes et al., 2015). The adipocyte storage and release activity is tightly regulated by endocrine signals: insulin promotes storage and noradrenalin promotes release. Therefore, adipocytes are able to integrate endocrine signals from other organs, but white adipocytes can also be endocrine producing cells. In this regard, WAT secretes a lot of adipokines, which are adipocyte-secreted cytokines involved in various endocrine functions. For instance, among the most known ones, we can find adiponectin, leptin, FGF21, BMPs, and cathepsins. Adipokines both act directly on adipose tissues, mediating for example adipocyte metabolism and function, but also at the systemic level, acting on different tissues and regulating appetite, insulin secretion, energy expenditure and much more (Fasshauer et al., 2015). There will be a paragraph especially dedicated to the main adipokines in the section II.3 (page 39).

II.2.2 BAT and beige adipose tissue

Compared to WAT, BAT is mainly characterized by adipocytes, which present an organization of lipids in multiple small droplets, a great accumulation of mitochondria and a central nucleus. These different characteristics reflect specific functions. While WAT is dedicated to the storage of lipids and to energy metabolism, BAT is focused on thermogenesis through dissipation of chemical energy and increased fatty acid oxidation (Fenzl et al., 2014). This process, first discovered in hibernating animals and infants (Rylander et al., 1972), is called “uncoupled respiration” and it is mediated by the uncoupling protein-1 (UCP1). Beige

or brite adipose tissue is defined as the groups of UCP1-expressing adipocytes that are located outside traditional brown fat depots and frequently amongst white adipocytes.

Like brown adipocytes, beige adipocytes can convert energy from lipids into heat and develop in response to various stimuli like cold exposure (Harms et al., 2013). Despite their similarities, beige and brown adipocytes are considered distinct cell types, with distinct developmental origins: BAT develops embryonically, while beige fat is induced in white adipose tissue postnatally (Petrovic et al., 2010; Wu et al., 2012). The development of beige fat is not clear yet, but generally it is both thought to originate from a white-to-brown trans-differentiation and from a *de novo* differentiation from specific precursor cells (Xue et al., 2007; Wu et al., 2012; Ikeda et al., 2018). Some speculations have been made on the possibility of beige adipocytes as interchangeable between their thermogenic and energy storage cells, ascribing their plasticity and therefore adaptability to different fat depots in which they reside (Wu et al., 2013).

II.2.2.1. Origins and development of peripheral adipose tissues

The peripheral adipose tissues start developing during the prenatal period, around the 14th and 16th week of pregnancy, and at birth the white adipose tissue (WAT) accounts for around 16% of total body weight, with the brown adipose tissue (BAT) constituting around 2-5% (Frühbeck, 2008).

Both WAT and BAT origin from the mesoderm, but they are known to come from different mesenchymal stem cell lineages. BAT adipocytes are closely related to skeletal muscle precursor cells, since they were shown to share a common progenitor expressing the myogenic lineage marker Myf5 (Seale et al., 2008). On the other hand, white and beige adipocytes originate from Myf5 negative progenitors (Petrovic et al., 2010; Wu et al., 2012). Once passed this developmental difference however, the consequent adipogenic differentiation process is mediated by common transcriptional factors, mainly PPARγ and C/EBPα (Farmer, 2006).

From adipocyte stem cells to mature adipocytes, several stages of development take place, mostly regulated by a cascade of transcription factors (Lefterova et al., 2009). In particular, the peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs), more specifically C/EBPα, β and δ play fundamental roles in adipocytes maturation. PPARγ is induced during adipogenesis and it is necessary and sufficient for adipocyte differentiation, while C/EBPs were also among the first transcription factors to be discovered as fundamental to the adipocyte differentiation (Wang et al., 1995; Tanaka et al., 1997; Yang et al., 2005).

II.2.3. BMAT

The bone marrow (BM) is a soft, sponge-like tissue richly innervated and highly vascularized located within the bone cavities. It contains stem cells, progenitors and mature cells from various different lineages (Reagan et al., 2016). Structurally, the BM consists of two distinct but interdependent compartments: the hematopoietic system and the bone marrow stroma. The hematopoietic system is composed of hematopoietic stem cells (HSCs), red blood cells, white blood cells and platelets. The bone marrow stroma includes reticular cells, fibroblasts, endothelial cells, adipocytes and bone marrow stromal cells/skeletal stem cells (BMSCs). Thus, the BM is a complex and dynamic tissue composed by an elaborate extracellular matrix in which bone marrow adipocytes (BMAds) are enclosed along with hematopoietic and mesenchymal cells. The bone marrow adipose tissue (BMAT), which represents almost the totality of the BM volume in adults, has been first identified from autopsy samples by a german pathologist named Franz Ernst Christian Neumann. In 1868, he noticed how during aging « large yellow cells » replaced the red marrow in the human distal skeleton (Rosen et al., 2009). Afterwards, several observations were made on the presence of adipocytes within the BM (Piney, 1922) and by the 1980s the BMAT had not only been recognized as a constant feature of the BM and a separate adipose tissue, but also its potential correlation with bone mass started to be investigated, both on animal models and observations in humans (Meunier et al., 1971). More recently, the advent of magnetic resonance imaging (MRI) allowed to study the human BMAT non-invasively and added more knowledge on BMAT differences based on gender, ethnic and age variations (Karampinos et al., 2018; Paccou et al., 2019).

II.2.3.1 Origins and development of BMAT

In humans, BMAT is virtually absent at birth and develops postnatally starting from the distal ends of long bones. So, from the early beginning and throughout life, the red hematopoietic marrow is replaced by adipocytes within the BM, giving rise to the yellow marrow. It starts at the distal bones and, with a centripetal pattern, it develops first within the epiphyses of long bones and then it expands to the diaphyses (Blebea et al., 2007) (figure 9). By the age of 25, BMAT accounts for about 70% of the BM volume in healthy adult individuals and almost 10% of the total fat mass (Suchacki et al., 2016). This red-to-yellow conversion gradually continues even after reaching maturity both in men and women and it goes on all through the rest of life.

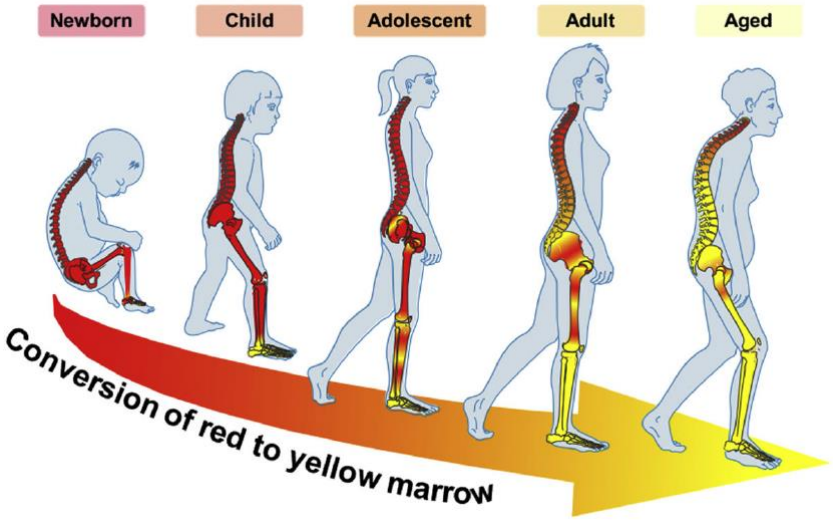


Figure 9. The conversion of red to yellow marrow during aging. From Li et al, Bone, 2018.

As a confirmation of the continuous BMAT expansion, even in older individuals its increase has been observed in some age-related and pathological conditions like osteoporosis or diabetes (Justesen et al., 2001 a). Around menopause, women experience a strong BMAT expansion due to estrogen deficiency. This brings women around 65 years old to develop almost 10% more BMAT than men (Griffith et al., 2012). This condition is also well recapitulated in animal models of ovariectomy, where the absence of estrogens brings to an increased BMAT volume correlated with osteoporosis and increased fracture risk (Beekman et al., 2019).

II.2.3.2 Bone marrow mesenchymal stromal cells

The origin of BMAds is known to be different from the other fat depots in our body. More specifically, within the BM there is a subgroup of multi-potent and self-renewal cells with the ability to give rise to bone, cartilage, the stroma and BMAds and those are known as Skeletal Stem Cells (SSCs) or Bone Marrow Mesenchymal Stromal Cells (BMSCs) (Bianco et al., 2015). Those are all skeletal lineages, connected to one another via specific developmental processes. Especially regarding BMAds, in a review from 2016, Tencerova and Kassem have well described BM adipocyte differentiation and regulation from BMSCs within the BM. Several transcription factors are well characterized as regulator of BMSC differentiation (Tencerova et al., 2016). Osteogenic cells depend on the expression of the transcription factors Runt-related protein-2 (Runx2) and Osterix-1 (Osx-1), while adipogenic cells require the expression of PPARγ and CEBPα (Figure 10).

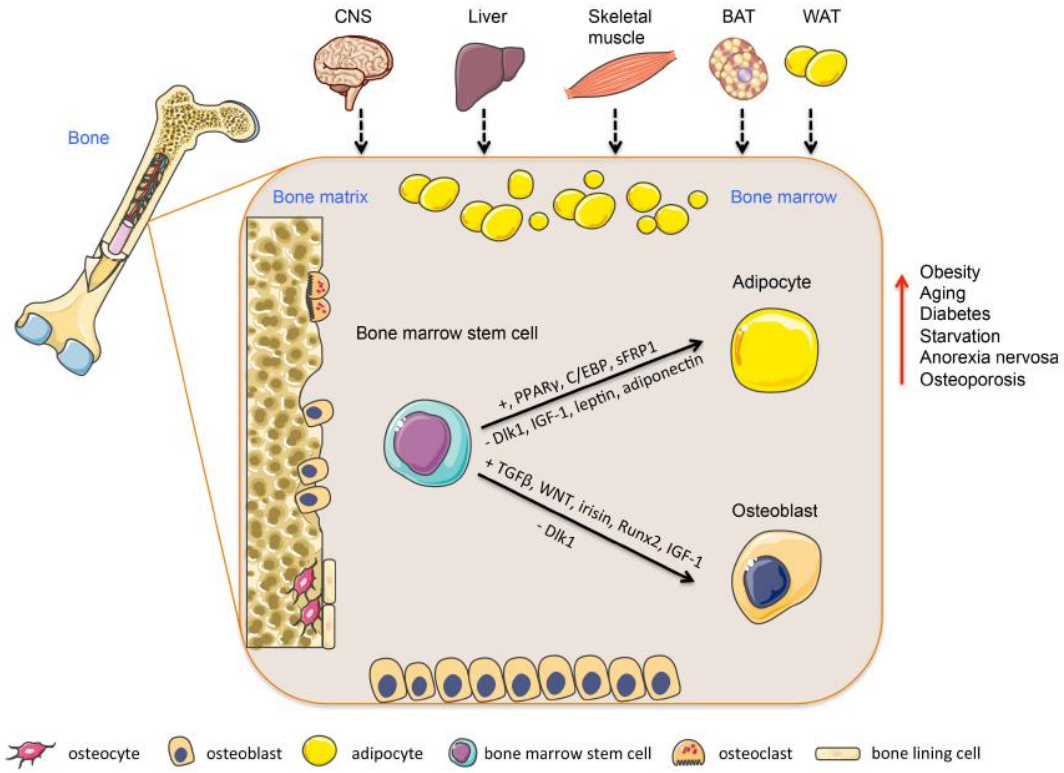


Figure 10. Regulation of bone marrow stem cells differentiation into adipocytes or osteoblasts.
The bone marrow is a heterogeneous organ, consisting of various different cell types. Mesenchymal stromal cells give rise mainly to osteoblasts and adipocytes through a well-regulated process by several transcription factors and secreted molecules. From Tencerova & Kassem, 2016

Interestingly, Ge and colleagues determined the importance of the ERK/MAPK signalling in the regulation of BMSCs differentiation into osteoblasts or adipocytes, identifying Runx2 and PPARγ as fundamental for this process (Ge et al., 2016). In particular, they showed how phopshorylation of Runx2 or PPARγ can, respectively, stimulate osteoblast differentiation and block adipogenesis. Mouse models have also showed this osteo/adipo balance: postnatal Runx2 deletion induces a decreased bone mass and a parallel increased BMAT, showing the importance of Runx2 postnatally to maintain the balance between bone and adipocyte formation (Tosa et al., 2019). In the same time, suppression of PPARγ in mesenchymal stem cells enhances osteoblast differentiation and inhibits adipogenesis *in vitro* (Sun et al., 2013).

Unravel BMSCs heterogeneity: single cell RNA sequencing

BMSCs represent a very heterogeneous cell population. Nowadays probably one of the most powerful approach to analyse this heterogeneity is represented by single cell RNA sequencing (scRNAseq). Using optimized next-generation sequencing (NGS) technologies, scRNAseq examines the sequence information from individual cells and it quantifies their transcriptome. Determining the differences in gene expression between individual cells has the potential to identify rare populations that cannot be detected from an analysis of pooled cells. In the case of BMSCs, since they represent a small portion of the entire

BM, a step of enrichment is needed before performing the scRNAseq analysis. There are two possible ways to do so: a negative and a positive selection. The negative selection involves the sorting of the cells we do not want to analyse and remove them by FACS sorting prior to the scRNAseq. Usually the hematopoietic cells are sorted using specific markers like CD45, for the hematopoietic lineage (except erythrocytes), Ter119, for the erythroid lineage and CD31 for the endothelial lineage. This selection indeed allows an unbiased screening for the BMSCs population, but it also may under-represent a minority cell type. On the other hand, the positive selection actually involves staining of the cells of interest by lineage specific CRE lines crossed with tdTomato strains and then capture them by FACS sorting. This selection allows the discovery of small cell populations otherwise lost in the process, but it is also biased by the selection choice and it may miss unknown cell types. Both types of selection have been used recently to describe various sub-populations of BMSCs, allowing to broaden knowledge on BMSCs heterogeneity. For example, using the negative selection approach, Baryawno and colleagues were able to identify 17 bone marrow stroma cells clusters (Baryawno et al., 2019). Among them, a cluster of Lepr+ mesenchymal stromal cells has been identified with pre-adipocytic features. Another cluster representing MSC-descendent osteolineage cells (OLCs) was identified, which expressed Runx2, the master regulator transcription factor controlling the commitment of BMSCs to OLCs. Another interesting scRNAseq analysis performed using a positive selection is the one from Helbling and colleagues, who were able to isolate pure cellular populations of CXCL12 abundant reticular cells (CARc), platelet-derived growth factor receptors (PDGFR)-α^{+}Sca1^{+}cells (PαSc), arterial and sinusoidal endothelial cells (AECs and SECs) from the BM using FACS sorting (Helbling et al., 2019). They were subsequently able to obtain cell-subset specific transcriptomic fingerprints for phenotypic detection and analysis of spatial localization of those populations. Doing so, they were able to obtain a global transcriptomic profiling of the bone marrow stromal microenvironment, identifying specific signatures regarding BMSCs during development, in adult mice and also with aging. Another interesting study has been performed by Zhong and colleagues, who used a positive selection approach taking advantage of a Col2.3-Cre Rosa26 <lsl-tdTomato> (Col2:Td) mouse model, which was previously reported to label bone marrow mesenchymal lineage cells (Zhong et al., 2020). They identified 9 different clusters and thanks to the analyses of lineage-specific markers, they were also able to identify the ones with gene signatures for osteoblasts, osteocytes, adipocytes and chondrocytes. Last but not least, Tikhnova and colleagues, also using a positive selection thanks to a lineage-specific Cre line, were able to isolate LepR^{+} cells and Col2.3^{+} cells, which both marked osteoblasts (Tikhonova et al., 2019). Indeed, scRNAseq analysis in those populations allowed to identify four clusters, two of them expressing adipogenesis-associated markers and the other two osteogenesis-associated markers. The Col2.3^{+} cells were transcriptionally split into three populations, one of them expressing osteogenic as well as chondrocyte-specific genes, potentially representing cells undergoing osteogenic transdifferentiation. To conclude, while many markers have been suggested and used in mice, there is none who has been unanimously accepted and some

already in use may also be expressed in other cell types. The need of a single accepted combination of markers to identify BMSCs is more and more clear and this technique is a promising step to finally find one.

II.2.3.3. Features and regulation of BMAT

More and more data have been collected over the years, giving the possibility to better characterize BMAT and to distinguish different types of BMAds. In 1976, Tavassoli and his group described two different types of adipocytes within the BM depending on their histological staining: adipocytes within the red marrow stained positively for the performic acid-Schiff staining (PFAS), while those within the yellow marrow were negative (Tratwal et al., 2020). Lately, the use of osmium staining technique has added some new notion about murine BMAT organization, confirming how two distinct adipocyte populations can be identified. Those two populations have been named the constitutive MAT (cMAT) and the regulated MAT (rMAT) (Scheller et al., 2015) (Figure 11). The distinction has been made based on their localization (the cMAT localizes in the distal part of the BM, the rMAT in the proximal one), the size of their adipocytes (the cMAT is characterized by large and round adipocytes, the rMAT by smaller ones interspersed with red marrow), their regulation by cold exposure (the rMAT decreases while the cMAT remains unchanged), their lipid content (increased cMAT unsaturation compared to rMAT) and their gene expression profile (Craft et al., 2019).

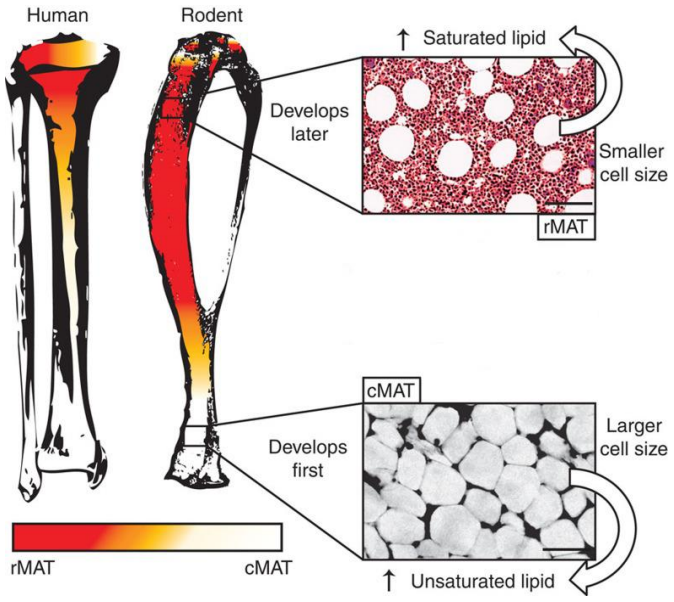


Figure 11. rMAT vs cMAT. In the human and mouse tibia, cMAT is present in the distal portion of the bone. The red marrow contains rMAT adipocytes. cMAT is the first to develop, with an increased proportion of unsaturated fatty acids and larger adipocytes compared to rMAT, which develops throughout life and is characterized by more saturated adipocytes with a smaller size. Adapted from Scheller et al, Nat. Comm., 2015.

As its name says, the rMAT's most important feature is probably its greater propensity to be regulated compared to the much more stable cMAT in cold exposure situation. In conditions of estrogen deficiency both rMAT and cMAT are significantly increased, but rMAT expansion is way more extensive (Lecka-Czernik et al., 2017). This is true for many other conditions, like high fat diet-induced obesity

(Scheller et al., 2016) or caloric restriction (Cawthorn et al., 2014), making the rMAT the most studied BMAT component to investigate concerning BMAT changes in different contexts.

II.2.3.4. *in vitro* and *in vivo* approaches for BMAT investigation

Both *in vitro* and *in vivo* models have been used and are currently exploited and ameliorated to better characterize the BMAds and understand their role in the BM environment and in the context of numerous pathologies where the BMAT is altered. A recent review from the Methodologies Working Group of the International Bone Marrow Adiposity Society has well summarized all the most commonly used models nowadays (Tratwal et al., 2020).

In vitro models

BMAds are located within a complex microenvironment inside the bone, where their recovery as primary mature adipocytes for culture experiments is complicated and still in need of a well-established method. This is why for the vast majority BMAds are studied in culture as the result of *in vitro* adipogenic differentiation from BMSCs. *In vitro models* of BMAT can be divided in two main categories: 2D and 3D *in vitro* cultures. Usually the BM from samples isolated from both human or animal sources is plated and expanded in 2D, in culture plastic dishes and the resulting adherent monolayer of stromal cells is considered to be made of BMSCs. Those cells are then induced to differentiate into adipocytes using specific adipogenic media and eventually they will give rise to multilocular or unilocular adipocytes depending on their level of maturation. Finally, the obtained BMAds can be validated as adipocytes using Oil Red O staining, or gene or protein expression analyses. Even if less representative of the BM microenvironment, 2D BMSCs cultures have been commonly used for a long time, mostly because they are easier to obtain and also because, until recently, a 3D culture model had not yet been described. The work of Tratwal and colleagues has well summarized the variability among the existing BMSCs isolation and 2D adipogenic differentiation protocols (Tratwal et al., 2020). First of all, it is important to consider the BMAT site of isolation, since not all BMAT sources are the same. Long bones (mostly tibias) and tail vertebrae are the most common sites used to obtain murine BMAT samples. Isolating BMAT from tibias is usually preferred, mostly due to their highest adipocytic content and their low presence of trabecular bone. Therefore, the BM from tibias is usually collected through a gentle flushing or centrifugation after cutting the epiphyses. Another fundamental aspect for BMSCs adipogenic differentiation is the choice of medium to use. Many different cocktails have been described in the literature and the most commonly used components are:

- *Dexamethasone*, a corticosteroid that strongly induces the expression of the transcriptional regulator C/EBP-α;
- *Rosiglitazone,* an anti-diabetic drug, acts as a potent ligand for PPARγ inducing the adipogenic differentiation;
- *Isobutylmethylxanthine (IBMX),* a phosphodiesterase inhibitor which ultimately activates PPARγ expression;
- *Indomethacin,* a cyclooxygenase-2 (COX-2) inhibitor that significantly upregulates PPARγ expression;
- *Insulin,* an adipogenic hormone which induces the differentiation of pre-adipocytes to mature adipocytes;
- *Hydrocortisone,* a natural glucocorticosteroid which inhibits the expression of bone differentiation markers and increases the expression of adipocyte differentiation markers.

Obviously, not all of them are used at once, usually a mix of three components is frequently adopted (See Table 4).

Table 4. Variability of in vitro murine bone marrow stromal cell adipogenic differentiation protocols.
From Tratwal et al, 2020. Refs. 147: Sreejit et al, Cell Tissue Res. (2012); 84: Yu et al, Cell Stem Cell. (2018); 156: Abdallah et al, Bone. (2018); 160: Zhu et al, Nat Protoc. (2010); 56: Fan et al, Cell Metab. (2017); 135: Fairfield et al, Bone (2019); 5: Ambrosi et al, Cell Stem Cell (2017).

References	(147)	(84)	(156)	(160)	(56) (135)			(5)	
Maintenance									
Medium	DMEM:F12	α-MEM	RPMI	α-MEM	α-MEM or DMEM			60% DMEM low Glc: 40%MCDB	
Serum	20% FBS	10% FBS	10% FBS	10% FBS	20% or 10% FBS			2% FBS	
Other	0.1 mM NEAA	200 μM NEAA						ITS, linoleic acid, dexa, AA, EGF, LIF, PDGFBB, bFGF	
Adipogenic									
Medium	DMEM:F12	α-MEM	DMEM	α-MEM	α-MEM or DMEM			60% DMEM low Glc: 40%MCDB	
Serum	20% FBS	10% FBS	9% horse serum	10% FBS	20% or 10% FBS			2% FBS	
Other		200 μM NEAA							
IBMX (μM)	500	500	450	0.5	500			0.5	
Dexa or Hydrocortisone (μM)	1	0.5	0.25	1	1			1	
Indomethacin (μM)	100	60						50	
Insulin (μg/ml)	5		5	0.01	10	10	10	5	5
Rosiglitazone (μM)			1		1	1			
T3 (nM)								1	1
Differentiation time	2 weeks	3 weeks	12 days	2 weeks	2–4 days	3–4 days	4 days	48 h	5 days

NEAA, non-essential amino acids; dexa, dexamethasone; ITS, insulin-transferrin-selenium mix; AA, L-ascorbic acid 2-phosphate; EGF, epidermal growth factor; LIF, leukemia inhibitory factor; PDGFBB, platelet-derived growth factor BB; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum.

While the most commonly used cocktail is composed by dexamethasone, IBMX and Insulin, this medium results in strong induction of adipogenic differentiation and may mask subtle differences that otherwise could have been observed. This is why for our adipogenic differentiation experiments, we decided to choose a cocktail characterized by Indomethacin, Hydrocortisone and IBMX added to the serum-containing medium.

3D *in vitro* models of the BM are less common, but surely more close to the real microenvironment in which BMAds are located and allow a better understanding of BMAds behaviour *in vivo.* One of the few 3D models recently published is the silk-based scaffold and BMSCs-derived BMAds from Michaela R. Reagan and her group (Fairfield et al., 2018). This silk-based scaffold is described in comparison with 2D cultures in the classic plastic dishes. Those scaffolds are robust, allow for human and murine BMSCs differentiation into BMAds and mostly induce a healthier environment for BMSCs-derived BMAds and the activation of their signalling pathways. Other 3D *in vitro* models have been described in the literature, but they have been developed mostly for obesity and WAT modelling rather than BMAT (Emont et al., 2015; Murphy et al., 2019). Another circumstance in which recreating the bone marrow niche via 3D models has been of fundamental importance is cancer and the related malignant marrow diseases. As well resumed by Ham and colleagues, various 3D models are currently in use to develop strategies and eventually treatments, mostly regarding multiple myeloma, leukemia and all the bone-metastatic tumors (Ham et al., 2019).

In vivo models

I*n vivo* models of BMAT usually rely on rodents: mostly mice, rats and rabbits. *In vivo* BMAT is usually studied in long bones (tibia and femur), but also within the vertebrae. Mice are probably the most used animal model to study bone marrow adiposity. To investigate and quantify BMAT, several techniques are commonly used. First, BMAds can be assessed through histological analyses of bone sections like H&E staining, which can provide informations on the adipocytes number, size. If combined with histomorphometry, it can also add essential data regarding BMAds spatial localization within the BM. Second, and probably the most promising technique to study BMAT, x-ray microfocus computed tomography (µCT) is currently considered one of the most powerful tools to examine mineralized tissues. More recently, with the development of contrast-enhancing staining agents, the 3D imaging of soft tissues has become more and more feasible. The possibility to use this technique to study BMAT has been first illustrated by Scheller and colleagues, who used osmium-tetroxide (OsO4) to visualize and quantify BMAT amount and distribution in murine long bones (Scheller et al., 2014). After that, OsO4 staining has been widely used in this field and it is now considered the gold standard technique to image and study BMAT. Despite its specific binding to lipids, which makes this contrast agent ideal to be used for BMAT imaging, OsO4 is highly toxic and needs to be handled cautiously (Makarovsky et al., 2007). A huge step forward in the use of µCT to study BMAT has been achieved by Kerckhofs and colleagues at the Catholic University of Leuven, who recently described a new contrast agent named Hafnium-based Wells-Dawson polyoxometalate (Hf-POM), capable to simultaneously visualize mineralized and non-mineralized skeletal tissues (Kerckhofs et al., 2018). This new technique not only allows the concurrent 3D visualization of bone, blood vessels and BMAT, but it also provides the possibility to perform histology and immunostaining

afterwards, since it does not alter the sample. Moreover, Hf-POM-based contrast-enhanced microfocus computed tomography (CE-CT) allows to not only quantify BMAT volume within the bone, but it also allows the measurement of BMAds number and diameter. Unlike OsO4, Hf-POM is non-toxic and therefore safer to manipulate (see figure 12 for representative images of BMAT obtained by OsO4 and Hf-POM). The research group of Prof. Kerckhofs has gone way further in the analysis and development of other contrast agents to allow a better visualization of soft tissues. Their most recently published paper reveals four additional Wells-Dawson POMs, showing potential great advances for 3D imaging and contrast-enhanced X-ray computed tomography (de Bournonville et al., 2020).

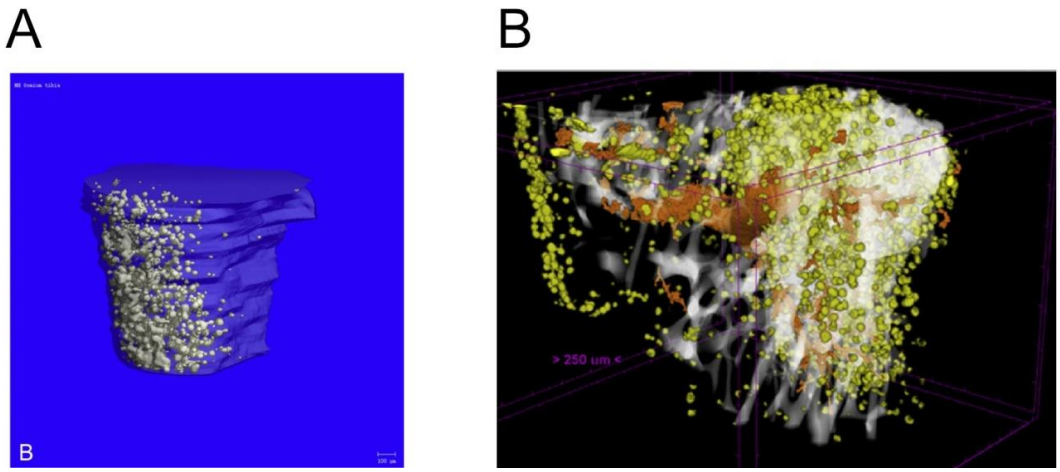


Figure 12. Representative images of BMAT obtained by A) OsO4 and B) µCT analyses.
In both reconstruction we can see the shadow of the bone containing the BMAT (A) or even the trabecular detail (B). Interestingly, with the Hf-POM staining we are also able to distinguish vessels (in orange) and observe their 3D organozation correlated to bone (in white) and adipocytes (in yellow). From Scheller et al, nat comm. 2015 and Kerckhofs et al, Biomaterials, 2018).

Moreover, BMAT can be further examined through the analysis of adipocyte-specific markers like PPARγ, FABP4, Adiponectin or Perilipin: changes in their expression profile through RT-qPCR could bring important informations to the investigation. Although it is not sufficient per se, this technique can provide useful information if combined to other research data.

II.3. The relationship between bone and the adipose tissues

II.3.1. The bone as an endocrine organ

All along the last century, a series of new discoveries have highlighted endocrine functions of the skeleton in addition to its structural role, bringing out its involvement in energy metabolism. Proteins secreted from bone, and in particular from osteoblasts, osteocytes and osteoclasts, exert a paracrine regulation on osteoblastogenesis, osteclastogenesis and angiogenesis. Moreover, it also secretes hormones involved in the energy homeostasis like FGF23 and Lipocalin-2 (Han et al., 2018). FGF23 is secreted by

osteoblasts and osteocytes within the skeleton and plays important roles in modulating phosphate homeostasis: it inhibits phosphate reabsorption and vitamin D3 production in the kidney and suppresses parathyroid hormone (PTH) synthesis in the parathyroid gland, which reduces the circulating phosphate levels (Han et al., 2018). Lipocalin-2 is also secreted by osteoblasts and it inhibits food intake by binding to the melanocortin 4 receptor in the hypothalamus. Moreover, it regulates glucose tolerance, insulin sensitivity, and insulin secretion to maintain glucose homeostasis (Han et al., 2018).

Interestingly, another protein synthetized by osteoblasts called osteocalcin (OCN) has a positive effect on insulin resistance: OCN-deficient mice are hyperglycaemic, hypoinsulinaemic, have low beta cell mass, decreased insulin sensitivity, increased fat mass and decreased energy expenditure (Lee et al., 2007). Since OCN is an osteoblast-secreted protein, these findings once again demonstrate that bone is an endocrine organ affecting energy metabolism, in particular regulating insulin secretion and glucose homeostasis through OCN (Figure 13, (Ducy, 2011)).

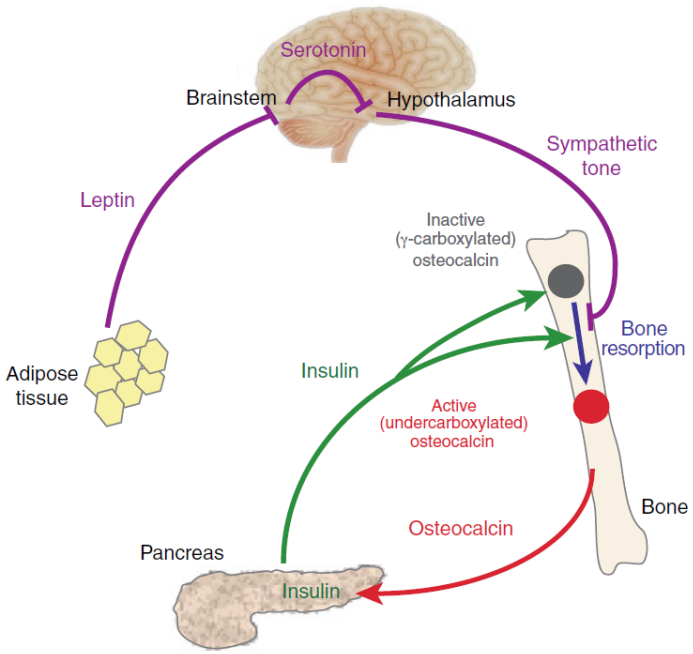


Figure 13. representation of the cross-talk between bone and energy metabolism. From Ducy et al, Diabetologia, 2011.

Interestingly, the recently published work of Diegel and colleagues showed controversial results regarding OCN and its role in energy metabolism (Diegel et al., 2020). Indeed, they showed how mice with a new OCN-encoding genes double KO (generated by CRISPR/Cas9-mediated gene editing) have no alteration of serum glucose levels and male fertility. This absence of a metabolic phenotype, previously described in OCN-deficient mice (Lee et al., 2007; Oury et al., 2011), is surprising and not yet explained by the authors.

Another paper published around the same time by Moriishi and colleagues also showed an absence of a metabolic phenotype in OCN-deficient mice, this time generated by the replacement of OCN-encoding

genes by the *neo* gene (Moriishi et al., 2020). This lack of reproducibility, which is extremely important for science, will be probably clarified in the future with further investigations.

The notion of the skeleton as an endocrine organ should not be astonishing, since any apparatus that undergoes endocrine regulations usually is capable to put into action feedback loops to control them in return. Here we introduce the relationship between bone and fat, which is in close contact with the skeleton and acts on it via the secretion of different factors like adipokines.

II.3.2. Peripheral adipose tissues and secreted adipokines

For a long time, WAT and BAT have been appreciated for their functions of energy storage, insulation, protection and non-shivering thermogenesis, while their role in energy metabolism as an endocrine organ has been recognised only later on with the discovery of their principal secreted adipokines: leptin and adiponectin. Leptin and adiponectin, respectively identified in 1994 (Zhang Y et al., 1994) and 1995 (Scherer et al., 1995), probably represent the two most studied adipocyte-secreted factors and are also among the most important mediators of the adipose tissue crosstalk.

Adiponectin

First discovered in 1995 as an adipocyte complement-related protein of 30 kDa (Acrp30), adiponectin is a secreted protein predominantly produced by the adipose tissue (Scherer et al., 1995; Hu et al., 1996). Adiponectin is the major marker of mature adipocytes. Its structure is characterized by an N-terminal variable region followed by a conserved collagenous domain and a C-terminal globular domain (Shapiro et al., 1998) and it forms low molecular weight trimers, intermediate molecular weight hexamers and high molecular weight dodeca- to octadecamers (Fig. 14).

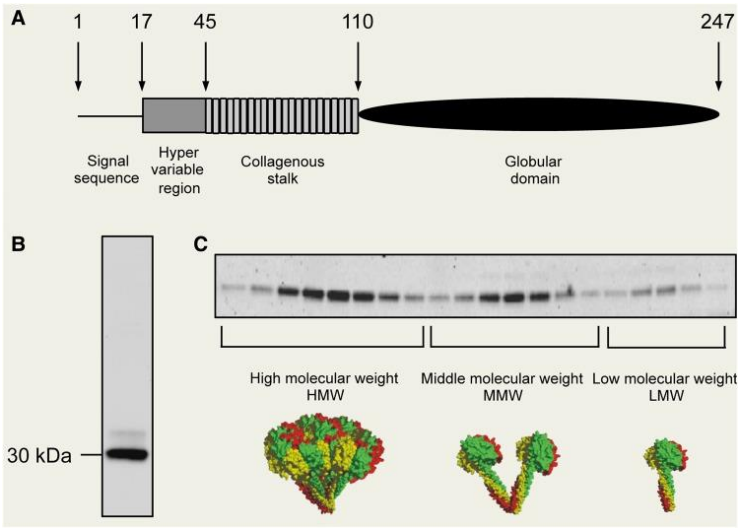


Figure 14. The biochemical properties of Adiponectin.
A) The domain structure of mouse adiponectin. B) Mouse adiponectin exists as a major 30 kDa band in reducing and denaturing SDS-PAGE. C) Native adiponectin shows three distinct complex forms, high molecular weight (HMW, 12–18mer), middle molecular weight (MMW, hexamer), and low molecular weight (LMW, trimer) From (Wang et al., 2016).

As it can be easily understood, the native form of adiponectin is highly in contrast with the simple outcome of an SDS-PAGE obtained in reducing conditions. This is really important, considering that different forms of adiponectin can have distinct biological effects (Waki et al., 2003; Pajvani et al., 2004). First findings on the biological function of adiponectin came out in 2001, where this adipokine was shown to be implicated in glucose metabolism, enhancing insulin action (Berg et al., 2001; Yamauchi et al., 2001). Now, we know that adiponectin can also increase β-oxidation in skeletal muscle and suppress lipid accumulation in the liver (Lee et al., 2012; Combs et al., 2014). Various studies have also demonstrated that circulating adiponectin is reduced in subjects presenting insulin resistance, obesity, type 2 diabetes, or cardiovascular disease (Arita et al., 1999; Weyer et al., 2001; Genoux et al., 2020). A decreased adiponectin secretion has been observed in diabetic obese individuals (Weyer et al., 2001). Oshima and colleagues showed that adiponectin has a positive effect on bone mass, activating osteoblasts and suppressing osteoclasts activity (Oshima et al., 2005). Interestingly, BMAT is also capable of secreting adiponectin and contributing to its increase at the serum levels as already discussed before, in particular in a condition like caloric restriction where we also observe an inverse correlation between BMAT and bone (Cawthorn et al., 2014). Circulating adiponectin indeed has various effects on many different tissues via its receptors and the key sites of adiponectin's action are adipose tissue, heart, kidney, liver and pancreas. Without entering too much into details, a summary of its functions is presented in figure 15 (Straub et al., 2019).

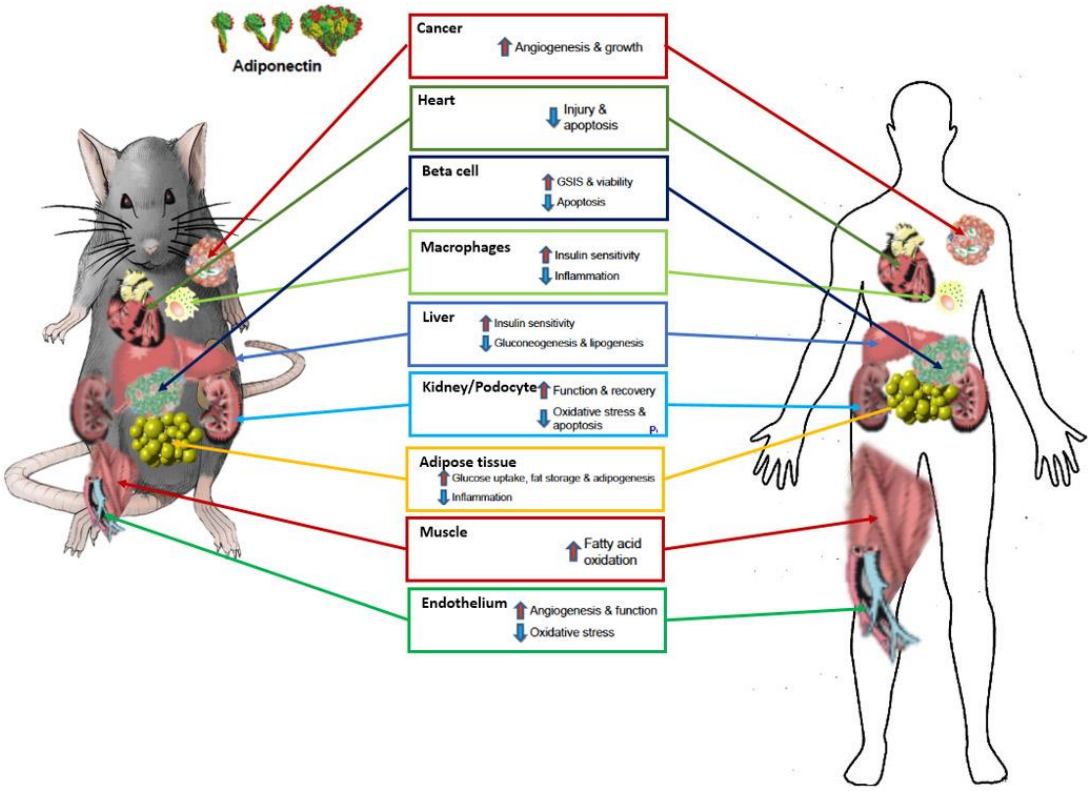


Figure 15. Target tissues and biological activity of adiponectin.
The high molecular weight multimer (HMW) of adiponectin is the most biologically active form, targeting a diverse set of tissues and cell types and regulating important metabolic processes. Adiponectin's effects range from anti-inflammatory and anti-apoptotic to insulin sensitizing. From Straub et al, 2019.

Two isoforms of the adiponectin receptor exist: AdipoR1, which shows an ubiquitous expression, and AdipoR2, more limited to the liver (Yamauchi et al., 2013). Adiponectin receptors are also present on osteoblast and osteoclasts (Berner et al., 2004) suggesting that this adipokine might be a link between bone and fat metabolism. In fact, the possible involvement of adiponectin in the regulation of bone metabolism had already been suggested due to clinical studies showing its inverse correlation with BMD (Biver et al., n.d.). Indeed, *in vitro* studies, animal studies and clinical observations have extensively improved our knowledge on the role of adiponectin on bone metabolism and they were well summarized in the review published by China and colleagues in 2018 (Pal China et al., 2018).

Various *in vitro* studies have been performed on bone cells: adiponectin has been shown to be osteoanabolic, leading to increased proliferation, differentiation and mineralization of osteoblasts. However, in contrast with those observations, this adipokine has also been described as an inhibitor of osteoblasts differentiation, reported to increase RANKL/OPG ratio and therefore favoring osteoclastogenesis and bone resorption (Pal China et al., 2018). As for the *in vivo* reports, animal studies in *Adiponectin*$^{-/-}$ mice fail to provide a clear explanation of the role of this adipokine on bone. Some studies on *Adiponectin*$^{-/-}$ mice reported a reduced BMD compared to WT mice (Naot et al., 2016), however it was also shown that adiponectin-deficient mice were protected from ovariectomy-induced bone loss and also seem to have a better bone quality (Wang et al., 2013). Studies performed on mice overexpressing the adiponectin receptor AdipoR1 displayed an increased bone mass and decreased bone resorption, while transgenic mice having high levels of circulating adiponectin showed reduced BMD and bone biomechanical parameters (Oshima et al., 2005; Jiang et al., 2011). Lastly, clinical studies on both men and women showed a possible inverse correlation between circulating adiponectin and BMD in men, but without consistent results concerning women (Pal China et al., 2018). Evidently, we are still far from understanding the effect of adiponectin on bone and further research needs to be done.

Leptin

Leptin is a 16 kDa adipocyte-derived protein first discovered in 1994 (Zhang Y et al., 1994). It is encoded by the *ob* gene and it acts at the level of the hypothalamus through the leptin receptor (Ob-R) (L et al., 1995). The main function of leptin is to regulate food intake and body weight (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Friedman et al., 1998). The more we accumulate fat in our depots, the more the levels of circulating leptin are high, feeding back to the brain to limit food intake (Friedman, 2002). As a demonstration, most obese patients and animals exhibit increased levels of circulating leptin (Frederich et al., 1995; Considine et al., 1996; Lee et al., 1997). This actually suggests the development in diet-obese patients of a leptin resistance, very similar to the one observed for insulin in the case of T2D, though the mechanisms that lead to leptin resistance are still unclear (Sáinz et al., 2015). Leptin also has an effect on bone: both directly on bone cells and indirectly through its action in the brain,

in particular in the central nervous system (CNS) (Karsenty et al., 2012; Reid et al., 2018). The leptin receptor (LepRb) is expressed in primary osteoblasts and also in osteoblastic and chondrocytic cell lines, indicating a direct effect of leptin on bone. Various data show an anabolic effect of leptin on osteoblasts, displaying an increased osteoblast differentiation and bone matrix synthesis. Leptin also appears to regulate osteoclastogenesis (Reid et al., 2018). Leptin receptors are present on chondrocytes and it seems that leptin plays a key role in regulating their proliferation and differentiation in the growth plate. Leptin receptors are also highly expressed in the hypothalamus, where leptin exerts its indirect effects on bone influencing levels of thyroid hormones, growth hormone, cortisol and sex steroids, which all have effects on bone (Reid et al., 2018). The work of Takeda and colleagues in 2002 elegantly identified the neuronal networks required for leptin anti-osteogenic function, demonstrating that the SNS is a negative regulator of bone formation (Takeda et al., 2002). Thus, the effects of leptin on bone are conflicting and mediated both directly and indirectly through the brain. The balance between the direct effect on bone and the one leptin plays on it indirectly through the brain are well summarized by figure 16 (from Reid et al, 2018). Those two effects determine leptin's effect on bone physiology, even though a lot of work still needs to be done to better characterize it.

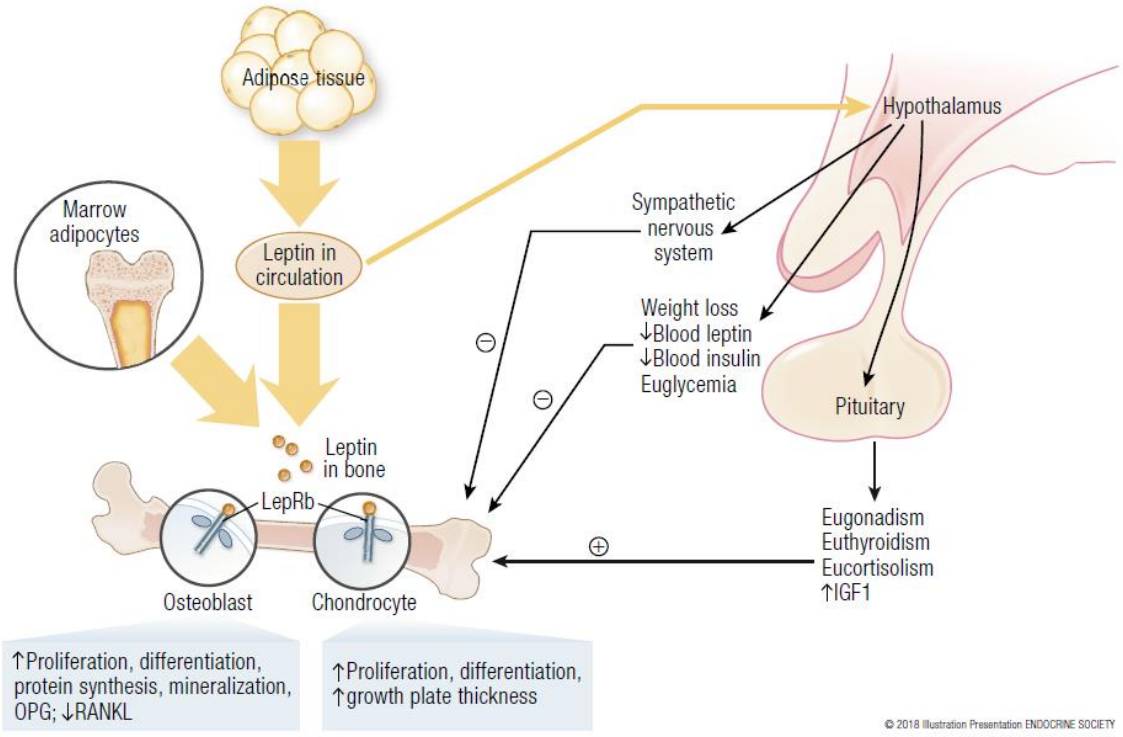


Figure 16. Pathways by which leptin, originating in adipose tissue, bone marrow adipocytes, and osteoblasts, influences bone. From Reid et al, 2018

II.3.3. BMAT as an endocrine organ

Even though a lot has been accomplished in the past years to investigate BMAT and its physiological role, we are still a long way off its complete understanding. However, if we consider that only recently the BMAT has been in the spotlight of various different research projects compared to other tissues, we are already in possession of a discrete amount of data to start speculating about it.

Local functions

The bone marrow is a closed system and it is only logical to imagine a local function of this tissue, especially concerning hematopoiesis and bone metabolism.

1.BMAT and bone

Regarding the relationship between bone and BMAT, marrow adipocytes are linked to bone forming cells via the same progenitor cell and a complex regulatory system mediates the maturation in one or the other cell type. In addition, BMAds with their paracrine function can influence bone homeostasis locally through their secreted factors. Indeed, they were shown not only to secrete adipokines like leptin or adiponectin, both linked to bone metabolism, but also a series of mediators like IL-6 or RANKL known for having a negative effect on osteoblasts and bone homeostasis (Veldhuis-Vlug et al., 2017). Lastly, they were also shown to secrete free fatty acids, acting as energetic substrate for bone and hematopoietic cells. BMAds are also exploited by neoplastic cells to survive and proliferate. (Figure 17 from (Veldhuis-Vlug et al., 2017)).

In particular regarding the role of BMAds in providing FFAs as an energetic substrate, a recently published review from Rendina-Ruedy and Rosen nicely summarized the current knowledge in this regard (Rendina-Ruedy et al., 2020) : various data showed the presence of lipid droplets within osteoblasts and/or osteocytes, suggesting the possibility of BMAds acting as energy storage and mobilise FFAs to bone cells when they are in demand of energy. Being in constant need for energy to sustain its homeostasis, the skeleton consumes mostly glucose. Glucose is a relative ease source of fuel, coming from the extracellular fluids and being rapidly used to generate ATP through glycolysis. However, FFAs have more store energy per mole than glucose, and they could represent a relevant source of energy, for example in states of glucose starvation (Rendina-Ruedy et al., 2020).

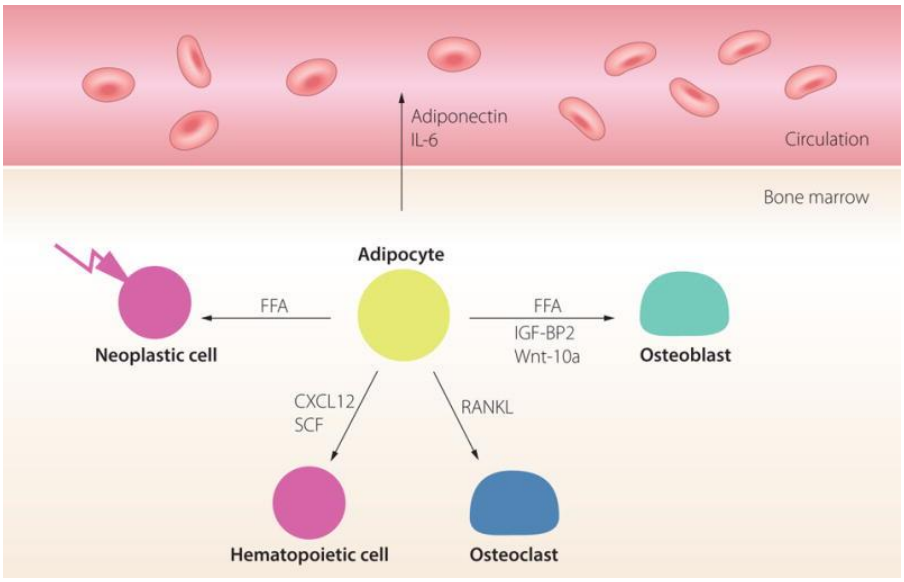


Figure 17. Function of the marrow adipocyte.
The BMAT secretes adipokines and also a series of mediators like IL-6 or RANKL known for having a negative effect on osteoblasts and bone homeostasis. From Veldhuis-Vlug and Rosen, J Intern Med, 2018.

BMAT is strongly correlated with skeletal health. Studies on healthy populations show that BMAT increases with age both in men and women, with men having generally more BMAT (Baum et al., 2018; Justesen et al., 2001). An inverse correlation of BMAT volume and bone mineral density (BMD) has been observed in healthy Caucasian women (Shen et al., 2007), middle-aged men and women (Shen et al., 2012) and also in both young and older individuals (Di Iorgi et al., 2008; Wren et al., 2011). Nevertheless, during puberty, the amount of marrow fat is positively associated with total bone mineral content in girls (L Newton et al., 2013) and, even if men have greater BMAT mass compared to women, they also have greater bone mass. These observations highlight a complex relationship between BMAT and bone, which cannot be probably described just as an inverse correlation.

Nonetheless, in addition to data regarding healthy individuals, both in rodents and humans an increased BMAT is associated with aging, a natural condition, which is notably related to a decline in skeletal health. It is mostly observed in women going through menopause, but it is also present in men. With aging and loss of sex hormones, an increase in BMAT is mostly evident and it is frequently associated with a loss in bone quality and the presence of osteoporosis (Justesen et al., 2001; Cohen et al., 2012; Schwartz et al., 2013; Li et al., 2014; Woods et al., 2020).

An increased BM adiposity has also been observed in circumstances where the BMD is altered due to pathological conditions, here are the major cases :

- *Anorexia nervosa*, an eating disorder strongly associated with loss of body weight, reduction of body fat, a strong increase in fracture risk, a significant bone loss and a decreased bone turnover (Schorr et

al., 2018). Despite the loss of peripheral body fat, this condition is curiously associated with a marked increase in BMAds (Bredella et al., 2009). At the hormonal level, patients with anorexia nervosa present a series of adaptations in order to minimize energy expenditure. One of the first adjustments set up by the organism to face chronic starvation and economize energy is the process of reproduction, via a functional hypothalamic amenorrhea. This condition leads to an estrogen deficiency which, as in post-menopausal women, is associated with an increased BMAT. Women with anorexia nervosa also have higher levels of cortisol and adiponectin and decreased levels of Igf-1 and leptin, which seem to be important determinants of increased BMAT, however it is not clear yet if there is a link between those factors and the increased BMAT (Devlin et al., 2010; Cawthorn et al., 2014; Sebo et al., 2019). Interestingly, part of the bone loss and the increased BMAT seem to be reversible and this happens when patients resume a normal nutrition and they recover their body weight (Paccou et al., 2019). Notably, a recently published paper compared the regional characteristics of BMAT in hips of underweight and weight-recovered anorexic women (Badr et al., 2019). Using magnetic resonance spectroscopy they observed a negative correlation between total hip BMD and BMAT with discrete regional patterns and significant correlations between bone marrow adiposity, lipid unsaturation levels, total hip BMD, and the body fat percentage. Overall, their results show regional specificities in the bone marrow fat profile, alterations and impaired bone health that appear to persist after recovery (Badr et al., 2019).

- *Type 1 diabetes*, a metabolic condition also known as juvenile diabetes, which is a chronic pathology where the pancreas produces no insulin. Insulin is the hormone needed to internalise glucose inside our cells and therefore producing energy. Patients with type 1 diabetes show also a dramatic bone loss and an increased risk fracture (Starup-Linde et al., 2019), which is followed by an ambiguous BMAT phenotype: mouse models for this pathology always have an increased BMAT (Botolin et al., 2007), while patients do not display a defined BMAT phenotype (McCabe, 2007; Slade et al., 2012).

- *Type 2 diabetes and obesity*, type 2 diabetes (T2D) is an adult-onset metabolic condition, where patients present high blood sugar, insulin resistance, and relative lack of insulin. Obesity is a pathological condition in which patients accumulate an excess of body fat, which may lead to a negative effect on body health. While not all T2D patients are overweight, a vast majority of obese individuals also have T2D (Verma et al., 2017). BMAT phenotype in obese people is not quite clear: BM fat is found unchanged (Ermetici et al., 2018) or increased (Bredella et al., 2011) compared to control in patients, while high-fat feeding mice always show a markedly increased BMAT (Doucette et al., 2015; Tencerova et al., 2018). Interestingly, a recently published review from Benova and Tencerova finely summarizes the changes induced by obesity on the BM homeostasis (Benova et al., 2020). Indeed, this condition induces a general inflammation that challenges immune responses throughout the body. This has consequences on the BM,

altering its cellular composition and also BMSCs differentiation capacity, contributing to an alteration in bone homeostasis and the production of secretory factors within the BM (Benova et al., 2020). In T2D marrow fat is also reported unchanged (Baum et al., 2012) or increased (Ferland-McCollough et al., 2018) depending on the bone site. In both pathological cases, fracture risk is always increased, however BMD is frequently unchanged or increased (Schwartz, 2016; Walsh et al., 2017).

- *Pharmacologic treatments.* Thiazolidinediones (TZDs) are oral anti-diabetes agents that act mainly as insulin-sensitizers by activating PPARɣ. For example the PPARɣ-agonist Rosiglitazone, part of the TZDs family, is known to increase BM adiposity and fracture risk in treated patients and, moreover, to stimulate murine BMSCs differentiation into adipocytes over osteoblasts (Rzonca et al., 2004; Kahn et al., 2006).

- *Immobilization,* frequently due to decreased mobility or forced bed rest, is known to have an overall impact on our organism (Pavy-Le Traon et al., 2007). Usually with immobilization comes reduced bone density, which has been frequently observed in astronauts after long periods in space. Another consequence of forced rest is BMAT increase (Trudel et al., 2009).

Consequently, an increased bone marrow adiposity with a parallel bone loss is also observed in animal models of aging, ovariectomy, caloric restriction, skeletal unloading or following treatement with rosiglitazone or glucocorticoid (Rharass et al., 2018). At the same time, several examples of genetically modified mice have also shown the same inverse correlation, displaying an increased BMAT at the expenses of the bone tissue (see table 5).

Table 5. Genetically modified mice showing an inverse correlation between bone and BMAT.

Mouse model	BMAT phenotype	Bone phenotype	Mechanism	Refs
Prx1 Cre - PTH1R fl/fl	Increased BMAT	Decreased bone mass	Increased adipogenic and decreased osteogenic differentiation	(Fan et al., 2017)
Pgc1α -/-	BMAT accumulation	Bone loss	Increased adipogenic and decreased osteogenic differentiation	(Yu et al., 2018)
Pkd1 cKO in OBs	Increased BMAT	Osteopenia	Enhanced adipogenic differentiation and impaired osteoblastogenesis	(Xiao et al., 2010)
Rnf146fl/fl - Osx-Cre	Increased fat stores (included BMAT)	Osteopenia	RNF146 suppresses adipogenesis and enhances osteoblastogenesis	(Matsumoto et al., 2017)

The existence of this strong relationship between BMAT and bone has induced very recently the group of Zou and colleagues to investigate the effect of postnatal adipocyte elimination on bone cells (Zou et al., 2020). Using an adiponectin-cre line crossed with mice expressing an inducible primate diphtheria toxin receptor (DTR), they showed how the ablation of adiponectin-expressing cells in the bone marrow leads to a quick and profound increase in systemic bone mass. This rapid increase in bone mass seems to be due to bone morphogenetic protein receptor induction combined with epidermal growth factor receptor stimulation. Those results show that indeed BMAT regulates skeletal mass and, even if further analyses need to be performed, the authors suggest the possibility of using these experiences to an eventual application in pathological bone loss conditions.

2.BMAT and hematopoiesis

Regarding hematopoiesis, about ten years ago, Naveiras and colleagues showed how an adipocyte-rich marrow in mice exhibits altered hematopoiesis with reduced hematopoietic stem cells (HSCs) and progenitors compared to a less fatty marrow (Naveiras et al., 2009). Interestingly, when irradiated to mimic hematopoietic failure, 8-week-old mice display a shift from red to yellow marrow, with a red to yellow conversion passes from 6% to 53% (tibia). This is also the case in leukemia patients post-chemotherapy, where the more adipocytes in the bone marrow the less hematopoietic cells were observed (from O. Naveiras, oral communication at the BMA meeting 2020). During her talk at the BMA2020, Olaia Naveiras also showed unpublished *in vitro* and *in vivo* experiments demonstrating again the negative effect of marrow adipocytes on hematopoietic progenitors, showing how the degree of adipocyte maturation inversely correlates with hematopoietic proliferation. A paper published in 2017 seemed to be contradictory, stating that bone marrow adipocytes are a niche component that promotes hematopoietic regeneration through the secretion of the stem cell factor (SCF) (Zhou et al., 2017). However, in the light of what has been discovered by Naveiras and her group, this suggests that, through their maturation, marrow adipocytes probably lose the ability to secrete the SCF and this leads to their negative effect on hematopoietic cells. This means that the support for hematopoiesis is provided rather by stem cells or progenitors than mature adipocytes within the BM (from O. Naveiras, oral communication at BMA 2020). This could maybe be confirmed by co-culture experiments of murine BMSCs or mature adipocytes and murine hematopoietic stem cells.

Systemic functions

To understand the physiological role of the BMAT, we cannot consider its function only locally within the bone marrow cavity, but it is necessary to further take into account its broader role in the systemic energy homeostasis. It is clear that BMAT secretes factors, which are capable not only to influence locally the tissues in close contact to it, but also systemically. Indeed, BMAT is recognized as an endocrine tissue,

capable of secreting adipokines. It also expresses the insulin receptor and responds to insulin-sensitizing anti-diabetic drugs (thiazolidinediones). These data clearly link BMAT with energy metabolism (Sulston et al., 2016; Li et al., 2019 a). This connection can also be perceived in BMAT response to physiological and pathological changes in energy balance. With ageing, over-nutrition or malnutrition for example the volume of BMAT changes and, even though a clear mechanism has yet to be found, it is evident that BMAT is not isolated and only interacting directly with its adjacent tissues. In this regard, Cawthorn and colleagues showed for the first time that BMAT is indeed an endocrine organ capable of secreting adiponectin, at least in particular conditions like caloric restriction (Cawthorn et al., 2014). In particular, they observed an increased serum adiponectin associated with an increased BMAT in patients with anorexia nervosa. To investigate the capacity of the BMAT to secrete adiponectin, they cultured explants of rabbit and human WAT and BMAT and analyzed the secreted adiponectin within the medium. They observed a much higher level of adiponectin secreted from the BMAT than the WAT, confirming the endocrine ability of the BMAT in secreting adiponectin and its greater contribution to it compared to the WAT (Figure 18A).

Additionally, Suchacki and colleagues recently investigated the role of BMAT in energy metabolism showing that it can potentially influence glucose homeostasis at the systemic level (Suchacki et al., 2020). In this work, they revealed BMAT as a molecularly distinct fat depot from WAT, BAT and beige adipose tissue, with a lower expression of key insulin signalling components (Figure 18B). Consequently, they also showed how BMAT resists insulin-stimulated glucose uptake compared to WAT and, again, how it exhibits an increased basal glucose uptake even compared to muscle or WAT (Figure 18C). This paper clearly states the unique nature of BMAT from the other fat depots and it unravels its distinctive role in glucose homeostasis.

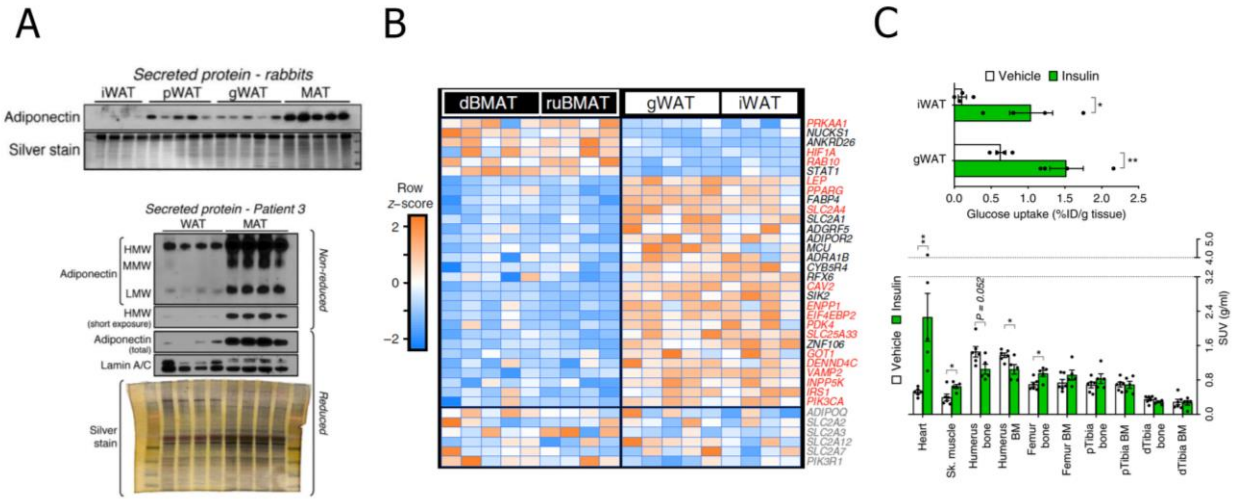


Figure 18. BMAT as an endocrine organ and a regulator of glucose homeostasis.
A) Immunoblots and silver stain of conditioned media from WAT and MAT explants from one rabbit (representative of 7 animals) and from one of 3 patients. B) Heatmaps of transcripts differentially expressed between BMAT and WAT in a rabbit cohort and C) glucose uptake in iWAT, gWAT and other tissues (including BMAT) of insulin- or vehicle-treated mice. A) from Cawthorn et al, Cell Metab, 2014. B) and C) from Suchacki et al, Nat Comm, 2020.

AIMS OF THE THESIS

This PhD project originated from the identification by our research group of PiT2 as a new regulator of endochondral and intramembranous ossification and an essential determinant for bone quality and strength (Beck-Cormier et al., 2019). PiT2 was originally described as a Na-Pi co-transporter of the Slc20 family, largely expressed in mammals and encoded by the *Slc20a2* gene. Our research group documented that PiT2-deficient mice have shorter long bones, reduced cortical thickness, reduced bone mineral density and bone mineral content, and severe reduction of bone biomechanical properties.

Strikingly, PiT2-deficient mice displayed normal *in vivo* osteoblastic bone formation, together with normal differentiation and mineralization properties of primary osteoblasts in vitro. In addition, osteoclastic bone resorption *in vivo* was unaltered in mutant mice, and *in vitro* mineralization process of chondrocytes from PiT2-deficient mice was normal. These observations suggested that the observed *in vivo* bone phenotype in PiT2 KO mice may originate from the absence of PiT2 in cells from a non-skeletal origin.

Considering the strong relationship existing between bone and the adipose tissues, both BMAT and peripheral adipose tissues, we decided to characterize the adipose tissues and their impact on skeletal health in the absence of PiT2.

To do so, **the first objective of this work was to characterize the effect of PiT2 deficiency on adipose tissues**, both BMAT and peripheral adipose tissues, by using *in vivo* (PiT2 KO mice) and *in vitro* approaches (PiT2-deficient bone marrow stromal cells (BMSCs) and knockdown of PiT2 in adipocyte-like 3T3-L1 cells).

The second objective of this work aimed at elucidating the molecular mechanisms of action of PiT2 by identifying new partners that could explain its function in bone or adipose tissues. To do so, we performed a yeast two-hybrid screen, co-immunoprecipitation assays and expression analyses to validate the interaction of PiT2 with potential proteic partners and determine their expression pattern.

RESULTS

I. Article 1: PiT2 deficiency protects against age-induced fat gain

The issue

As described in the introduction, the skeletal phenotype of the PiT2-deficient mice cannot be accounted by alterations in either cellular differentiation or mineralization of primary osteoblasts and chondrocytes. Given the strong relationship between bone and BMAT described in the literature, we hypothesized that the skeletal phenotype of PiT2 KO could, at least partially, result from anomalies of the adipose tissues and decided to characterize them in our model.

Methodological approach

In vivo (PiT2KO mice) and *in vitro* (PiT2-deficient bone marrow stromal cells (BMSCs) and knockdown of PiT2 in adipocyte-like 3T3-L1 cells) approaches have been used to characterize the effect of PiT2 deficiency on adipose tissues. Contrast-Enhanced high-resolution µCT (CE-CT) analyses have allowed the quantification of BMAT volume within PiT2-deficient tibias in young and adult PiT2KO mice. BMSCs from tibias of young PiT2KO mice were characterized by FACS analyses and their adipogenic differentiation assessed *in vitro*. For FACS analyses, BMSCs have been quantified as $Ter119^-$ $CD45^-$ $Sca1^+$ $CD44^+$ $CD105^+$. Markers have been chosen among the most commonly used to identify the murine BMSCs population in the literature (Boxall et al, Stem Cells Int, 2012). Adipogenic progenitors have been quantified as $Ter119^-$ $CD45^-$ $CD31^-$ $Sca1^+$ $CD24^-$, as previously described (Ambrosi et al, Cell Stem Cell, 2017). 3T3-L1 cells have been used to determine the effect of the absence of PiT2 on mature adipocytes. Lastly, the metabolic phenotype of the PiT2KO mice have been analysed by performing glucose and insulin tolerance tests and ELISA assays for the main adipokines and insulin on serum from PiT2WT and PiT2-deficient mice at different time points. The food intake was also determined, along with the mRNA expression of the PiT proteins and the major adipogenic markers on whole tibia for the BMAT, on peripheral adipose tissues (iWAT and gWAT) and on culture cells at different time points of differentiation.

Results

In this article, we show that the absence of PiT2 induces an increased BMAT in young PiT2KO mice, which however is not accounted by major alteration in the BMSCs population nor its increased adipogenic potential. We also show that the absence of PiT2 protects against the expected increase in adipose tissue volume during aging, both regarding peripheral AT and BM adiposity, without inducing any major metabolic changes.

Conclusion

This work showed that PiT2 is a potential regulator of adipose tissues' homeostasis, being involved in their age-related increase without altering the energetic metabolism. Further analyses need to be performed in order to broaden our knowledge on the role of PiT2 in the context of skeletal health. This work has nevertheless expanded our comprehension of the role of PiT2 and identified it as a new potential regulator of obesity. The next step will be to perform a high fat diet protocol on PiT2KO mice. This model of induced obesity will give us the possibility to challenge both the BMAT and the pAT in order to test whether the absence of PiT2 will protect from the obesity-induced fat depots increase. Another experiment we are planning to perform is a protocol of caloric restriction on PiT2KO mice. This protocol, as for the HFD, will give us the possibility to challenge both fat depots in the absence of PiT2, but with an opposite outcome: indeed, with a caloric restriction protocol a dramatic decrease in peripheral fat depots is expected, while the BMAT is usually increased.

PiT2 deficiency protects against age-induced fat gain

Journal:	*Journal of Bone and Mineral Research*
Manuscript ID	Draft
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Frangi, Giulia; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie Guicheteau, Marie; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie Pyka, Gregory; UCLouvain, Biomechanics lab, Institute of Mechanics, Materials, and Civil Engineering; KU Leuven, Department of Materials Engineering Kerckhofs, Greet; UCLouvain, Biomechanics lab, Institute of Mechanics, Materials, and Civil Engineering; UCLouvain, Institute for Experimental and Clinical Research; KU Leuven, Department of Material Science and Engineering Feyeu, Magalie; Structure Federative de Recherche Francois Bonamy, MicroPiCell Véziers, Joëlle; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie; CHU Nantes, PHU 4 OTONN; Structure Federative de Recherche Francois Bonamy, SC3M facility Guihard, Pierre; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie Halgand, Boris; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie Source, Sophie; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie Guicheux, Jerome; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie; Centre Hospitalier Universitaire de Nantes, Pôle Hospitalo-Universitaire 4 (PHU4) - Ostéo-articulaire - Tête et Cou - Odontologie - Neurochirurgie - Neuro-traumatologie (OTONN) Prieur, Xavier; Institut du thorax, Inserm U1287; Université de Nantes, UFR Odontologie Beck, Laurent; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie Beck-Cormier, Sarah; Inserm UMR 1229, RMeS Regenerative Medicine and Skeleton; Université de Nantes, UFR Odontologie
Keywords:	Genetic animal models < ANIMAL MODELS, Bone-fat interactions < SYSTEMS BIOLOGY - BONE INTERACTORS, Stromal/Stem Cells < CELLS OF BONE
Abstract:	Skeletal health is strongly linked to adipose tissues through their endocrine relationship in which bone influences adipocyte function and

	adipocyte-derived cytokines (adipokines) regulate bone cells. We recently showed that the sodium-phosphate co-transporter PiT2/SLC20A2 is an important determinant for bone mineralization, strength and quality. While PiT2 is broadly expressed, nothing is known about its function in adipose tissues. Here, we identify impaired adipose tissues in the PiT2-/- mice. The absence of PiT2 in juveniles leads to a dramatic increase in the bone marrow adipose tissue (BMAT) that does not originate from an increased adipogenic differentiation of bone marrow stromal cells. Despite the BMAT increase, plasma levels of Adiponectin were unchanged, contrasting with what is observed in mice during caloric restriction when BMAT is also increased. In contrast to young mice, we show a severe reduction of the BMAT volume in adult PiT2-/- mice compare to controls. Furthermore, we show that the absence of PiT2 leads to reduced peripheral white adipose tissues that do not increase with age, without major metabolic alterations. Altogether, our data highlight a novel function of PiT2, as a modulator of age-related fat gain.

PiT2 deficiency protects against age-induced fat gain

Giulia Frangi[1,2], Marie Guicheteau[1,2], Grzegorz Pyka[3,4,], Greet Kerckhofs[3,4,5,6], Magalie Feyeux[7], Joëlle Veziers[1,2,8,9] , Pierre Guihard[1,2], Boris Halgand[1,2,8], Sophie Source[1,2], Jerôme Guicheux[1,2,8], Xavier Prieur[10] Laurent Beck[1,2] and Sarah Beck-Cormier[1,2*]

[1] INSERM, UMR 1229, Regenerative Medicine and Skeleton (RMeS), Université de Nantes, Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation, Nantes-Atlantique (ONIRIS), Nantes, FRANCE

[2] Université de Nantes, Unité de Formation et de Recherche (UFR) Odontologie, Nantes, FRANCE

[3] Biomechanics lab, Institute of Mechanics, Materials, and Civil Engineering, UCLouvain, Louvain-la-Neuve, BELGIUM

[4] Department of Materials Engineering, KU Leuven, Leuven, BELGIUM

[5] IREC, Institute of Experimental and Clinical Research, UCLouvain, Woluwé-Saint-Lambert, BELGIUM

[6] Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, BELGIUM;

[7]MicroPICell facility, SFR-Santé, INSERM, CNRS, UNIV Nantes, CHU Nantes, Nantes, FRANCE

[8]Centre Hospitalier Universitaire (CHU) Nantes, Pôle Hospitalo-Universitaire 4 (PHU4) - Ostéo-articulaire - Tête et Cou - Odontologie - Neurochirurgie - Neuro-traumatologie (OTONN), Nantes, FRANCE

[9] INSERM, UMS 016, CNRS 3556, Structure Fédérative de Recherche François Bonamy, SC3M Facility, CHU Nantes, Université de Nantes, Nantes, F-44042, FRANCE

[10] Institut du Thorax, INSERM, CNRS, Université de Nantes, Nantes, FRANCE

*** Correspondence to:**

Sarah Beck-Cormier, INSERM U1229 – RMeS, Faculté de Chirurgie Dentaire, 1, place Alexis Ricordeau 44042 Nantes cedex 1, France. Tel: +33240412821, email: sarah.beck@univ-nantes.fr

Supplemental data

Supplemental Figure 1

Supplemental Table 1

Disclosures

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Abstract

Skeletal health is strongly linked to adipose tissues through their endocrine relationship in which bone influences adipocyte function and adipocyte-derived cytokines (adipokines) regulate bone cells. We recently showed that the sodium-phosphate co-transporter PiT2/SLC20A2 is an important determinant for bone mineralization, strength and quality. While PiT2 is broadly expressed, nothing is known about its function in adipose tissues. Here, we identify impaired adipose tissues in the *PiT2*$^{-/-}$ mice. The absence of PiT2 in juveniles leads to a dramatic increase in the bone marrow adipose tissue (BMAT) that does not originate from an increased adipogenic differentiation of bone marrow stromal cells. Despite the BMAT increase, plasma levels of Adiponectin were unchanged, contrasting with what is observed in mice during caloric restriction when BMAT is also increased. In contrast to young mice, we show a severe reduction of the BMAT volume in adult *PiT2*$^{-/-}$ mice compare to controls. Furthermore, we show that the absence of PiT2 leads to reduced peripheral white adipose tissues that do not increase with age, without major metabolic alterations. Altogether, our data highlight a novel function of PiT2, as a modulator of age-related fat gain.

Key words: PiT2/Slc20a2, bone marrow adipose tissue, peripheral adipose tissue, genetic animal model

Introduction

PiT2 is part of the Slc20 family of sodium (Na)-phosphate (Pi) co-transporters, encoded by the *Slc20a2* gene. While, its paralog PiT1 has long been considered as the fundamental Pi transporter for bone mineralization, recent *in vivo* studies showed that PiT1 is dispensable for an adequate mineralization process[1-6]. In contrast, our group and others have shown the involvement of PiT2 in bone quality, mineralization and strength[7,8]. However, despite the skeletal alterations observed in PiT2-deficient mice (*PiT2*$^{-/-}$), the number and the activity of osteoblasts and osteoclasts are similar between the mutant and *PiT2*$^{+/+}$ (WT) mice. Moreover, primary osteoblast and chondrocyte cultures showed no defects in either cellular differentiation or mineralization, suggesting that the observed *in vivo* bone phenotype could result from the consequence of PiT2 deficiency in cells of non-skeletal origin[8].

Skeletal health is strongly linked to adipose tissues for at least two reasons: through their endocrine functions and the fact that osteoblasts and bone marrow adipocytes (BMAds) both derive from bone marrow stromal cells (BMSCs). Furthermore, since the last decade, a great interest has emerged on bone marrow adipose tissue (BMAT) and its intimate relationship with skeletal health[9]. Several studies showed an inverse correlation between BMAT volume and bone quality, describing an increased bone marrow adiposity in conditions like aging, osteoporosis and other pathologies where the bone mass is decreased[10-15]. In addition, BMAds have been shown to contribute to the increased serum adiponectin in caloric restriction[16]. While inverse correlation is well described in the literature, less is known about its causal factors.

Given the skeletal phenotype observed in the *PiT2*$^{-/-}$ mice and the strong relationship existing between fat and the skeleton, we hypothesized that in addition to the skeletal defects previously described[8], adipose tissues are also altered in the absence of PiT2.

Materials & Methods

Mice

C57BL/6NTac-Slc20a2tm11a (EUCOMM) Wtsi (*Slc20a2*$^{+/-}$, hereafter named *PiT2*$^{+/-}$) heterozygous mice were obtained from the European Mouse Mutant Archive (EMMA) and maintained and genotyped at the University of Nantes. Experiments on mice were conducted according to the French and European regulations on care and protection of laboratory animals (EC Directive 86/609, French Law 2001-486 issued on June 6, 2001). Animal care and maintenance were provided through the University of Nantes accredited animal facility at the "Unité de Thérapeutique Expérimentale" (UTE). Genotyping was performed by PCR as described[8].

WT and *PiT2*$^{-/-}$ mice were analysed at 3 and 16 weeks, 6 months and 1 year old. Three-week-old mice were used for *in vitro* experiments (adipogenic differentiation, flow cytometry), 16-week-old for glucose and insulin tolerance tests. Experimental groups were homogeneous and composed of equivalent animals based on gender, age, and genotype.

Contrast-enhanced high resolution microfocus computed tomography (CE-CT)

Mice were anesthetized with ketamine/xylazine administered intraperitoneally and were perfused transcardially with Phosphate Buffered Saline (PBS) and then 4% Paraformaldehyde (PFA; Sigma Aldrich, USA). Long bones were removed and fixed in 4% PFA for 24 hours at 4°C and stored in PBS at 4°C. Before polyoxometalate-staining[17], the distal end of the bones was removed to allow better diffusion of the contrast agent into the bone marrow compartment. Samples were incubated in the staining solution during 48 hours at 4°C while shaking gently.

CE-CT acquisition and image processing were performed as previously described [17]. Briefly, samples were imaged using a Phoenix Nanotom S (GE Measurement and Control Solutions, Germany) and analysed using DataViewer (Bruker MicroCT, Belgium) for the reorientation of the CE-CT datasets and CTAn (Bruker MicroCT) for the assessment of the fat volume in the proximal

and distal tibias Ad.V relative to Ma.V (bone marrow without trabecular and cortical bones) was determined at 2-µm resolution in a 2mm (proximal) or 4mm (distal) region beginning right underneath the growth plate. Segmentation and morphological assessment of adipocytes was performed as previously described[17].

Tibia explants

Tibia from P21 *PiT2*$^{-/-}$ and WT mice were dissected free of the surrounding soft tissue. After stripping away the periosteum and removing the epiphyses, tibias were cultured at 37°C in 5% CO2 humidified incubator in 96-well plates with 100µl of culture medium (α-MEM supplemented with 0.2% Bovine Serum Albumin, 2mM Glutamine, 10 Units/ml Penicillin and 10µg/ml Streptomycin). After 24 hours of culture, the conditioned media was used to perform an ELISA assay on mouse Adiponectin (Mouse Adiponectin ELISA kit, Catalog number 80569, Crystal Chem).

BMSCs culture

BMSCs were harvested from of 3-week-old *PiT2*$^{-/-}$ and WT tibias. After removing the epiphyses, bones were flushed to isolate total bone marrow cells and BMSCs were expanded in growth media consisting of α-MEM (Eurobio, France) supplemented with 15% fetal bovine serum (FBS), 100IU/mL penicillin, 100mg/mL streptomycin, 2mM Glutamine. At confluence, BMSCs were cultured in adipogenic-inducing media consisting of α-MEM (Eurobio, France) supplemented with 15% FBS, 100IU/mL penicillin, 100mg/mL streptomycin, 2mM Glutamine, 0.5µM 3-isobutyl-1-methylxanthine (IBMX), 0.5µM Hydrocortisone and 60µM Indomethacin (Sigma Aldrich).

3T3-L1 culture and PiT2 knockdown

3T3-L1 cells were maintained in medium A (DMEM glucose 4.5g/L, 10% calf serum, 1% Penicilin/streptomycin, 1% glutamin). Cells were seeded at a density of 20,000 cells/ml in medium B (DMEM glucose 4.5g/L, 10% fetal calf serum, 1% Penicilin/streptomycin, 1% glutamin) and

adipocyte differentiation was induced 2 days after confluence in medium B with 2 µM insulin, 500 mM IBMX and 1 µM dexamethasone (differentiation media induction, DMI). On day 3, medium was changed to medium B supplemented with 2µM insulin and from day 6, wells were switched back to medium B.

For siRNA experiments, at day 6 of differentiation in T75 flask, cells were dissociated with TrypleE express (Thermofisher) and resuspended in medium B (12 ml/Flask). The siRNA mix (20 nm siRNA, 9 µl lipofectamin RNAiMAX (Thermofisher), 191 µl of OptiMEM (Thermofisher), was added to a collagen IV precoated well of 12-well plate, and 1 ml of differentiated adipocyte suspension was added for reverse transfection. Seventy-two hours after, cells were dry-frozen for RNA analysis.

Oil Red O staining and relative quantification

Cells were fixed in 2% paraformaldehyde for 15min, washed with water, incubated with 60% isopropanol for 5min and stained with newly filtered Oil Red O solution for 10min at room temperature. To quantify staining, Oil Red O was extracted from the cells with isopropanol and absorbance of the solution was measured at a wavelength of 520nm to determine the relative amount of dye. Determination of the number of adipocytes (Oil Red O positive cells) and the number of total cells (Hoechst positive cells) was performed by using the High Content Screening Arrayscan (ThermoScientific). Image acquisitions (361 images per well) were performed using the Cellomics ArrayScan VTI/HCS Reader (ThermoScientific) using x5 magnification. Images analysis was performed with CellProfiler[18].

Flow cytometry

Flow cytometry was performed on a BD LSRFortessa™ (BD Biosciences), FACS data were collected using DIVA (Becton Dickinson) and analysed using FlowJo software (Tree Star). For flow cytometry analyses, BMSCs were isolated by flushing tibias of P21 mice, flushed bones were

crushed with a mortar and treated with collagenase for 20min at 37°C to retrieve any remaining BMSCs. The reaction was stopped by adding α-MEM complemented with 15% FBS and the cell suspension was passed through a 70µm cell strainer (BD Falcon, MA, USA) to remove bone fragments. The cells were then added to the previously flushed bone marrow with isolated BMSCs. Cells were centrifuged at 1200rpm for 5min at RT, the pellet was re-suspended in Red Blood Cell Lysis Buffer (Sigma, Product No. R 7757) to eliminate red blood cells and centrifuged again at 1200rpm for 5min at RT. The pellet was re-suspended and cells counted. Five millions cells were stained with antibodies (Supp. Table1) for 30min on ice. Compensation was performed using OneComp^{TM} eBeads Compensation Beads (ThermoFisher Scientific Inc.). BMSCs were analysed as Ter119^{-} CD45^{-} Sca1^{+} CD44^{+} CD105^{+}[19,20] and adipogenic progenitors as Ter119^{-} CD45^{-} CD31^{-} Sca1^{+} CD24^{-}[21].

Histology

Immediately following sample collection, peripheral adipose tissues were fixed in 4%PFA for 24 hours. Five-µm paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and scanned using a Hamamatsu Nano-Zoomer HT (Hamamatsu Photonics KK, Hamamatsu City, Japan) digital scanner at magnification x20.

RNA isolation and RT-qPCR

Total RNA was prepared with TRIzol Reagent (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA was reverse transcribed and analysed on a Bio-Rad CFX96 detection system using SYBR Select Master Mix (Applied Biosystems, Warrington, UK). mRNA levels were normalized relative to beta-glucuronidase (*GusB*) and Pinin (*Pnn*) expression and quantified using the ΔΔCT method[22]. RT-qPCR primers were: Pinin Fw-ACCTGGAAGGGGCAGTCAGTA and Rv-ATCATCGTCTTCTGGGTCGCT, GusB Fw-CTCTGGTGGCCTTACCTGAT and Rv- CAGTTGTTGTCACCTTCACCTC, PiT1 Fw-

TGTGGCAAATGGGCAGAAG and Rv-AGAAAGCAGCGGAGAGACGA, PiT2 Fw-CCATCGGCTTCTCACTCGT and Rv AAACCAGGAGGCGACAATCT, FABP4 Fw-GAATTCGATGAAATCACCGCA and Rv-CTCTTTATTGTGGTCGACTTTCCA, Adipoq Fw-TCTCCTGTTCCTCTTAATCCTGCC and Rv-CATCTCCTTTCTCTCCCTTCTCTC.

Metabolic studies

For food intake measurement, mice were individually housed for a week and their daily food intake was measured for 5 consecutive days with normal chow supplied *ad libitum*.

ELISA assays were used to determine serum insulin, leptin, adiponectin. The following kits were used: the Mouse Adiponectin ELISA kit (Catalog number 80569), Ultra Sensitive Mouse Insulin ELISA Kit (Catalog number 90080) and Mouse Leptin ELISA Kit (Catalog number 90030) from Crystal Chem. Manufacturer's recommended protocols were used for serum measurements.

For glucose tolerance test (GTT) and insulin tolerance test (ITT) analyses, mice were fasted for at least 6 hours. At time 0, a single dose of glucose (2g/kg, Sigma Aldrich, USA) or insulin (0.75IU/kg, Actrapid; Novo Nordisk, France) was administered by *i.p.* injection and blood glucose levels were monitored using a glucometer.

Statistics

Data are expressed as mean S.E.M. GraphPad 5.0 software was used to perform Mann&Whitney and two-way ANOVA tests. A p value of less than 0.05 was considered statistically significant (exact numbers are indicated in the figures).

Results

Abnormal regulation of the BMAT volume with age in *PiT2*$^{-/-}$ mice.

To first address the role of PiT2 in marrow adiposity, we investigated the bone marrow adipose tissue by using Hf-POM-based CE-CT analysis on tibias from 3- and 16-week-old *PiT2*$^{+/+}$ (WT littermate) and *PiT2*$^{-/-}$ mice. The 3D visualization and characterization of the bone marrow adipocytes showed an increased BMAT volume within the proximal tibia of 3-week-old *PiT2*$^{-/-}$ mice compared to WT mice (Fig. 1A, B), consistent with the increased mRNA gene expression of the mature adipocyte markers, *Adiponectin* and *FABP4* (Fig. 1C). Absence of *PiT2* mRNA expression was verified in the whole tibia from *PiT2*$^{-/-}$ mice by RT-qPCR (Fig. 1C). No difference in the expression of PiT1 was observed, excluding a possible PiT1-driven compensatory mechanism (Fig. 1C). To determine if the increased BMAT at 3 weeks was associated with an increased secretion of Adiponectin, we cultured tibia explants from 3-week-old *PiT2*$^{-/-}$ and WT mice and analysed adiponectin secretion into the conditioned media. We observed no differences between both genotypes (Fig. 1D).

In contrast to BMAT volume at 3 weeks, CE-CT analyses showed a decreased BMAT volume in *PiT2*$^{-/-}$ proximal tibias compared to the WT at 16 weeks (Fig. 1A, B). Remarkably, BMAT volume in *PiT2*$^{-/-}$ proximal tibias was not different between 3 and 16 weeks, illustrating a blunting of the age-induced BMAT increase in the absence of PiT2 that is normally observed in WT mice (Fig. 1B).

Quantification of the distal BMAT showed no difference between genotypes, both at 3 and 16 weeks. In addition, the age-related increase in the distal BMAT was observed both in WT and *PiT2*$^{-/-}$ mice (Fig. 1B). These results demonstrate that PiT2 deficiency led to a specific alteration of proximal BMAT volume during aging, while the distal BMAT remained unaffected.

PiT2 deficiency does not alter BMSCs adipogenic differentiation

To further investigate the cause of increased BMAT in 3-week-old *PiT2*$^{-/-}$ mice, we quantified the adipogenic progenitors and explored the adipogenic differentiation capacity of BMSCs. Since adipocytes derive from BMSCs, we quantified these cells as Ter119- CD45- Sca1+ CD44+ CD105+ by flow cytometry. The results showed no difference in the proportion of BMSCs in the *PiT2*$^{-/-}$ samples compared to the WT (Fig. 2A). Next, analyses of adipogenic progenitors as Ter119^{-} CD45^{-} CD31^{-} Sca1^{+} CD24^{-} showed that the proportion of adipogenic progenitors in the *PiT2*$^{-/-}$ mice was equal to that of WT mice (Fig. 2A).

Adipogenic differentiation analyses of BMSCs isolated from tibias of 3-week-old *PiT2*$^{-/-}$ and WT mice did not show differences between mutant and WT cells, neither by quantification of Oil Red O positive cells nor by *Adiponectin* and *FABP4* mRNA expression analyses (Fig. 2B-D). As observed *in vivo*, PiT1 expression showed no differences between *PiT2*$^{-/-}$ and WT cells (Fig. 2D). Altogether, these results revealed that the absence of PiT2 did not alter neither BMSCs populations nor adipogenic differentiation in young mice.

Decreased peripheral adipose tissues volume in *PiT2*$^{-/-}$ mice without major metabolic alterations

Since *PiT2*$^{-/-}$ mice have reduced body weight[7,8,23], we addressed whether peripheral white adipose tissue (WAT) weights were altered. After normalising to body weight, we showed that gonadal WAT (gWAT) and inguinal WAT (iWAT) weights were lower in *PiT2*$^{-/-}$ mice compared to WT (Fig. 3A), despite no differences in food intake (Fig. 3B). H&E staining of adipose tissues showed no histological difference between *PiT2*$^{-/-}$ and WT mice (Fig. 3C). As observed in the whole tibias, absence of *PiT2* mRNA expression was verified and PiT1 expression was not altered in *PiT2*$^{-/-}$ WATs (Fig. 3D). Analyses of adipogenic marker expression profile demonstrated similar *Adiponectin* and *FABP4* patterns among the genotypes, while a significantly upregulated expression of *Glut4* was observed in *PiT2*$^{-/-}$ compared to WT (Fig. 3D). To further determine the cellular effects of PiT2 deletion in mature adipocytes, we used a siRNA-mediated knockdown of PiT2 in the

3T3-L1 cells. PiT2 expression was reduced of approximately 76% (Supp. Fig. 1). Knockdown of PiT2 did not modify expression of *Adiponectin* and slightly increase *FABP4* and *Glut4* mRNA expression (Supp. Fig. 1).

Next, given the strong relationship between WAT and glucose metabolism, we evaluated the impact of PiT2 deficiency on energy metabolism. We first showed that serum Leptin levels were lower in *PiT2*$^{-/-}$ mice compared to WT (Fig. 3E), consistent with the decreased peripheral fat weight. No differences were observed in serum Adiponectin levels at any age (Fig. 3F), suggesting a normal Adiponectin secretion from the adipose tissues despite the absence of PiT2.

While fasted blood glucose and serum insulin levels in adult *PiT2*$^{-/-}$ mice were significantly decreased (Fig. 3G, H), glucose tolerance and insulin sensitivity were not affected by the absence of PiT2 (Fig. I, J). However, glucose-stimulated insulin secretion was higher, suggesting a more efficient response to glucose load, consistent with the lower glucose levels. Altogether, these data suggest that PiT2 deficiency led to a reduction in WAT weight that is not associated with any adipose tissue dysfunction nor major metabolic abnormalities.

Discussion

We have shown here for the first time that PiT2 is a regulator of both bone marrow and peripheral white adipose tissues, being involved in their age-related increase.

In young *PiT2*$^{-/-}$ mice, the BMAT volume is increased while bone quality is reduced[8]. This phenotype is in line with some studies showing that the BMAT negatively regulate bone mass[24-26]. In genetic models, where the BMAT volume is increased and bone mass is reduced, a lineage switch of BMSC toward the adipocytes at the expense of osteoblasts is often observed[27-30]. *In vitro*, we did not evidence any alteration of adipogenic differentiation of PiT2-deficient BMSCs and the number and the activity of osteoblasts *in vivo* were similar to the ones observed in WT mice[8], indicating that other unknown molecular or cellular mechanisms are involved. In contrast to young

PiT2$^{-/-}$ mice, the decreased BMAT volume and the reduced bone quality in adult mutants are not in agreement with the inverse correlation between BMAT and bone volumes evidenced in numerous models[27-31]. Interestingly, studies in the BMAT-deficient mice carrying loss-of-function mutations in the kit receptor gene suggested that the absence of BMAT did not have any relevant effect on ovariectomy-induced bone loss[32,33]. While the osteoblast area is increased, the authors showed that it was not sufficient to increase bone mass because of the increased in osteoclast area. However, we previously showed that osteoclastic activity was similar in *PiT2*$^{-/-}$ and WT mice, suggesting that still unknown cellular or paracrine mechanisms would be at work in *PiT2*$^{-/-}$ mice.

In contrast to the proximal BMAT, the distal BMAT did not really change between *PiT2*$^{-/-}$ and WT mice and it normally increased with age as expected. This was consistent with the fact that the proximal BMAT is more susceptible to remodeling and changes compared to the distal one[34,35].

The deletion of PiT2 in vivo led to reduced peripheral white adipose tissues. WAT analysis of *PiT2*$^{-/-}$ mice does not suggest that PiT2 deficiency strongly alters the adipocytes homeostasis and energetic metabolism. However, the robust increased expression of Glut4 in gWAT and iWAT in *PiT2*$^{-/-}$ mice is consistent with the decreased fasting glycemia and may have beneficial metabolic effects in pathological conditions such as obesity[36]. Hence, to further define the effect of PiT2 deficiency on weight gain and adipose tissue homeostasis, challenging *PiT2*$^{-/-}$ mice with high fat diet would certainly be relevant. Indeed, we do not have enough information yet to predict whether PiT2 deficiency would protect or worsen the metabolic consequences of high fat diet feeding.

The deletion of *Slc20a2* does not lead to an overexpression of the PiT2 paralog PiT1, neither in the whole tibia, nor in adipose tissues, both *in vivo* and *in vitro*. This excludes a possible compensation mechanism driven by PiT1 and highlights a specific function of PiT2 in the regulation of fat weight at baseline. The possibility that mechanisms independent of the Pi-transport function of PiT2 are involved in this new function is tempting. We hypothesize that these mechanisms may be similar to those that require interaction of specific protein partners with the 4th intracellular loop, as it has been demonstrated for PiT1 when specifically invalidated in

chondrocytes[5] or in hepatocytes[37]. Interestingly enough, the absence of PiT1 in hepatocytes inhibits the dissociation of its protein partner USP7 with IRS1, leading to the inhibition of HFD-induced obesity. Therefore, considering the structural and functional similarities between PiT1 and PiT2, it is quite possible that PiT2 may control the volume variations of AT in pathological contexts (caloric restriction or obesity), independently of PiT1 and of its Pi-transport function. This of course requires further detailed investigation.

Acknowledgments

This work was supported by grants from INSERM, Region des Pays de la Loire (AdipOs) and Société Française de Rhumatologie (PITAMO). GF received a doctoral fellowship from Université de Nantes. We thank the UTE-IRS-UN Animal Facility, MicroPiCell imaging facility, and SC3M Histology facility of the SFR Sante François Bonamy (UMS INSERM 016/CNRS 3556) (Nantes, France), and Sébastien Boni for LentiVec (Angers, France). We thank the Cytometry Facility Cytocell from Nantes for expert technical assistance. The authors gratefully acknowledge Carla Geeroms (Department of Materials Engineering, KU Leuven, Leuven, BELGIUM), Céline Menguy (Inserm U1087, Nantes, France) and Séverine Marionneau (Inserm U1232, Nantes) for technical help. The authors also gratefully acknowledge Dr Christophe Chauveau (MABLab, Boulogne-sur-mer, France) and Dr Céline Colnot (IMRB, Paris) for helpful discussions.

Authors' roles: Study design: GF, LB, XP, and SBC. Data collection: GF, MG, GP, PG, GK, MF, JV, BH, SS and SBC. Data analysis and interpretation: GF, GP, GK, MF, XP, LB and SBC. Drafting manuscript: GF, XP, LB and SBC. Revising manuscript content: GF, JG, XP, LB and SBC. Approving final version of manuscript: all authors. SBC takes responsibility for the integrity of the data analysis.

References

1. Suzuki A, Ammann P, Nishiwaki-Yasuda K, Sekiguchi S, Asano S, Nagao S, Kaneko R, Hirabayashi M, Oiso Y, Itoh M, Caverzasio J. Effects of transgenic Pit-1 overexpression on calcium phosphate and bone metabolism. J Bone Miner Metab. 2010 Mar 1;28(2):139–48.

2. Bourgine A, Pilet P, Diouani S, Source S, Lesoeur J, Beck-Cormier S, Khoshniat S, Weiss P, Friedlander G, Guicheux J, Beck L. Mice with hypomorphic expression of the sodium-phosphate cotransporter PiT1/Slc20a1 have an unexpected normal bone mineralization. PLoS ONE. 2013;8(6):e65979.

3. Crouthamel MH, Lau WL, Leaf EM, Chavkin NW, Wallingford MC, Peterson DF, Li X, Liu Y, Chin MT, Levi M, Giachelli CM. Sodium-dependent phosphate cotransporters and phosphate-induced calcification of vascular smooth muscle cells: redundant roles for PiT-1 and PiT-2. Arterioscler Thromb Vasc Biol. 2013 Nov 1;33(11):2625–32.

4. Yadav MC, Bottini M, Cory E, Bhattacharya K, Kuss P, Narisawa S, Sah RL, Beck L, Fadeel B, Farquharson C, Millán JL. Skeletal Mineralization Deficits and Impaired Biogenesis and Function of Chondrocyte-Derived Matrix Vesicles in Phospho1(-/-) and Phospho1/Pit1 Double Knockout Mice. J Bone Miner Res. 2016 Jan 15;31(6):1275–86.

5. Couasnay G, Bon N, Devignes C-S, Source S, Bianchi A, Véziers J, Weiss P, Elefteriou F, Provot S, Guicheux J, Beck-Cormier S, Beck L. PiT1/Slc20a1 Is Required for Endoplasmic Reticulum Homeostasis, Chondrocyte Survival, and Skeletal Development. J Bone Miner Res. 2019 Feb;34(2):387–98.

6. Beck-Cormier S, Beck L. The Need of a Paradigm Shift to Better Understand PiT1 and PiT2 Biology: Response to "Why Is There No PiT1/SLC20A1 Pathogenic Variants Yet Linked to Primary Familial Brain Calcification?." J Bone Miner Res. 2020 Apr;35(4):825–6.

7. Yamada S, Wallingford MC, Borgeia S, Cox TC, Giachelli CM. Loss of PiT-2 results in abnormal bone development and decreased bone mineral density and length in mice. Biochem Biophys Res Commun. 2018 Jan 1;495(1):553–9.

8. Beck-Cormier S, Lelliott CJ, Logan JG, Lafont DT, Merametdjian L, Leitch VD, Butterfield NC, Protheroe HJ, Croucher PI, Baldock PA, Gaultier-Lintia A, Maugars Y, Nicolas G, Banse C, Normant S, Magne N, Gérardin E, Bon N, Source S, Guicheux J, Beck L, Williams GR, Bassett JHD. Slc20a2, Encoding the Phosphate Transporter PiT2, Is an Important Genetic Determinant of Bone Quality and Strength. J Bone Miner Res. 2019 Jun;34(6):1101–14.

9. Bravenboer N, Bredella MA, Chauveau C, Corsi A, Douni E, Ferris WF, Riminucci M, Robey PG, Rojas-Sutterlin S, Rosen C, Schulz TJ, Cawthorn WP. Standardised Nomenclature, Abbreviations, and Units for the Study of Bone Marrow Adiposity: Report of the Nomenclature Working Group of the International Bone Marrow Adiposity Society. Front Endocrinol (Lausanne). 2019;10:923.

10. Justesen J, Stenderup K, Ebbesen EN, Mosekilde L, Steiniche T, Kassem M. Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. Biogerontology. 2001;2(3):165–71.

11. Schorr M, Klibanski A. Anorexia nervosa and bone. Current Opinion in Endocrine and Metabolic Research. 2018 Dec;3:74–82.

12. Yeung DKW, Griffith JF, Antonio GE, Lee FKH, Woo J, Leung PC. Osteoporosis is associated with increased marrow fat content and decreased marrow fat unsaturation: A proton MR spectroscopy study. J. Magn. Reson. Imaging. 2005;22(2):279–85.

13. Wren TAL, Chung SA, Dorey FJ, Bluml S, Adams GB, Gilsanz V. Bone Marrow Fat Is Inversely Related to Cortical Bone in Young and Old Subjects. J Clin Endocrinol Metab. 2011 Mar;96(3):782–6.

14. Gao Y, Zong K, Gao Z, Rubin MR, Chen J, Heymsfield SB, Gallagher D, Shen W. Magnetic Resonance Imaging–Measured Bone Marrow Adipose Tissue Area Is Inversely Related to Cortical Bone Area in Children and Adolescents Aged 5–18 Years. Journal of Clinical Densitometry. 2015 Apr;18(2):203–8.

15. Beekman KM, Veldhuis-Vlug AG, Heijer den M, Maas M, Oleksik AM, Tanck MW, Ott SM, van t Hof RJ, Lips P, Bisschop PH, Bravenboer N. The effect of raloxifene on bone marrow adipose tissue and bone turnover in postmenopausal women with osteoporosis. Bone. Elsevier Inc; 2019 Jan 1;118(C):62–8.

16. Cawthorn WP, Scheller EL, Learman BS, Parlee SD, Simon BR, Mori H, Ning X, Bree AJ, Schell B, Broome DT, Soliman SS, DelProposto JL, Lumeng CN, Mitra A, Pandit SV, Gallagher KA, Miller JD, Krishnan V, Hui SK, Bredella MA, Fazeli PK, Klibanski A, Horowitz MC, Rosen CJ, MacDougald OA. Bone Marrow Adipose Tissue Is an Endocrine Organ that Contributes to Increased Circulating Adiponectin during Caloric Restriction. Cell Metabolism. Elsevier Inc; 2014 Aug 5;20(2):368–75.

17. Kerckhofs G, Stegen S, van Gastel N, Sap A, Falgayrac G, Penel G, Durand M, Luyten FP, Geris L, Vandamme K, Parac-Vogt T, Carmeliet G. Simultaneous three-dimensional visualization of mineralized and soft skeletal tissues by a novel microCT contrast agent with polyoxometalate structure. Biomaterials. Elsevier Ltd; 2018 Mar 1;159:1–12.

18. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biology. 2006;7(10):R100.

19. Boxall SA, Jones E. Markers for Characterization of Bone Marrow Multipotential Stromal Cells. Stem Cells Int. 2012;2012(5):1–12.

20. Rostovskaya M, Anastassiadis K. Differential Expression of Surface Markers in Mouse Bone Marrow Mesenchymal Stromal Cell Subpopulations with Distinct Lineage Commitment. Shi X-M, editor. PLoS ONE. 2012 Dec 7;7(12):e51221.

21. Ambrosi TH, Scialdone A, Graja A, Gohlke S, Jank A-M, Bocian C, Woelk L, Fan H, Logan DW, Schürmann A, Saraiva LR, Schulz TJ. Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration. Stem Cell. Elsevier Inc; 2017 Jun 1;20(6):771–6.

22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec 1;25(4):402–8.

23. Jensen N, Schrøder HD, Hejbøl EK, Thomsen JS, Brüel A, Larsen FT, Vinding MC, Orlowski D, Füchtbauer E-M, Oliveira JR, Pedersen L. Mice knocked out for the primary brain calcification associated gene *Slc20a2* show unimpaired pre-natal survival but retarded growth and nodules in the brain that grow and calcify over time. Am J Pathol. American Society for Investigative Pathology; 2018 May 24;:1–41.

24. Kremer R, Gilsanz V. Fat and Bone: An Odd Couple. Front Endocrinol (Lausanne). 2016 Mar 7;6(1):1.

25. Paccou J, Penel G, Chauveau C, Cortet B, Hardouin P. Marrow adiposity and bone: Review of clinical implications. Bone [Internet]. Elsevier Inc; 2018 Feb 27;118(C):8–15. Available from: http://dx.doi.org/10.1016/j.bone.2018.02.008

26. Li Q, Wu Y, Kang N. Marrow Adipose Tissue: Its Origin, Function, and Regulation in Bone Remodeling and Regeneration. Stem Cells Int. 2018;2018:7098456.

27. Fan Y, Hanai J-I, Le PT, Bi R, Maridas D, DeMambro V, Figueroa CA, Kir S, Zhou X, Mannstadt M, Baron R, Bronson RT, Horowitz MC, Wu JY, Bilezikian JP, Dempster DW, Rosen CJ, Lanske B. Parathyroid Hormone Directs Bone Marrow Mesenchymal Cell Fate. Cell Metabolism. Elsevier Inc; 2017 Jan 25;:1–25.

28. Yu B, Huo L, Liu Y, Deng P, Szymanski J, Li J, Luo X, Hong C, Lin J, Wang C-Y. PGC-1a Controls Skeletal Stem Cell Fate and Bone- Fat Balance in Osteoporosis and Skeletal Aging by Inducing TAZ. Stem Cell. Elsevier Inc; 2018 Aug 2;23(2):193–5.

29. Matsumoto Y, La Rose J, Lim M, Adissu HA, Law N, Mao X, Cong F, Mera P, Karsenty G, Goltzman D, Changoor A, Zhang L, Stajkowski M, Grynpas MD, Bergmann C, Rottapel R. Ubiquitin ligase RNF146 coordinates bone dynamics and energy metabolism. J Clin Invest. 2017 Jun 30;127(7):2612–25.

30. Zou W, Rohatgi N, Brestoff JR, Li Y, Barve RA, Tycksen E, Kim Y, Silva MJ, Teitelbaum SL. Ablation of Fat Cells in Adult Mice Induces Massive Bone Gain. Cell Metabolism. Elsevier Inc; 2020 Nov 3;32(5):801–6.

31. Tratwal J, Labella R, Bravenboer N, Kerckhofs G, Douni E, Scheller EL, Badr S, Karampinos DC, Beck-Cormier S, Palmisano B, Poloni A, Moreno-Aliaga MJ, Fretz J, Rodeheffer MS, Boroumand P, Rosen CJ, Horowitz MC, van der Eerden BCJ, Veldhuis-Vlug AG, Naveiras O. Reporting Guidelines, Review of Methodological Standards, and Challenges Toward Harmonization in Bone Marrow Adiposity Research. Report of the Methodologies Working Group of the International Bone Marrow Adiposity Society. Front Endocrinol (Lausanne). 2020 Feb 28;11:112.

32. Iwaniec UT, Turner RT. Failure to generate bone marrow adipocytes does not protect mice from ovariectomy-induced osteopenia. Bone. Elsevier Inc; 2013 Mar 1;53(1):145–53.

33. Keune JA, Wong CP, Branscum AJ, Iwaniec UT, Turner RT. Bone Marrow Adipose TissueDeficiency Increases Disuse-Induced Bone Loss in Male Mice. Scientific Reports. Nature Publishing Group; 2017 Apr 6;:1–12.

34. Scheller EL, Doucette CR, Learman BS, Cawthorn WP, Khandaker S, Schell B, Wu B, Ding S-Y, Bredella MA, Fazeli PK, Khoury B, Jepsen KJ, Pilch PF, Klibanski A, Rosen CJ, MacDougald OA. Region-specific variation in the properties of skeletal adipocytes reveals

regulated and constitutive marrow adipose tissues. Nat Commun. Nature Publishing Group; 2015 Jul 30;6:1–13.

35. Scheller EL, Khandaker S, Learman BS, Cawthorn WP, Anderson LM, Pham HA, Robles H, Wang Z, Li Z, Parlee SD, Simon BR, Mori H, Bree AJ, Craft CS, MacDougald OA. Bone marrow adipocytes resist lipolysis and remodeling in response to ß-adrenergic stimulation. Bone. Elsevier Inc; 2019 Jan 1;118(C):32–41.

36. Moraes-Vieira PM, Saghatelian A, Kahn BB. GLUT4 Expression in Adipocytes Regulates De Novo Lipogenesis and Levels of a Novel Class of Lipids With Antidiabetic and Anti-inflammatory Effects. Diabetes. 2016 Jun 21;65(7):1808–15.

37. Forand A, Koumakis E, Rousseau A, Sassier Y, Journe C, Merlin J-F, Leroy C, Boitez V, Codogno P, Friedlander G, Cohen I. Disruption of the Phosphate Transporter Pit1 in Hepatocytes Improves Glucose Metabolism and Insulin Signaling by Modulating the USP7/IRS1 Interaction. CellReports. The Author(s); 2016 Aug 23;:1–14.

Figure Legends

Figure 1. Deregulation of BMAT volume in *PiT2*$^{-/-}$ mice. (A) Representative 3D visualization, using Hf-POM-based CE-CT, of the adipocytes in the bone marrow compartment of the proximal tibia (2mm height from the growth plate) of 3- and 16-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice. Scale bar = 250µm. (B) Quantification of the volume fraction of the adipocytes in the VOI of the proximal and distal tibia of 3- and 16-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice (n=6 and 11 per genotype, respectively). (C) Relative *PiT1*, *PiT2*, *Adiponectin* and *FABP4* mRNA expression in the whole tibia from 3- (n=4-6 per genotype) and 16- (n=8-11 per genotype) week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice. (D) Secreted adiponectin from the tibia explants from 3-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice (n=16 *PiT2*$^{+/+}$ and n=13 *PiT2*$^{-/-}$). Data are means ± SEM, Mann&Whitney test, **p<0.05*, ***p<0.01*; *: versus 3 weeks, #: versus WT at the same age.

Figure 2. Characteristics of BMSCs in *PiT2*$^{-/-}$ mice. (A) FACS quantification of BMSCs (Ter119^{-} CD45^{-} Sca1^{+} CD44^{+} CD105^{+}) and adipogenic progenitors (Ter119^{-} CD45^{-} CD31^{-} Sca1^{+} CD24^{+}) in the bone marrow of tibias from 3-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice (n=6 per genotype). (B-C) Representative images and quantitative analysis of oil-red O (ORO) positive cells after adipogenic differentiation of *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ BMSCs. Scale bar = 100µm. Graphs showing the percentage of ORO+ cells related to the total number of Hoechst-positive cells (upper graph) and ORO absorbance at 520nm (lower graph). (D) Relative *PiT1*, *PiT2*, *Adiponectin* and *FABP4* mRNA expression at 0, 7 and 14 days of differentiation, n=4-7 per genotype, n=3 independent experiments. Data are means ± SEM, **p<0.05* (versus WT at each time point).

Figure 3. PiT2 deficiency leads to reduced peripheral adipose tissues but no major metabolic alterations. (A) Weight of iWAT, gWAT and kidney relative to body weight of *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice at 3 weeks (n=9-12 per genotype), 16 weeks (n=9-16 per genotype), 6 months (n=17-25 per genotype) and 1 year (n=3-4 per genotype). (B) Food intake normalized by the body weight of 16-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice (n=16-23 per genotype). (C) H&E staining on iWAT and gWAT

sections from 16-week-old *PiT2*$^{+/+}$ (n=5) and *PiT2*$^{-/-}$ (n=3) mice. Scale bar = 125µm (D) Relative *PiT1*, *PiT2*, *Adiponectin*, *FABP4* and Glut4 mRNA expression in *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ iWAT (n=3-5 per genotype) and gWAT (n=5-18 per genotype). (E) Serum Leptin (n=6 per genotype) and (F) serum Adiponectin (n=6-12 per genotype) levels in *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice at 3 weeks, 16 weeks and 6 months. (G) fasting blood glucose (n=8-10 per genotype) and (H) serum Insulin (n=6-9 per genotype) from 16-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice . (I) GTT with its AUC and (J) ITT on 16-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice (n=8-10 per genotype). (K) GSIS test on 16-week-old *PiT2*$^{+/+}$ and *PiT2*$^{-/-}$ mice (n=6 per genotype). Data are means ± SEM, **p<0.05*, ***p<0.01, vs PiT2*$^{+/+}$ mice by Mann&Whitney test. Two-way Anova test has been performed on GTT, ITT and GSIS tests.

Supplemental Figure 1. Effects of PiT2 deficiency in mature adipocytes. Relative mRNA expression of *PiT2*, *FABP4*, *AdipoQ* and *Glut4* in mature adipocytes transfected with either the *PiT2*-targeting or scramble siRNA (n=4 experiments, each one performed in triplicates). Data are means ± SEM, **p<0.05* (versus Scramble).

Figure 1
Frangi *et al.*

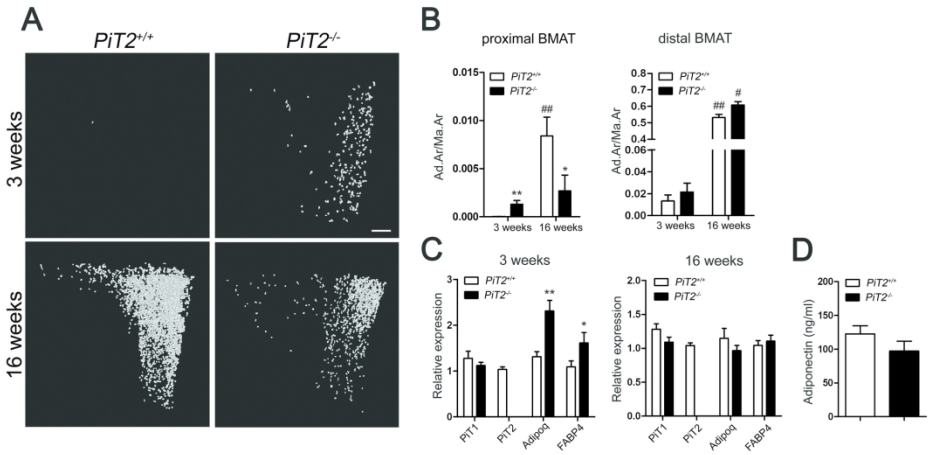


Figure 1. Deregulation of BMAT volume in PiT2-/- mice. (A) Representative 3D visualization, using Hf-POM-based CE-CT, of the adipocytes in the bone marrow compartment of the proximal tibia (2mm height from the growth plate) of 3- and 16-week-old PiT2+/+ and PiT2-/- mice. Scale bar = 250µm. (B) Quantification of the volume fraction of the adipocytes in the VOI of the proximal and distal tibia of 3- and 16-week-old PiT2+/+ and PiT2-/- mice (n=6 and 11 per genotype, respectively). (C) Relative PiT1, PiT2, Adiponectin and FABP4 mRNA expression in the whole tibia from 3- (n=4-6 per genotype) and 16- (n=8-11 per genotype) week-old PiT2+/+ and PiT2-/- mice. (D) Secreted adiponectin from the tibia explants from 3-week-old PiT2+/+ and PiT2-/- mice (n=16 PiT2+/+ and n=13 PiT2-/-). Data are means ± SEM, Mann&Whitney test, *p<0.05, **p<0.01; *: versus 3 weeks, #: versus WT at the same age.

202x122mm (300 x 300 DPI)

Figure 2
Frangi *et al.*

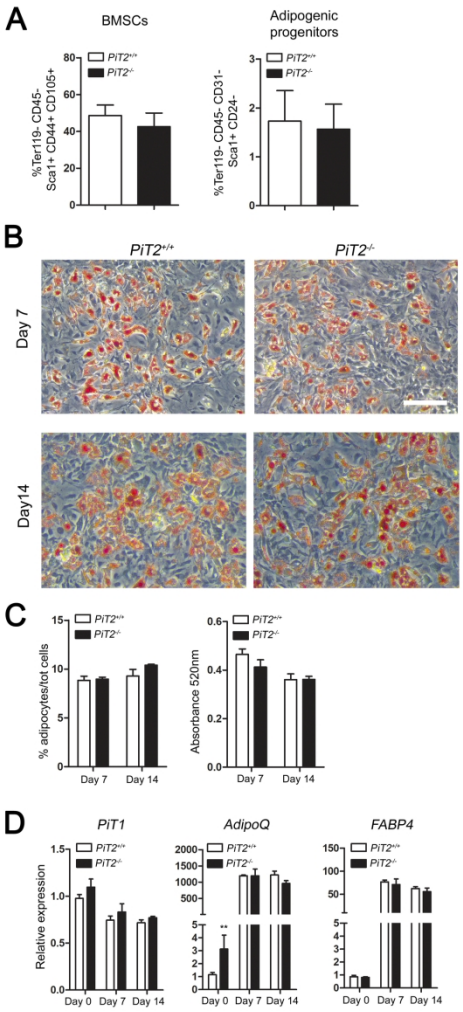


Figure 2. Characteristics of BMSCs in PiT2-/- mice. (A) FACS quantification of BMSCs (Ter119- CD45- Sca1+ CD44+ CD105+) and adipogenic progenitors (Ter119- CD45- CD31- Sca1+ CD24+) in the bone marrow of tibias from 3-week-old PiT2+/+ and PiT2-/- mice (n=6 per genotype). (B-C) Representative images and quantitative analysis of oil-red O (ORO) positive cells after adipogenic differentiation of PiT2+/+ and PiT2-/- BMSCs. Scale bar = 100µm. Graphs showing the percentage of ORO+ cells related to the total number of Hoechst-positive cells (upper graph) and ORO absorbance at 520nm (lower graph). (D) Relative PiT1, PiT2, Adiponectin and FABP4 mRNA expression at 0, 7 and 14 days of differentiation, n=4-7 per genotype, n=3 independent experiments. Data are means ± SEM, *p<0.05 (versus WT at each time point).

114x246mm (300 x 300 DPI)

Figure 3
Frangi *et al.*

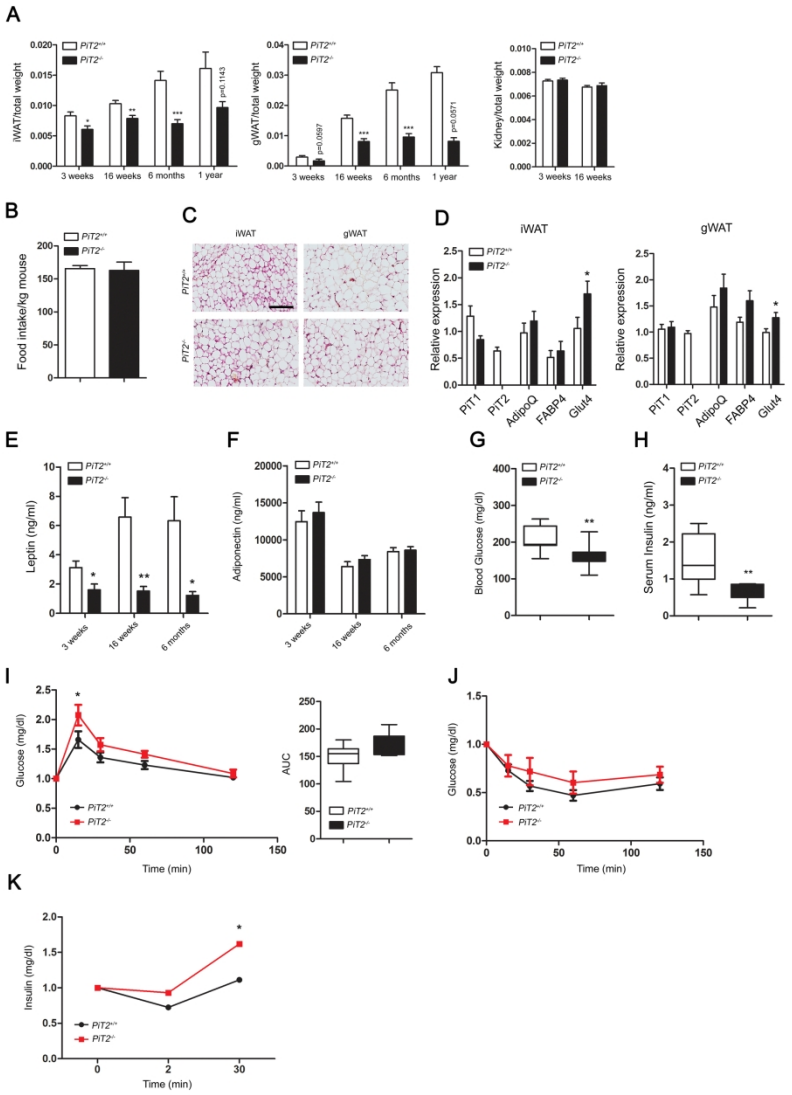
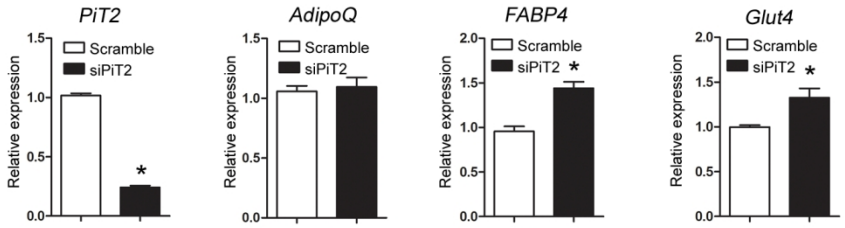


Figure 3. PiT2 deficiency leads to reduced peripheral adipose tissues but no major metabolic alterations. (A) Weight of iWAT, gWAT and kidney relative to body weight of PiT2+/+ and PiT2-/- mice at 3 weeks (n=9-12 per genotype), 16 weeks (n=9-16 per genotype), 6 months (n=17-25 per genotype) and 1 year (n=3-4 per genotype). (B) Food intake normalized by the body weight of 16-week-old PiT2+/+ and PiT2-/- mice (n=16-23 per genotype). (C) H&E staining on iWAT and gWAT sections from 16-week-old PiT2+/+ (n=5) and PiT2-/- (n=3) mice. Scale bar = 125µm (D) Relative PiT1, PiT2, Adiponectin, FABP4 and Glut4 mRNA expression in PiT2+/+ and PiT2-/- iWAT (n=3-5 per genotype) and gWAT (n=5-18 per genotype). (E) Serum Leptin (n=6 per genotype) and (F) serum Adiponectin (n=6-12 per genotype) levels in PiT2+/+ and PiT2-/- mice at 3 weeks, 16 weeks and 6 months. (G) fasting blood glucose (n=8-10 per genotype) and (H) serum Insulin (n=6-9 per genotype) from 16-week-old PiT2+/+ and PiT2-/- mice . (I) GTT with its AUC and (J) ITT on 16-week-old PiT2+/+ and PiT2-/- mice (n=8-10 per genotype). (K) GSIS test on 16-week-old PiT2+/+ and PiT2-/- mice (n=6 per genotype). Data are means ± SEM, *p<0.05, **p<0.01, vs PiT2+/+ mice by Mann&Whitney test. Two-way Anova test has been performed on GTT, ITT and GSIS tests.

202x286mm (300 x 300 DPI)

Supplemental Figure 1
Frangi *et al*.

A



138x75mm (300 x 300 DPI)

Supp. Table 1. Antibodies used for cytometry analysis

Antibodies:	**Source:**	**Identifier:**
APC Rat Anti-Mouse CD31	BD Biosciensces	Cat#: 551262
BV510 Rat Anti-Mouse TER-119	BD Biosciensces	Cat#: 563995
BV510 Rat Anti-Mouse CD45	BD Biosciensces	Cat#: 563891
APC Rat Anti-Mouse CD44	BD Biosciensces	Cat#: 559250
BV421 Rat Anti-Mouse Ly-6A/E	BD Biosciensces	Cat#: 562729
PE Rat Anti-Mouse CD24	BD Biosciensces	Cat#: 561079
BV650 Rat Anti-Mouse CD105	BD Biosciensces	Cat#: 740609

II. Additional results not included in the paper

In the context of my PhD project not all the experiments I performed were included in the scientific paper I had the chance to compile. As you know, a PhD is rarely just the result of what gets published in the very end. It is, instead, characterized by multiple attempts, failed experiments and supplementary results we get to spend our time performing, improving and not always exploiting for our manuscript. In the following section I will present most of the additional experiments and related results not included in the scientific papers I mentioned before, which nonetheless contributed to the development of the project.

II.1.BMSCs co-differentiation assay

The issue

Soon after confirming the increased BMAT volume in 3-week-old PiT2-deficient mice, the first thing we wanted to do was to investigate the role of BMSCs and their adipogenic potential in our model. It is well known that osteoblasts and adipocytes share a common progenitor: the bone marrow mesenchymal stromal cell (BMSC). It has been often proposed in the literature that the increased BMAT observed in cases of bone loss could be caused by a shift in the commitment of BMSCs from the osteogenic to the adipogenic pathway. It is in this context that we were interested in performing *in vitro* experiments to test whether an alteration in the BMSCs commitment could explain the increased BMAT present in PiT2KO mice, which presented a skeletal phenotype as described in the introduction. We were particularly interested in the work of Ghali and colleagues, who described an *in vitro* model to culture BMSCs in an osteogenic medium added with Dexamethasone to obtain in the same culture dish adipocytes and mineralizing osteoblasts (Ghali et al., 2015). This technique allows to evaluate the effects of one cell type on another, enabling to assess the influence of adipocytes on osteoblasts and vice versa. Our idea was to exploit this system to test whether in the absence of PiT2, the proportion of adipocytes compared to osteoblast would have changed and, if so, if this had an impact on osteoblast mineralization.

Methodological approach

BMSCs were harvested from tibias of 3-week-old PiT2KO and WT mice. The mice were sacrificed and their hindlimbs dissected. After removing the epiphyses, bones were flushed to isolate total bone marrow cells and BMSCs were expanded in growth media consisting of α-MEM (Eurobio, France) supplemented with 15% FBS, 100 IU/mL penicillin, 100 mg/mL streptomycin, 2mM Glutamine. When confluence was reached, BMSCs were cultured in the co-differentiation medium consisting of the standard osteogenic medium supplemented with 100nM of dexamethasone as described in Ghali et al, 2015. Lipid droplets

were stained with Oil Red O (ORO) and quantified with an high content screening (HCS) approach and bioinformatics as the number of ORO+ cells on the total number of cells (Hoechst +).

Results

We performed two separate experiments where we analysed the adipocyte formation via ORO staining and quantification of the number of adipocytes (ORO+) on the total cells (Hoechst +) per plate via a high content screening approach (HCS). We observed no significant differences in the presence or the absence of PiT2. We decided not to continue with those experiments due to the heterogeneity of the results and the small amount of adipocytes (<10%), which made the approach not suitable for our purpose (Figure 19).

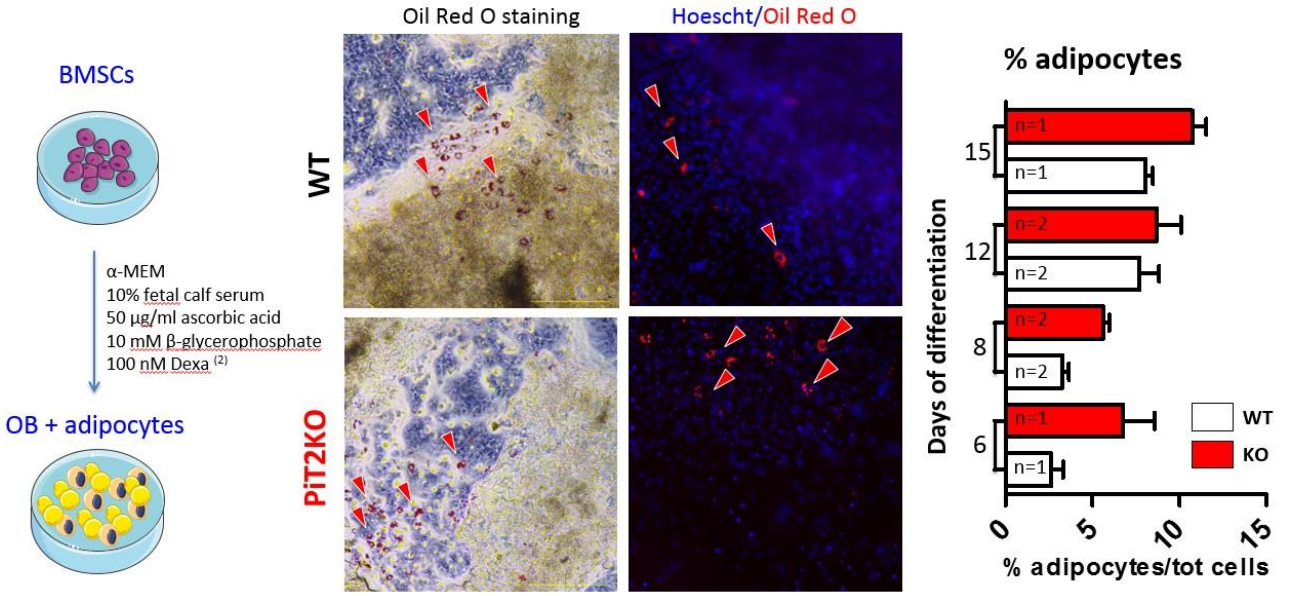


Figure 19. Osteo-adipogenic co-differentiation of PiT2WT and KO BMSCs from 8-week old mice.
Representative images of Oil Red O (ORO) staining at 11 days of co-differentiation by using light microscopy (left) or fluorescence microscopy (High Content Screening Arrayscan (HCS, Thermo Scientific), right). Graph shows quantification of the % of ORO+ cells normalized to total Hoescht+ cells (ImageJ, 110438<n<266572 Hoescht+ cells per well). Red arrows: adipocytes. (n=1-2 on 2 independent experiments).

Conclusion:

Even though we decided to end these analyses, we did not come out of this experiments empty handed: we gained a fundamental know-how in working with BMSCs, from the harvesting procedure through the treatment and timing of confluence and maturation which considerably helped us in performing the adipogenic differentiation protocol we put in place soon after.

As a perspective, it would have been interesting to perform medium transfer experiments from cultures of BMSCs-derived adipocytes to osteoblasts in order to test whether secreted factors from PiT2-deficient adipocytes could have an effect on osteoblasts differentiation and/or activity and vice-versa.

II.2.Identification and investigation of potential protein partners of PiT2

The issue

The protein sequences of PiT1 and PiT2 share 60% identity. However, their fourth intracellular loop have only 34% sequence identity, versus 75% regarding the rest of the protein, suggesting that they could be involved in specific functions. As I mentioned in the introduction, we now know that the PiT1 protein is a multi-functional protein involved in a wide variety of biological phenomena. Some of these functions depend on the protein partner with whom PiT1 interacts. For example, our group has shown that PiT1 is a new regulator of the ER stress response and allows the survival of chondrocytes located in the hypoxic zone of the growth plate. This work indicates that this function is achieved through the interaction of PiT1 with the PDI protein, one of 20 potential protein partners of PiT1 identified by a yeast two hybrid screening.

In order to discover novel protein partners of PiT2 we also decided to perform a yeast two hybrid screening. This technique is based on the reconstitution of a functional transcription factor (TF) followed by the expression of a reporter gene in genetically modified yeast cells. Upon physical binding of your protein of interest (bait) to a protein fragment from the library (prey), the DNA Binding Domain (DBD) of the TF is brought in close proximity to its Activation Domain (AD). Reconstitution of the functional TF activates the transcription of the HIS3 reporter gene, which allows yeast cells to grow on a selective medium lacking histidine. The DNA of the positive clones is then sequenced and analysed to identify the protein partners (Fig.20).

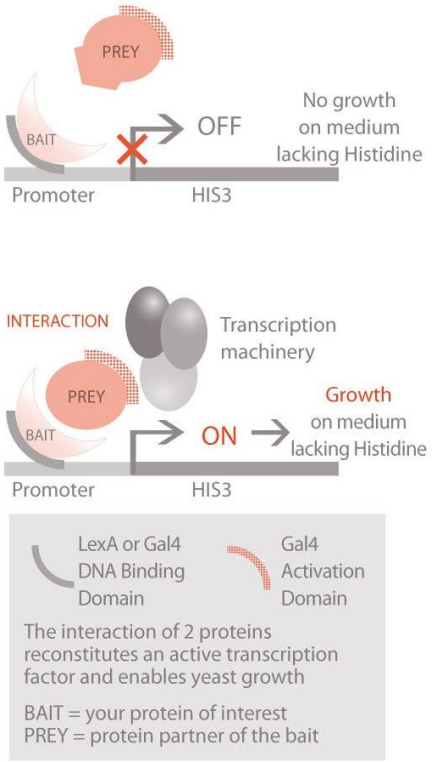


Figure 20. The yeast two-hybrid screen technique offers a sensitive and cost-effective mean to test the direct interaction between two targeted proteins, or to use one's favorite protein as a bait to screen libraries of proteins fragments prepared from the desired cell types, tissues or entire organisms. The identity of the interacting partners is then obtained by sequencing the corresponding plasmids in the selected yeast colonies. From https://www.hybrigenics-services.com/

Yeast experiments were performed by Hybrigenics (Paris, France). The library used for the screen was isolated from human placenta. The 4th intracellular loop (denominated iLoop2) of human PiT2 (from amino acids 236 to 482) was used as a bait for the screen. One hundred and twenty-four millions of interactions were analysed and, among them, 7 potential partners were identified with a very high confidence in the interaction (group A), 4 with a high confidence (group B) and 32 with a moderate confidence (group D). Eleven of them are expressed by adipocytes and have a role in regulating the function of this tissue. Analyses of the gene expression databases and of the literature have enabled us to select some potentially interesting partners. In the following table you will find the main potential partners we focused on, their level of confidence and their main characteristics:

Name	Y2H group	Cellular Localization	KO phenotype	References
Alix (PDCD6IP)	A	Cytoplasm	Small-sized brain, Reduced dendritic arborisation	(Laporte et al., 2017)
PAPP-A	A	Extracellular	↘ Mineralization (birth) and bone parameters (BMD, Cort.Th.) GTT and ITT : normal Expressed in fat	(Conover et al., 2004, 2013)
EFEMP1	A	Intracellular/secreted	Early ageing, decreased body mass and reduced fat	(McLaughlin et al., 2007)
Fhl2	B	Cytoplasm/Nucleus	↓BMAT, ↓BMD osteopenia	(Günther et al., 2005)

Alix

Alix, also known as Programmed Cell Death 6 Interacting Protein (PDCD6IP), is a protein involved in the endosomal sorting complex required for transport (ESCRT). It actually mediates the sorting of high-affinity phosphate transporters (PHT1) to the vacuole to limit their plasma membrane levels under phosphate-sufficient conditions in Arabidopsis thaliana (Cardona-Lopez et al 2015). In vertebrate, Alix is a marker of exosomes and is also found in small extracellular vesicles (EVs) from differentiated 3T3-L1 adipocytes (Durcin et al JEV 2017). We were mostly interested in Alix also for its fundamental role in the neuronal growth (Laporte et al., 2017) due to an ongoing collaboration with Franck Oury's group (INEM, Paris), who is interested in the role of PiT1 and PiT2 within the brain.

EFEMP1

EFEMP1 (EGF Containing Fibulin Extracellular Matrix Protein 1), also known as Fibulin 3, is a member of the fibulin family of extracellular matrix glycoproteins. Mutations in this gene are associated with Doyne

honeycomb retinal dystrophy and changes in *EFEMP1* expression are associated with the progression of numerous types of cancer (Livingstone et al, Biomolecules, 2020). EFEMP1KO mice display reduced reproductivity, herniation, early ageing and absence of subcutaneous fat (McLaughlin et al., Human Molecular Genetics, 2007). Furthermore, EFEMP1 negatively regulates chondrocyte differentiation and it is known to be involved in age-induced osteoarthritis (Hasegawa et al 2017; Wakabayashi et al, 2010). EFEMP1 is mainly an extracellular protein, however data from malignant mesothelioma investigations found a high immunoexpression of Fibulin 3 in neoplastic cells with nuclear and cytoplasmic localization (Caltabiano et al, 2018).

FHL2

FHL2 (Four And A Half LIM Domains 2) is part of the four-and-a-half-LIM-only protein family, which contain two highly conserved, tandemly arranged, zinc finger domains with four highly conserved cysteines binding a zinc atom in each zinc finger. Interestingly, FHL2KO mice display decreased BMAT (Brun et al Bone 2013), increased fat tissues (Lai et al JBMR 2006), decreased BMD and BMC (Govoni et al Calcified Tissues Int 2006), and osteopenia (Gunther et al EMBO J 2006). When we first observed the increased BMAT in 3-week-old PiT2KO mice followed by the decreased pAT we found very interesting that in Fhl2KO mice there was also an opposite effect on fat depots, with decreased BMAT and increased pAT.

FHL2 is known as a multifunctional protein: it impairs assembly of extracellular matrix proteins (Park et al., FASEB J 2008), it increases osteoblasts activity via Wnt-dependent Runx2 expression (Hamidouche et al FASEB J 2008; Brun et al Bone 2013; Gunther et al EMBO J 2006). It also mediates tooth development and human dental pulp cell differentiation into odontoblasts (Du et al, J Mol Hist 2016). In a recently published review the various interaction of Fhl2 with different proteins have been summarized, showing its functional implication in apoptosis, migration, and regulation of nuclear receptor function (Tran et al, 2016).

PAPP-A

PAPP-A, also known as Pappalysin-1, is a secreted metalloproteinase which cleaves insulin-like growth factor binding proteins (IGFBPs), in particular IGFBP-4 (Conover, Trends Endocrinol Metab. 2012). PAPP-A is associated with heparan sulphate-like GAG at the cell surface, enabling an efficient regulation of IGF-1 signaling. PAPP-A KO mice are consistently smaller than their WT littermates, showing a 40% reduction in body size and display a decreased mineralization at birth and also decreased bone parameters (BMD, Cort.Th.) (Conover et al., 2003; Conover et al., 2004), making of PAPP-A an important fetal growth regulatory factor *in vivo*. Those findings led to the proposal that PAPP-A promote somatic growth by increasing bioavailable IGFs (Oxvig, 2015; Fujimoto et al., 2017; Conover and Oxvig, 2018).

Interestingly, studies in mice indicate an important role for PAPP-A in regulating visceral fat depots (Conover et al. 2013; Bale et al. 2018), which furthermore brings us to hypothesize a potential interaction between PiT2 and PAPP-A which could possibly explain what we observe *in vivo*.

However, being extracellular, it is hard to imagine an interaction of this protein with PiT2. Even though truncated forms of PAPP-A exist (Boldt et al, 2004) and we could speculate the possibility of an interaction of one of them with PiT2.

Methodological approach

We have first confirmed the physical interaction of PiT2 with some of these protein partners through co-immunoprecipitation experiments. We also performed western blot analyses on proteins from whole tibias, qPCR analyses on the same tissue and on peripheral adipose tissues and different *in vitro* culture experiments to analyze their pattern of expression.

Results

We were able to confirm the physical interaction of PiT2 with 3 potential partners, in particular Alix, FHL2 and PAPPA (Figure 21). The interaction of PiT2 with other potential partners is still to be validated. Fhl2 caught our attention since it has been identified in the Y2H screen for iLoop1 from PiT1 (unpublished data). Real-time qPCR analyses on whole tibias from male and female WT and PiT2-deficient mice at 3 and 16 weeks did not show any significant differences between the genotypes (figure 22A for Fhl2, figure 23A for Alix and 23B for Efemp1, Figure 24 for PAPP-A). At 6 months, Fhl2 showed a significant increased expression within the whole tibias from male PiT2KO mice compared to the WT, with the same tendency in female samples (figure 22A). However, WB analysis performed on whole tibia from 6-month-old PiT2WT and KO females showed a decreased expression of Fhl2 in the absence of PiT2 (Figure 22C). mRNA expression of Fhl2, Alix and Efemp1 was also investigated in gWAT (Figure 22D for Fhl2, 23A for Alix and 23B for Efemp1) and no significant difference were observed for any of them.

In the course of our *in vitro* experiments using 3T3-L1 cells treated with siRNA against PiT1 or PiT2 or both, we showed an increased Fhl2 expression in the absence of PiT1, while no differences were observed in the absence of PiT2 or both transporters at the same time (figure 22B).

Conclusion

Certainly, these experiments are far from being concluded. Nonetheless, we were able to confirm the interaction of PiT2 at least with three among various potential partners tested. Moreover, the identification of Fhl2 as a potential partner of both PiT1 and PiT2 is intriguing. We already know that PiT1 and PiT2 form heterodimers, whose formation is regulated by variations of external Pi concentration (Bon et al., 2018), and Fhl2 could be a key partner interacting with both of them at the same time and/or with them

separately depending on the potential need. We still don't know what could be its function, but our results, starting from the confirmation of the interaction between Fhl2 and PiT2, are promising and surely need further investigation.

If I had the opportunity and time to devote myself more to this part of the project, I would have really appreciated going further in the analyses of the interaction between PiT2 and its potential partners. First, a complete profiling of both the mRNA and protein expression of the potential partners should be done in order to identify any possible changes in the absence of PiT2. Even though we did not observe major differences for now, we just performed qPCR analyses on the main tissues we were investigating in the mean time for other purposes. Morever, it would have been fundamental to confirm the interaction endogenously rather than in overexpressed tagged proteins conditions. Once these interactions were confirmed endogenously, the next step would be to investigate the consequences of their occurence or not. Unfortunately, the absence of PiT2 does not alter any cell proliferation or differentiation in any of the cell types we have studied (osteoblasts, chondrocytes, BMSCs, nor 3T3-L1). So, performing *in vitro* rescue experiments to detail the molecular mechanism of action of PiT2 with any of those protein partners were not possible. Nonetheless, another possibility would have been to generate genetically modified mouse models to specifically delete PiT2 and/or the potential partners within osteoblasts or adipocytes and have the possibility to investigate the outcome.

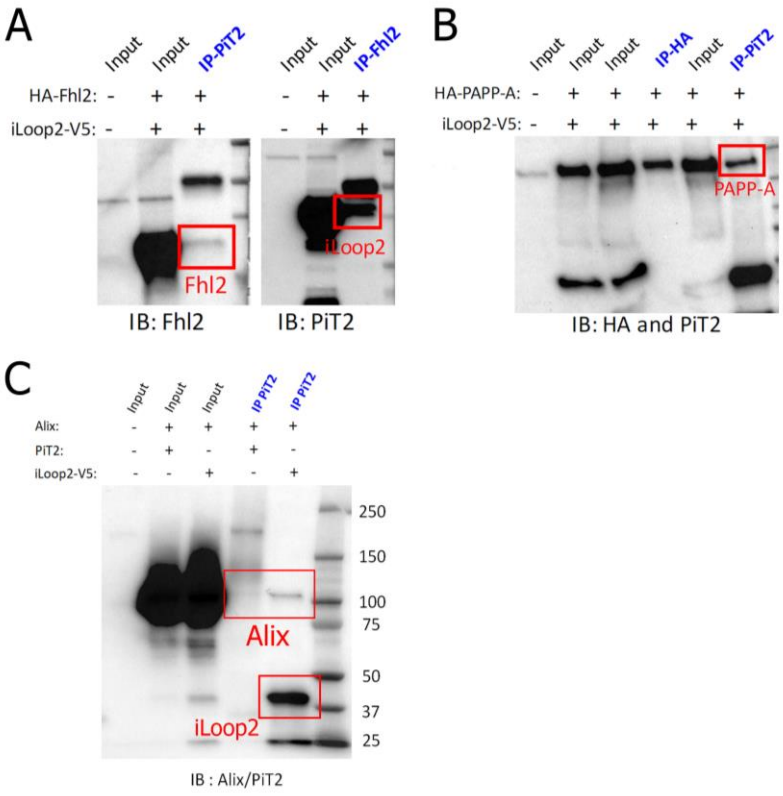


Figure 21. FHL2, PAPPA and Alix respectively physically interact with the 4th intracellular loop of PiT2.
A) Coimmunoprecipitation of PiT2 and Fhl2; B) Coimmunoprecipitation of PiT2 and PAPPA; C) Coimmunoprecipitation of PiT2/iloop2 and Alix. Alix ≈ 100 kDa, PAPPA ≈ 200 kDa, Fhl2 ≈ 32 kDa, Alix ≈ 100 kDa, PiT2 ≈ 75 kDa, iLoop2 ≈ 37 kDa. Primary antibody for PiT2 from Proteintech Group Inc. (Ref. 12820-1-AP), primary antibody for Fhl2 from Abcam (Ref. ab12327), primary antibody for PAPPA anti-HA from SIGMA (Ref. H6908), primary antibody for Alix from Covalab (Ref. pab0204).

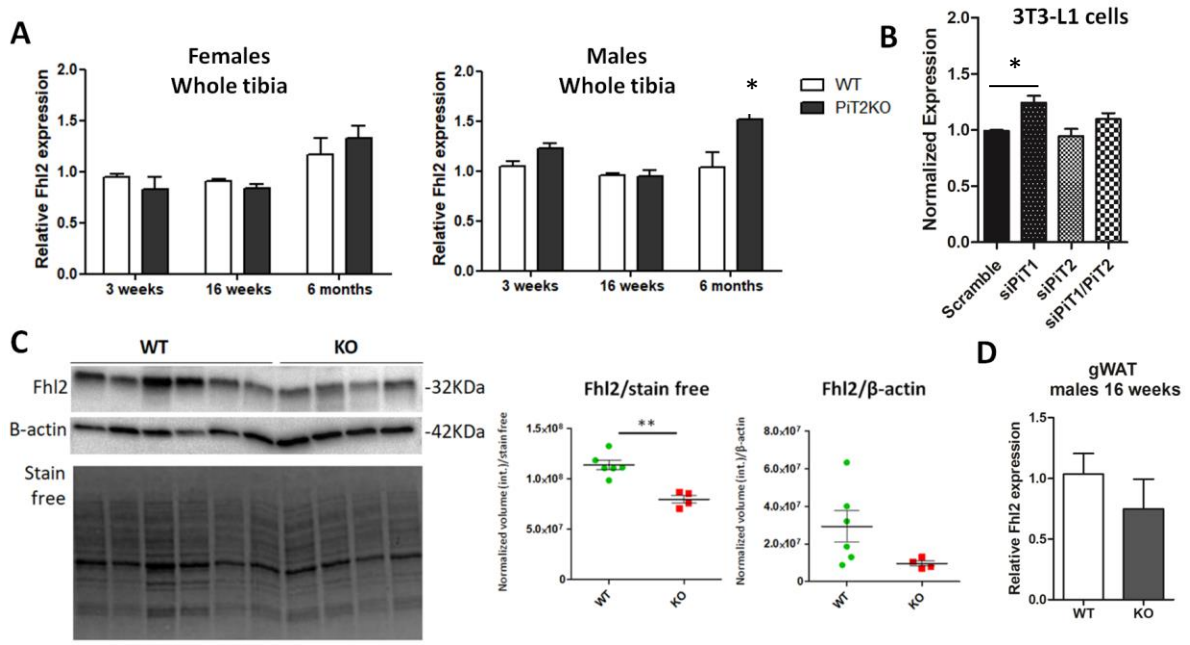


Figure 22. Fhl2 mRNA and protein expression in WT and PiT2KO mice.
*A) qPCR on whole tibia from 3 weeks(n=7 females and 5 males WT, n=4 females and n=5 males KO), 16 weeks (n=8 females and 7 males WT, n=11 females and 5 males KO) and 6 months (n=5 females and 6 males WT and n=5 females and males KO) old PiT2WT and KO male and female mice. B) Fhl2 mRNA expression in 3T3-L1 cells differentiated into adipocytes and treated 72h with siRNA for PiT1, PiT2 or the two together (n=4 independent experiences) C) WB on proteins from whole tibias from WT and PiT2KO female mice at 6 months of age (n=6WT and n=4 KO). The results are presented either in relation to total protein expression (Stain-Free Imaging technology, BioRad) or in relation to ß-actin expression. D) qPCR on gWAT from 16-week-old PiT2WT (n=13) and KO(n=7) mice. Statistics: Mann&Whitney test, data are means ± SEM, *p value<0.05.*

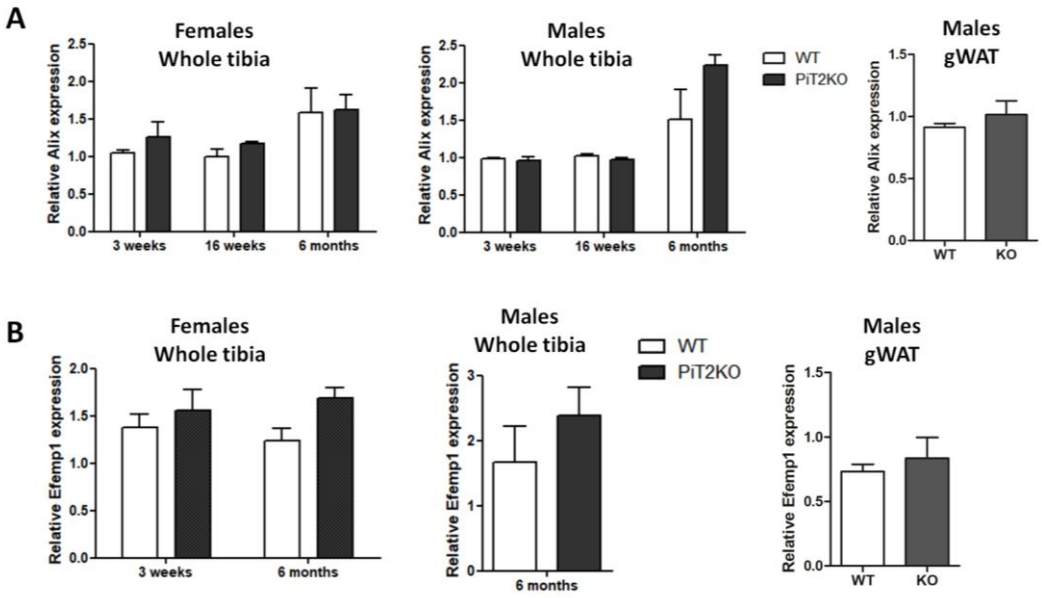


Figure 23. Alix and Efemp1 mRNA expression in whole tibia and gWAT from WT and PiT2KO mice.
A) Alix qPCR on whole tibia from 3 weeks(n=7 females and 5 males WT, n=4 females and n=5 males KO), 16 weeks (n=8 females and 7 males WT, n=11 females and 5 males KO) and 6 months (n=5 females and 6 males WT and n=5 females and males KO) old PiT2WT and KO male and female mice. qPCR on gWAT from 16-week-old WT(n=) and PiT2KO (n=) male mice. B) Efemp1 qPCR on whole tibia from 3 weeks(n=7 females and 5 males WT, n=4 females and n=5 males KO) and 6 months (n= 6 s WT and n=5 KO) old PiT2WT and KO male and female mice. Efemp1 qPCR analysis on gWAT from 16-week-old WT (n=13) and PiT2KO (n=7) male mice.

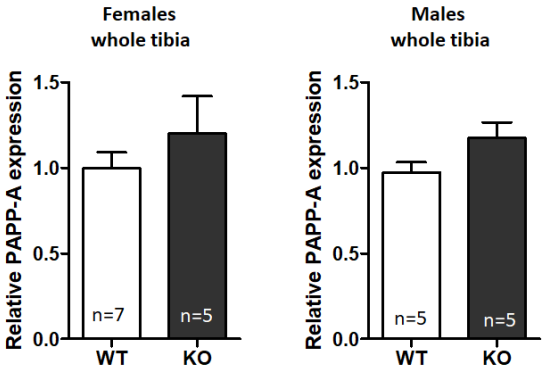


Figure 24. PAPP-A mRNA expression in whole tibia from 3-week-old PiT2WT and KO male and female mice.

III. Article 2: Phosphate-dependent FGF23 secretion is modulated by PiT2/Slc20a2

This article has been published in 2018 in the journal "Molecular Metabolism" (Bon et al., 2018).

The issue

As described in the introduction of this paper, extracellular Pi is capable of modulating the secretion of FGF23 in humans and animals. However, the underlying mechanisms are still unclear. The promising results described in Bon et al, JBC, 2018 led us to study the potential involvement of the PiT proteins in the control of the secretion of FGF23 by phosphate.

Methods

We have studied the regulation of FGF23 secretion in the PiT2KO mice. To this end, we fed these mice at 4 weeks of age with diets containing a low (0.05%), normal (0.55%) and high (1.65%) concentration of phosphate for one week and analyzed the blood and urine parameters characterizing phosphate homeostasis. We tried to generate mice with a specific deletion for PiT1 in the osteocytes (PiT1lox/lox;DMP1-Cre), however the targeting resulted inefficient, leading us to find another way. To do so, adenoviruses expressing the Cre recombinase to invalidate PiT1 were used *ex vivo* on bone shafts to evaluate the role of PiT1 in phosphate-dependent FGF23 secretion from bone. We also used the *ex vivo* bone shaft model on PiT2KO mice. The diaphyses of long bones were dissected and flushed prior to culture. The medium was changed to evaluate the effect of different concentrations of extracellular Pi on the secretion of FGF23 over 24 hours. Immunohistochemistry analyses were also performed on kidney sections from PiT2WT and KO mice in order to investigate the expression of αKlotho (the co-receptor mediating the effects of FGF23 in the kidney).

Results

Analysis of PiT2KO mice showed that the regulation of serum and urinary parameters such as Pi, Ca and PTH concentration are not altered under a normalized standard diet. However, we showed PiT2KO mice were no longer capable to increase serum FGF23 concentrations in response to augmentation of extracellular Pi. Thus, PiT2 is essential in the regulation of FGF23 secretion in response to Pi. Interestingly, the *ex vivo* long bone model has allowed us to confirm the importance of PiT2 in the regulation of FGF23 secretion by phosphate. This model suggests that the regulations observed in the *in vivo* model are probably due to a direct regulation on the bone, since in the organ system no endocrine loops are present. Unfortunately, the model of PiT1 invalidation in osteocytes *in vivo* did not show a sufficient deletion to be able to conclude on the results obtained on these mice. However, adenoviruses expressing the Cre

recombinase to invalidate PiT1 *ex vivo* allowed us to suggest that deletion of PiT1 alone is not sufficient blunt the FGF23 secretion in response to variation of extracellular Pi.

An interesting point was the fact that PiT2 KO mice have undisturbed phosphate homeostasis parameters (Beck-Cormier et al., 2019), consistent with the absence in humans of PiT2 mutations causing alterations of phosphate homeostasis. More intriguingly, when fed low Pi diets, Pi homeostasis parameters of both PiT2 WT and KO mice remained identical, despite the fact that FGF23 serum levels decreased in the WT and remained abnormally normal in the KO. A possible explanation for this discrepancy is the observation that mRNA and protein levels of αKlotho in the kidney was vastly reduced in PiT2 KO fed a low Pi diet, as compared to the WT. Hence, since αKlotho acts as a co-receptor for FGF23 receptor, its down-regulation in the KO mice would act as a compensatory mechanism and explain why FGF23 did not affect Pi homeostasis parameters in this condition.

Conclusion

This study presented the first direct evidence that PiT2 is fundamental for normal FGF23 secretion following dietary phosphate changes. These results illustrate that the Pi-dependent regulation of FGF23 is dependent upon normal bone PiT2 expression and highlights PiT2 as an important putative Pi sensor. However, the underlying mechanism linking PiT2 to the secretion of FGF23 remains to be uncovered.

Brief Communication

MOLECULAR METABOLISM

Phosphate-dependent FGF23 secretion is modulated by PiT2/Slc20a2

Nina Bon [1,2], Giulia Frangi [1,2], Sophie Sourice [1,2], Jérôme Guicheux [1,2,3], Sarah Beck-Cormier [1,2], Laurent Beck [1,2,*]

ABSTRACT

Objective: The canonical role of the bone-derived fibroblast growth factor 23 (Fgf23) is to regulate the serum inorganic phosphate (Pi) level. As part of a feedback loop, serum Pi levels control Fgf23 secretion through undefined mechanisms. We recently showed *in vitro* that the two high-affinity Na^+-Pi co-transporters PiT1/Slc20a1 and PiT2/Slc20a2 were required for mediating Pi-dependent signaling. Here, we addressed the contribution of PiT1 and PiT2 to the regulation of Fgf23 secretion.

Methods: To this aim, we used *PiT2* KO and *DMP1Cre; PiT1$^{lox/lox}$* fed Pi-modified diets, as well as *ex vivo* isolated long bone shafts. Fgf23 secretion and expression of Pi homeostasis-related genes were assessed.

Results: *In vivo, PiT2* KO mice responded inappropriately to low-Pi diets, displaying abnormally normal serum levels of intact Fgf23. Despite the high iFgf23 level, serum Pi levels remained unaffected, an effect that may relate to lower αKlotho expression in the kidney. Moreover, consistent with a role of PiT2 as a possible endocrine Pi sensor, the iFGF23/cFGF23 ratios were suppressed in *PiT2* KO mice, irrespective of the Pi loads. While deletion of *PiT1* in osteocytes using the *DMP1-Cre* mice was inefficient, adenovirus-mediated deletion of *PiT1* in isolated long bone shafts suggested that PiT1 does not contribute to Pi-dependent regulation of Fgf23 secretion. In contrast, using isolated bone shafts from *PiT2* KO mice, we showed that PiT2 was necessary for the appropriate Pi-dependent secretion of Fgf23, independently from possible endocrine regulatory loops.

Conclusions: Our data provide initial mechanistic insights underlying the Pi-dependent regulation of Fgf23 secretion in identifying PiT2 as a potential player in this process, at least in high Pi conditions. Targeting PiT2, therefore, could improve excess FGF23 in hyperphosphatemic conditions such as chronic kidney disease.

Keywords FGF23 secretion; Phosphate sensing; PiT1/Slc20a1; PiT2/Slc20a2; Bone

1. INTRODUCTION

Fibroblast growth factor 23 (Fgf23) is a bone-derived hormone, which acts in concert with its co-receptor αKlotho to regulate the serum inorganic phosphate (Pi) concentration and vitamin D metabolism [1,2]. Mutations in humans and mice preventing normal FGF23 bioactivity result in elevated serum Pi concentrations due to impaired urinary Pi excretion. Prolonged hyperphosphatemia can lead to life-threatening situations resulting from an inappropriate deposition of calcium-Pi crystals in cardiovascular tissue that occurs at high prevalence in diabetes, chronic kidney disease (CKD) and cardiovascular diseases (CVD) [3,4]. Conversely, elevated levels of biologically active FGF23 observed in inherited or acquired disorders of Pi homeostasis lead to renal Pi wasting, low serum Pi levels, and abnormal vitamin D metabolism [5,6]. The regulation of Fgf23 expression and/or secretion is controlled by numerous factors, including FGFR1 signaling [5,7], iron deficiency [8], and pro-inflammatory stimuli [9,10]. The intracellular cleavage of the biologically active intact form of FGF23 into N- and C-terminal fragments is also an important regulatory step. This regulation involves a balance between O-glycosylation of Thr^{178} by polypeptide N-acetylgalactosaminyltransferase 3 (GalNT3), providing a protection of furin-mediated cleavage, and phosphorylation of Ser^{180} by Family with sequence similarity 20, member C (Fam20C) that counteracts O-glycosylation [11].

Interestingly, the presence of regulatory feedback loops operating between FGF23 and Pi/vitamin D has been suggested [1,12]. In humans, serum FGF23 levels have been associated with the amount of dietary Pi [13,14], while intravenous infusion or acute duodenal Pi load were found to increase FGF23 levels in healthy humans [15]. Similarly, *in vivo* animal models have shown a relationship between dietary Pi loads and circulating Fgf23 levels [16,17], indicating that extracellular Pi induces the secretion of Fgf23. However, the mechanism by which Pi might regulate FGF23 production remains insufficiently defined and difficult to elucidate.

An interesting and possible mechanism is the direct regulation of FGF23 expression by Pi, in view of the recently accumulated data indicating that extracellular Pi acts as a signaling molecule [18,19]. While binding and/or cellular uptake of Pi by specialized Pi transporters are involved in

[1]Inserm, UMR 1229, RMeS, Regenerative Medicine and Skeleton, Université de Nantes, ONIRIS, Nantes, F-44042, France [2]Université de Nantes, UFR Odontologie, Nantes, F-44042, France [3]CHU Nantes, PHU 4 OTONN, Nantes, F-44042, France

*Corresponding author. INSERM U1229, RMeS, Faculté de Chirurgie Dentaire, 1, place Alexis Ricordeau 44042, Nantes cedex 1, France. E-mail: laurent.beck@inserm.fr (L. Beck).

Received December 23, 2017 • Revision received February 12, 2018 • Accepted February 15, 2018 • Available online 26 February 2018

https://doi.org/10.1016/j.molmet.2018.02.007

the Pi signal processing in lower organisms, no Pi-receptor or 'sensor' has yet been identified in mammals [20]. Remarkably, we recently investigated the role of the two high-affinity mammalian Na^+-Pi co-transporters PiT1/Slc20a1 and PiT2/Slc20a2 as Pi sensors in bone and showed that, *in vitro,* both PiTs were required for mediating Pi-dependent signaling [21], raising the question of their role in the modulation of the Pi-dependent FGF23 secretion.

To gain insights into the mechanism by which Pi could regulate FGF23 production, we explored the contribution of PiT1 or PiT2 to the regulation of Fgf23 expression and secretion by using *in vivo* and *ex vivo* murine models.

2. METHODS AND MATERIALS

2.1. Animals

The generation of *PiT1*$^{lox/lox}$ mice has been described previously [22]. Osteocyte-specific deletion of *PiT1* was performed by crossing *PiT1*$^{lox/lox}$ (129sv genetic background) to *DMP1-Cre* mice, generated using the 9.6 kb Dmp1 promoter [23]. *Slc20a2*$^{tm1a(EUCOMM)Wtsi}$ mice (thereafter named *PiT2* KO mice) on the C57BL/6 genetic background were obtained from the European Mouse Mutant Archive (EMMA). The mutant allele contains an IRES:lacZ trapping cassette and a splicing site disrupting *PiT2* gene expression [24]. Genomic DNA from tail was used for PCR genotyping. Animal care and maintenance were provided through the University of Nantes accredited animal facility at the Unité de Thérapeutique Expérimentale. Mice were housed under specific pathogen-free conditions and were fed with RM1 diet for maintenance and with RM3 diet for breeding (SDS Special Diets Services, France). This study conformed to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All procedures were approved by the Animal Care and Use Committee of the Région Pays de la Loire and conducted according to the French and European regulations on care and protection of laboratory animals (EC Directive 86/609, French Law 2001-486 issued on 6 June 2001) and the National Institutes of Health Animal Welfare (project #02286.02). The mice were weaned at post-natal day 21 (P21) to a normal Pi diet containing 0.7% calcium and 0.55% phosphorus. To induce Fgf23 regulation, the mice were fed at P28 with either a customized low-Pi (<0.05% phosphorus) or normal-Pi (0.55% phosphorus) or high-Pi (1.65% phosphorus) diet for 7 days (ssniff Spezialdiäten GmbH, Soest, Germany).

2.2. Serum and urine parameters

Serum phosphorus and calcium were analyzed using the Phosphorus Liqui-UV Test and the Calcium CPC LiquiColor Test kits according to the manufacturer's protocol (Stanbio Laboratory, Boerne, TX). Serum intact Fgf23 concentrations were assessed using ELISA kit according to the manufacturer's protocol (Kainos Laboratories, Tokyo, Japan). Serum PTH and C-terminal Fgf23 levels were determined using ELISA kits for mouse intact PTH and mouse/rat C-term Fgf23, respectively (Immutopics). Urine Pi, calcium, and creatinine concentrations assays were performed with Olympus AU400 Chemistry Analyzer.

2.3. Gene expression analysis

Total RNA was isolated from tissues using the Trizol® Reagent (Thermo Fisher Scientific) (for diaphysis) or using the Nucleospin® RNA II kit (Macherey—Nagel, Germany) (for soft tissues) according to the manufacturer's instructions. RNA was reverse transcribed using SuperScript® VILO™ (for diaphysis) or SuperScript®III (for soft tissues) (Thermo Fisher Scientific) as per manufacturer's instructions. Real-time PCR (qPCR) was performed on a Bio-Rad CFX96 using SYBR®Select Master Mix (Thermo Fisher Scientific). Primer efficiency was determined using a standard curve with a 1:4 dilution and specificity of amplification was verified from the melting curve analysis. Expression of target genes were normalized to pinin (*Pnn*) and beta-glucuronidase (*GusB*) expression levels, and the relative gene expression levels were calculated as previously described [25].

2.4. Immunohistochemistry (IHC)

Paraffin embedded kidney sections were processed for IHC as previously described [22]. Primary antibody for Klotho (1:100, KM2076, TransGenic Inc.) was incubated overnight at 4 °C. Secondary anti-mouse biotinylated goat antibody (1:500, Dako) was used and staining was performed using 3,3-diaminobenzidine chromogen (Dako) for 5 min and counterstaining with Mayer's hematoxylin. Stained sections were then mounted with Eukitt® and scanned using a Hamamatsu NanoZoomer HT (Hamamatsu Photonics KK) digital scanner at a 20× magnification.

2.5. Long bone shafts preparation

Femur, tibia, and humerus bones from P35 mice were dissected free of the surrounding soft tissue and flushed. After stripping away the periosteum, the long bone shafts were trimmed into two pieces for weighing. One half of each bone type was cultured overnight at 37 °C in 5% CO_2 humidified incubator in a well of a 96-well plate with 100 µl of culture medium containing Dulbecco's Modified Eagle's Medium (DMEM) high glucose GlutaMAX™ (catalogue no 31966, ThermoFisher Scientific, Saint-Aubin, France) supplemented with 0.2% Bovine Serum Albumine (Sigma—Aldrich, St Louis, MO, USA), 10 mM HEPES, and 50 µg/ml gentamycin. To study Pi-dependent Fgf23 secretion, the long bone shafts were washed three times with pre-warmed 0.9% NaCl solution and incubated during 24 h in stimulation medium. The stimulation medium consisted of phosphate-free high glucose GlutaMAX™ DMEM (catalogue no 11971-025, ThermoFisher Scientific) supplemented with 0.2% BSA, 10 mM HEPES, 50 µg/ml gentamycin and 0, 1, 3, 7, or 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4. Intact and C-terminal Fgf23 concentrations were assessed in the supernatants using ELISA kits according to the manufacturer's protocol (Kainos Laboratories and Immuntopics, respectively) and normalized by bone mass.

2.6. Transduction

Adeno-CMV-iCre (catalogue no 1045 Vector Biolabs) was added to the media of *PiT1*$^{lox/lox}$ long bone shafts culture at a concentration of 10^7 virus particles per mL of media. Control samples were treated with Ad-CMV-GFP (catalogue no 1060 Vector Biolabs) at the same concentration. The media were replaced 24 h after transduction, and, 72 h post-transduction, the long bone shaft were harvested and analyzed for PiT1 deletion.

2.7. Statistics

Data are expressed as mean ± S.E.M. GraphPad 5.0 software was used to perform Mann—Whitney tests or analysis of variance. A p value of less than 0.05 was considered statistically significant. Unless otherwise stated, experiments were repeated at least three times (exact numbers are indicated in the figures).

3. RESULTS

3.1. Deletion of *PiT2* in mice results in impaired Pi-dependent Fgf23 secretion

To investigate the role of PiT2 in the Pi-dependent regulation of Fgf23 secretion *in vivo*, we fed four-week-old *PiT2* KO mice with low

(0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. RT-qPCR analysis confirmed the deletion of *PiT2* in tibias, while *PiT1* expression was increased in mutant tibias under low-Pi diet condition (Figure 1A). The expression of the Pi exporter *Xpr1* [26] in tibias and kidneys was not changed, nor was the expression of the Na-Pi cotransporters *Npt2a* and *Npt2c* in kidneys (not shown). When *WT* and *PiT2* KO were fed a low Pi diet, we observed the expected decrease in serum Pi levels (Figure 1B). However, feeding a high Pi diet failed to reveal an increase in serum Pi levels, an observation that was already made previously, which may relate to the existence of compensatory endocrine loops [27]. The urine Pi/creatinine ratio was consistent with the feeding scheme and revealed a massive increase when mice were fed a high Pi diet, and a massive decrease when mice were fed a low Pi diet. This was true for both mouse strains, with no observed difference between genotypes (Figure 1B).

In WT mice fed a low Pi diet, circulating intact Fgf23 (iFgf23) levels were decreased compared to normal Pi diets (Figure 1C), a finding that was consistent with the decrease in serum Pi and urine Pi/creatinine levels (Figure 1B). Remarkably, in *PiT2* KO mice fed low-Pi diet, the circulating iFgf23 levels were abnormally high, being similar to the iFgf23 levels in the normal-Pi diet condition (Figure 1C). Although the C-terminal Fgf23 (cFgf23) levels were not statistically different between *WT* and *PiT2* KO mice, we could observe a similar trend (Figure 1D), suggesting that increased iFgf23 does not originate from a decrease in Fgf23 cleavage. In support of this, we observed no difference in *Galnt3* and *Fam20C* gene expressions between *WT* and *PiT2* KO mice (not shown). Accordingly, the *Galnt3/Fam20C* mRNA expression ratio was similar in *WT* and *PiT2* KO mice (Figure 1E). Moreover, RT-qPCR analysis of *Fgf23* gene expression in the diaphysis (Figure 1A) and of *Fgfr1*, *Fgfr2*, *Fgfr3*, and *Fgfr4* in the kidneys revealed no statistical difference between *WT* and *PiT2* KO mice (not shown). Interestingly, when iFgf23/cFgf23 ratios were calculated for the different Pi diet conditions, no change was observed for *PiT2* KO mice, while this ratio increased as a function of Pi load in *WT* mice (Figure 1F). Overall, the response of *PiT2* KO mice to the variation of Pi load was blunted compared to the response of WT mice, further suggesting a role of PiT2 in the setting of Pi-dependent Fgf23 secretion.

Of note, although the serum parathyroid hormone (PTH) levels increased as a function of Pi diet content as expected, these levels were similar between *WT* and *PiT2* KO mice in all Pi diet conditions (Figure 1G). Therefore, the normal serum Pi level observed in *PiT2* KO in the low-Pi condition despite a high iFgf23 serum level cannot be attributed to abnormal serum PTH levels. Similarly, the expression of *Cyp27b1*, *Cyp24a1*, and *Vdr* genes was similar between *WT* and *PiT2* KO mice (not shown). Interestingly however, the gene expression of α*Klotho*, the mandatory co-receptor for mediating Fgf23 renal effects, was lower in *PiT2* KO mice in the low-Pi diet condition (Figure 1H). This was accompanied by a drastic reduction of the protein expression of αKlotho in the kidney (Figure 1I).

3.2. Deletion of *PiT1* in long bone shafts *ex vivo* does not support a role of PiT1 in Pi-dependent Fgf23 secretion from bone

We next investigated the role of PiT1 in the Pi-dependent regulation of Fgf23 secretion by crossing conditional *PiT1*$^{lox/lox}$ mice [22] with *DMP1-Cre* transgenic mice [23] to generate *DMP1-Cre; PiT1*$^{lox/lox}$ mutant mice. When analyzing the deletion of *PiT1* in *PiT1*$^{lox/lox}$*;DMP1-Cre* mice, we observed that *PiT1* was deleted in a number of non-skeletal tissues (Supplemental Figure 1A), revealing unintended deletions as was shown in a recent report [28]. In addition, although the deletion of *PiT1* was visible in bone tissues at the genomic level, the deletion at the mRNA level was very low (Supplemental Figure 1B). This latter result was confirmed using RT-qPCR analysis showing no decrease in *PiT1* gene expression in mutant mice (Supplemental Figure 1C). Accordingly, no difference was observed in circulating Pi and iFgf23 levels between *WT* and *DMP1-Cre; PiT1*$^{lox/lox}$ mice (Supplemental Figure 1D). Similarly, no difference was observed in urine Pi, urine calcium or serum calcium, and PTH levels in any Pi diet condition (not shown).

To overcome the inefficient targeting of *PiT1* using the *DMP1Cre* mice, we used *ex vivo* organ cultures of long bone shafts from *WT* and *PiT1*$^{lox/lox}$ mice transduced with Cre-expressing adenoviruses (*AdVCre*) (Figure 2A). We observed a 61—71% deletion of *PiT1* mRNA without upregulation of *PiT2* mRNA (Figure 2B), allowing us to assess the role of PiT1 in the Pi-dependent regulation of Fgf23 secretion. Despite this efficient deletion of PiT1, results showed that following Pi challenge, secreted iFgf23 and cFgf23 levels (Figure 2C), as well as the iFgf23/cFgf23 ratio (Figure 2D), were similar in *WT* and *PiT1*$^{lox/lox}$*; AdVCre* bones. Consistently, the expression of *Fgf23* in the diaphysis was similar in all conditions tested as were the expression of *Galnt3* and *Fam20C* and the *Galnt3/Fam20C* expression ratio (not shown). These data showed that despite an efficient deletion of PiT1 in bone, the role of PiT1 in the Pi-dependent regulation of Fgf23 could not be demonstrated, suggesting that more appropriate *in vivo* tools are required to successfully address this question.

3.3. The Pi-dependent modulation of Fgf23 secretion by PiT2 is bone-autonomous

We used the *ex vivo* long bone shaft approach to determine whether the role of PiT2 in modulating the Pi-dependent Fgf23 secretion was bone-autonomous. RT-qPCR analysis confirmed the deletion of *PiT2* in diaphyses, while *PiT1* expression was higher in mutant than *WT* tibias under high-Pi diet condition (Figure 3A). In *WT* bones, while the expression of *Fgf23* remained unchanged when extracellular Pi was changed (Figure 3B), the secretion of iFgf23 in the extracellular medium increased as a function of extracellular Pi concentration, as expected (Figure 3C). Strikingly, this was not the case anymore in *PiT2* mutant bones, where secreted iFgf23 remained low (Figure 3C). Interestingly, the secreted cFgf23 from *PiT2* KO mice displayed an inverse profile than the secreted iFgf23 and appeared higher in mutant than in *WT* bones (Figure 3C). Accordingly, the iFgf23/cFgf23 ratios were blunted in *PiT2* KO tibias (Figure 3D), as was observed in *in vivo* experiments (Figure 1F). The low iFgf23 secretion and iFgf23/cFgf23 ratios were consistent with the lower expression of *Fgf23* in mutant bones (Figure 3B). Finally, no differences in *Fam20C* and *Galnt3* gene expression, nor in *Galnt3/Fam20C* mRNA expression ratios, were evident between WT and mutant bones (not shown). Altogether, our data indicate that the expression of *PiT2* is mandatory for a normal regulation of Fgf23 secretion following changes in extracellular Pi concentrations. Interestingly, we recently showed that PiT2 was necessary for the Pi-dependent activation of the ERK1/2 MAPK pathway [21], a finding that may be relevant with the activation of the ERK1/2 MAPK pathway through FGFR1 that is known to stimulate Fgf23 secretion [29]. However, when blocking the Pi-dependent ERK1/2 phosphorylation with the MAPK inhibitor U0126 (Figure 3E) in isolated bone shafts, no change was observed in the Pi-dependent Fgf23 secretion, irrespective of the Pi load (Figure 3F). Moreover, similar expression of *Fgfr1* was found in WT and PiT2 KO mice (Figure 3G). These data suggested that mechanistic links other than the ERK1/2 pathway are likely at work between PiT2 and Fgf23 secretion.

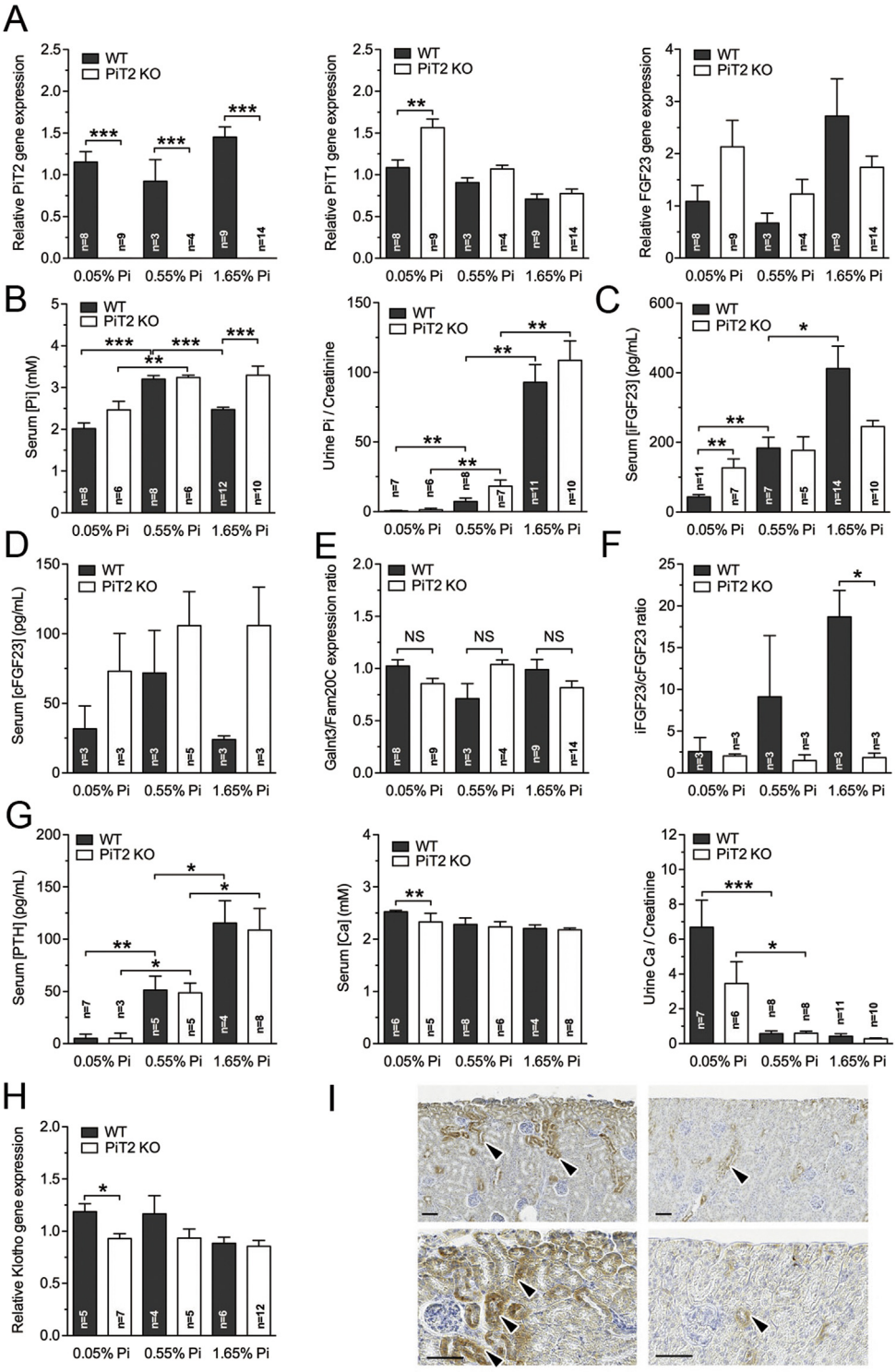


Figure 1: Deletion of *PiT2 in vivo* leads to inappropriate serum Fgf23 levels in low-Pi condition. *(A)* RT-qPCR analysis of *PiT2*, *PiT1* and *Fgf23* mRNA levels in flushed tibias isolated from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. *(B)* Serum and urine Pi values from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%), and high (1.65%) Pi diets for one week. *(C-D)* Serum intact Fgf23 (iFgf23) *(C)* and C-terminal Fgf23 (cFgf23) *(D)* levels (ELISA) from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *(E-F) Galnt3/Fam20C* mRNA ratios as determined by RT-qPCR analysis *(E)* and iFgf23/cFgf23 ratios *(F)* from flushed tibias from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. *(G)* Serum PTH levels *(Left)* and serum and urine Ca values *(Center and Right)* from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *(H)* RT-qPCR analysis of α*Klotho* mRNA levels in kidneys isolated from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. *(I)* Immunohistochemistry for αKlotho of kidney sections from *WT* or *PiT2* KO mice fed with low (0.05%) Pi diet for one week. *Black arrowheads*: αKlotho-positive staining. *Top,* low magnification; *Bottom,* high magnification, scale bar, 50 μm. Data are means ± S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS: not significant.

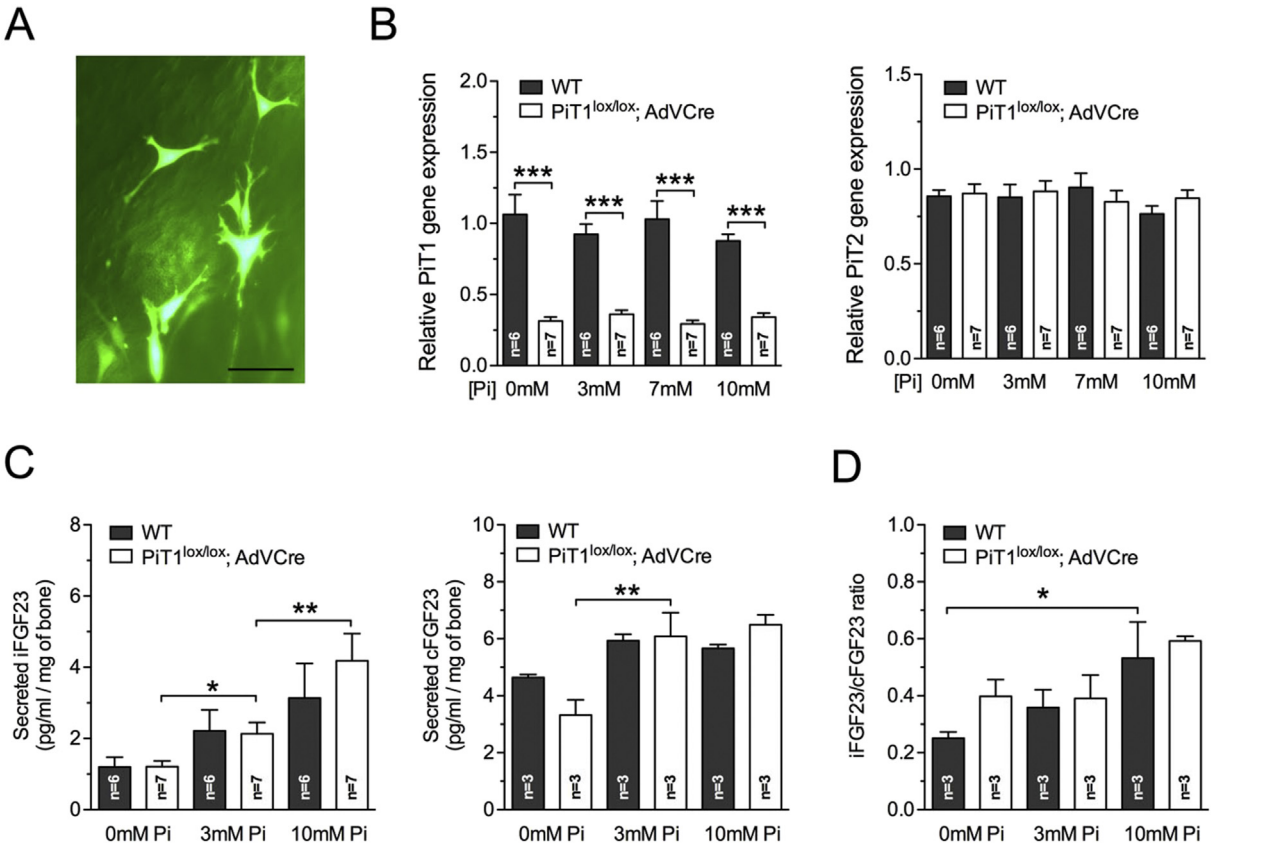


Figure 2: *Ex vivo* deletion of *PiT1* in long bone shafts does not support a role of PiT1 in Pi-dependent Fgf23 secretion from bone. *(A)* Expression of GFP in Ad-CMV-GFP-transduced bone shafts 48 h after transduction. *(B)* RT-qPCR analysis of *PiT1* and *PiT2* mRNA levels in flushed tibias isolated from *WT* or *PiT1*$^{lox/lox}$ transduced with Adeno-CMV-iCre (10^7 virus particles per mL), and stimulated for 24 h in medium containing 0, 3, 7, or 10 mM Pi, as indicated. *Pnn* and *GusB* genes were used as internal controls. *(C)* Secreted levels of intact Fgf23 (iFgf23) and C-terminal Fgf23 (cFgf23) from flushed tibias isolated from *WT* or *PiT1*$^{lox/lox}$ transduced with Adeno-CMV-iCre and stimulated for 24 h in medium containing 0, 3, or 10 mM Pi. *(D)* iFgf23/cFgf23 ratios from *WT* or *PiT1*$^{lox/lox}$ transduced with Adeno-CMV-iCre and stimulated for 24 h in medium containing 0, 3, or 10 mM Pi. Data are means ± S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4. DISCUSSION

This study presents the first direct evidence that PiT2 expression *in vivo* and *ex vivo* is essential for normal secretion of Fgf23 following dietary Pi changes. While iFgf23 levels were correlated to dietary Pi loads *in vivo* or extracellular Pi concentrations *ex vivo* in *WT* animals, the Pi-dependent regulation of Fgf23 secretion was blunted in *PiT2* KO mice and isolated bones, as was clearly showed when calculating iFgf23/cFgf23 ratios. Since similar Pi-dependent regulation of Fgf23 secretion was observed in whole mutant animals and isolated bone shafts from *PiT2* KO mice, the contribution of other organs or possible endocrine loops to this regulation appears unlikely. However, while this illustrates that Pi-dependent regulation of Fgf23 is dependent upon normal bone *PiT2* expression and highlights PiT2 as an important putative Pi sensor, the underlying mechanism linking PiT2 to the secretion of Fgf23 remains to be deciphered. Notably, the similar expression of *Fam20C* and *Galnt3* in *WT* and *PiT2* KO mice was not consistent with the difference observed in the Fgf23/cFgf23 ratios, suggesting that the regulation of Fgf23 cleavage by PiT2 might involve *Fam20C* and *Galnt3* independent mechanisms, as was suggested recently [30]. Moreover, although FGFR1-mediated stimulation of FGF23 secretion was illustrated through the activation of the ERK1/2 MAPK pathway [29], our data showed that the Pi-dependent activation of the ERK1/2 pathway was not involved in Fgf23 secretion from bone, further indicating that other mechanistic links are at work between PiT2 and Fgf23. One possibility is the involvement of PiT2-specific protein partners linking Pi signaling to Fgf23 secretion, as was shown recently for other PiT2-or PiT1-specific physiological functions [31,32]. Moreover, while our data clearly showed a role of PiT2 in the setting of Fgf23 secretion, most of the physiological parameters remained normal. This finding is consistent with the absence of identified PiT2 mutations in humans that would cause a disturbed Pi homeostasis and suggests the existence of mechanisms compensating the lack of PiT2. One of the possible compensatory mechanism involves the negative regulation of αKlotho that we uncovered in the *PiT2* KO kidneys, which may explain why mutant mice fed a low Pi diet and exhibiting abnormally normal Fgf23 levels retained normal serum and urine Pi levels. Other compensatory mechanisms, such as the existence of other Pi sensors, may also be involved. Along this line, although we could efficiently down-regulate PiT1 in isolated bone shafts and illustrated no role in Pi-dependent regulation of Fgf23 in this model, its actual role *in vivo* remains to be determined.

As an illustration of the complexity of these regulations, the effect of Pi-modified diets on FGF23 secretion in humans still remains a matter of debate [33,34]. Much of the recent focus on FGF23 has been driven by human studies showing that even a mild loss of kidney function is associated with considerable elevations of serum FGF23 levels, preventing hyperphosphatemia early in the course of

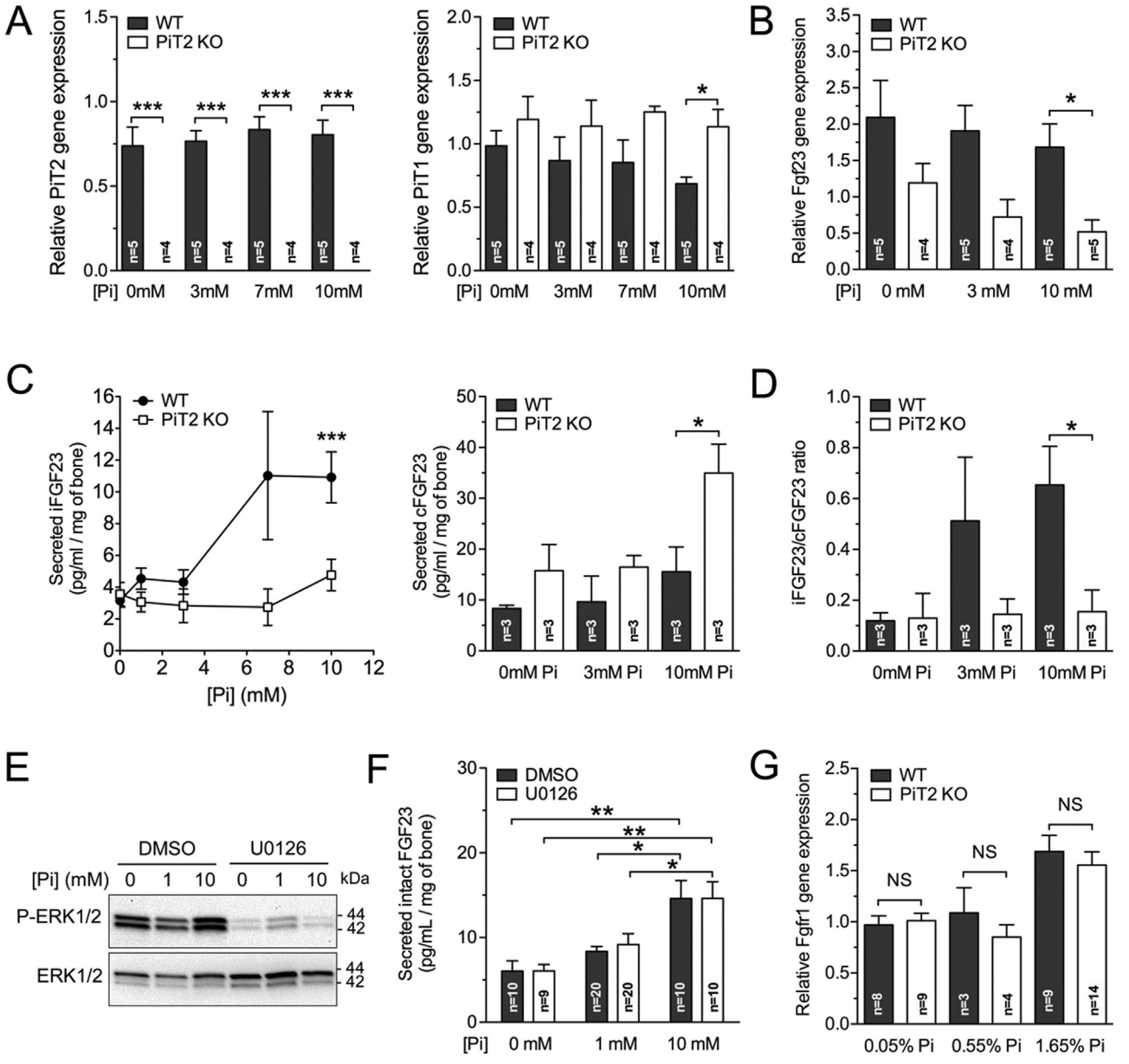


Figure 3: *Ex vivo* organ culture of long bone shafts from *PiT2* KO mice illustrates the bone-autonomous Pi-dependent role of PiT2 in regulating the FGF23 secretion. *(A)* RT-qPCR analysis of *PiT2* and *PiT1* mRNA levels in flushed tibias isolated from *WT* or *PiT2* KO mice and stimulated for 24 h in medium containing 0, 3, 7, or 10 mM Pi, as indicated. *Pnn* and *GusB* genes were used as internal controls. *(B)* RT-qPCR analysis of *Fgf23* mRNA levels in flushed tibias isolated as in *A*. *(C)* Secreted levels of intact (*Left*) and C-terminal (*Right*) Fgf23 from flushed tibias isolated from *WT* or *PiT2* KO and stimulated for 24 h in medium containing indicated Pi concentrations. *(D)* iFgf23/cFgf23 ratios from flushed tibias isolated from *WT* or *PiT2* KO and stimulated for 24 h in medium containing indicated Pi concentrations. *(E)* Western-blot analysis of ERK1/2 phosphorylation (P-ERK1/2) in flushed tibias from *WT* mice (C57BL/6 background) stimulated for 30 min with 0, 1 mM, or 10 mM extracellular Pi concentration, in the presence of DMSO or U0126, as indicated. Total ERK1/2 proteins were used as a loading control. *(F)* Secreted iFgf23 levels from flushed tibias from *WT* mice (C57BL/6 background) stimulated for 30 min with 0, 1 mM, or 10 mM extracellular Pi concentration, in the presence of DMSO or U0126, as indicated. *(G)* RT-qPCR analysis of *Fgfr1* mRNA levels in diaphysis from *WT* or *PiT2* KO mice fed with low (0.05%), normal (0.55%), and high (1.65%) Pi diets for one week. *Pnn* and *GusB* genes were used as internal controls. Data are means ± S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS: not significant.

the disease. Hence, several studies were designed to restrict dietary Pi in CKD patients to decrease circulating FGF23 levels. Although most studies showed a reduction in Pi excretion, the effects on FGF23 levels have been mixed, with some studies showing mild reduction, particularly when measuring intact FGF23 [33], and other studies revealing no change in FGF23 levels [34]. The role of PiT2 and possible other players in the setting of high FGF23 concentration following the early phase of CKD characterized by hyperphosphatemia, remains to be determined. This is particularly important in view of the detrimental effects of FGF23 in the later stages of the disease, which has fueled the search of strategies to lower serum FGF23 levels or block its unwanted actions [33]. In view of the emerging cross-talk between inflammation, iron deficiency, and bone mineral metabolism in controlling FGF23 secretion and/or expression [8–10,12,35], the possible involvement of PiT2 in these processes should now be considered.

In summary, here we provide the first experimental evidence that PiT2 regulates synthesis and secretion of FGF23 in response to high Pi load *in vivo* and in bone organ cultures *ex vivo*. These findings may identify PiT2 as a target for novel therapies to improve the excessive FGF23 secretion in hyperphosphatemic disorders such as chronic kidney disease.

AUTHOR'S CONTRIBUTION

N.B., S.B.C., and L.B. designed experiments, N.B., G.F., S.B.C., and S.S. performed experiments, N.B., S.B.C., and L.B. analyzed data, N.B. and L.B. wrote the manuscript, N.B., J.G., S.B.C., and L.B. made adjustments to the final paper version. All authors reviewed the results and approved the final version of the manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from Inserm, «Région des Pays de la Loire» (Nouvelle Equipe/Nouvelle Thématique and SENSEO). Nina Bon received a fellowship from « Région des Pays de la Loire » (SENSEO) and University of Nantes. Giulia Frangi received a fellowship from the «Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation». We gratefully thank the UTE IRS-UN animal facility of the SFR Santé F. Bonamy (UMS Inserm 016/CNRS 3556) (Nantes, France).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.02.007.

REFERENCES

[1] Kawai, M., Kinoshita, S., Ozono, K., Michigami, T., 2016. Inorganic phosphate activates the AKT/mTORC1 pathway and shortens the life span of an α-klotho-deficient model. Journal of the American Society of Nephrology: JASN 27(9): 2810—2824. https://doi.org/10.1681/ASN.2015040446.

[2] Erben, R.G., 2016. Update on FGF23 and Klotho signaling. Molecular and Cellular Endocrinology 432(C):56—65. https://doi.org/10.1016/j.mce.2016.05.008.

[3] Razzaque, M.S., 2011. Phosphate toxicity: new insights into an old problem. Clinical Science (London, England : 1979) 120(3):91—97. https://doi.org/10.1042/CS20100377.

[4] Lau, W.L., Pai, A., Moe, S.M., Giachelli, C.M., 2011. Direct effects of phosphate on vascular cell function. Advances in Chronic Kidney Disease 18(2):105—112. https://doi.org/10.1053/j.ackd.2010.12.002.

[5] Martin, A., Liu, S., David, V., Li, H., Karydis, A., Feng, J.Q., et al., 2011. Bone proteins PHEX and DMP1 regulate fibroblastic growth factor Fgf23 expression in osteocytes through a common pathway involving FGF receptor (FGFR) signaling. The FASEB Journal 25(8):2551—2562. https://doi.org/10.1096/fj.10-177816.

[6] Christov, M., Jüppner, H., 2013. Insights from genetic disorders of phosphate homeostasis. Seminars in Nephrology 33(2):143—157. https://doi.org/10.1016/j.semnephrol.2012.12.015.

[7] Xiao, Z., Huang, J., Cao, L., Liang, Y., Han, X., Quarles, L.D., 2014. Osteocyte-specific deletion of Fgfr1 suppresses FGF23. PLoS One 9(8):e104154. https://doi.org/10.1371/journal.pone.0104154.t003.

[8] Wolf, M., White, K.E., 2014. Coupling fibroblast growth factor 23 production and cleavage. Current Opinion in Nephrology and Hypertension 23(4):411—419. https://doi.org/10.1097/01.mnh.0000447020.74593.6f.

[9] David, V., Martin, A., Isakova, T., Spaulding, C., Qi, L., Ramirez, V., et al., 2015. Inflammation and functional iron deficiency regulate fibroblast growth factor 23 production. Kidney International, 1—12. https://doi.org/10.1038/ki.2015.290.

[10] Ito, N., Wijenayaka, A.R., Prideaux, M., Kogawa, M., Ormsby, R.T., Evdokiou, A., et al., 2015. Regulation of FGF23 expression in IDG-SW3 osteocytes and human bone by pro-inflammatory stimuli. Molecular and Cellular Endocrinology 399(C):208—218. https://doi.org/10.1016/j.mce.2014.10.007.

[11] Tagliabracci, V.S., Engel, J.L., Wiley, S.E., Xiao, J., Gonzalez, D.J., Nidumanda Appaiah, H., et al., 2014. Dynamic regulation of FGF23 by Fam20C phosphorylation, GalNAc-T3 glycosylation, and furin proteolysis. Proceedings of the National Academy of Sciences of the United States of America 111(15):5520—5525. https://doi.org/10.1073/pnas.1402218111.

[12] Martin, A., David, V., Quarles, L.D., 2012. Regulation and function of the FGF23/klotho endocrine pathways. Physiological Reviews 92(1):131—155. https://doi.org/10.1152/physrev.00002.2011.

[13] Ferrari, S.L., Bonjour, J.P., Rizzoli, R., 2005. Fibroblast growth factor-23 relationship to dietary phosphate and renal phosphate handling in healthy young men. The Journal of Clinical Endocrinology and Metabolism 90(3): 1519—1524. https://doi.org/10.1210/jc.2004-1039.

[14] Burnett, S.A.M., Gunawardene, S.C., Bringhurst, F.R., Jüppner, H., Lee, H., Finkelstein, J.S., 2006. Regulation of C-terminal and intact FGF-23 by dietary phosphate in men and women. Journal of Bone and Mineral Research 21(8): 1187—1196. https://doi.org/10.1359/jbmr.060507.

[15] Scanni, R., vonRotz, M., Jehle, S., Hulter, H.N., Krapf, R., 2014. The human response to acute enteral and parenteral phosphate loads. Journal of the American Society of Nephrology : JASN 25(12):2730—2739. https://doi.org/10.1681/ASN.2013101076.

[16] Saito, H., Maeda, A., Ohtomo, S.I., Hirata, M., Kusano, K., Kato, S., et al., 2005. Circulating FGF-23 is regulated by 1alpha,25-dihydroxyvitamin D3 and phosphorus in vivo. The Journal of Biological Chemistry 280(4):2543—2549. https://doi.org/10.1074/jbc.M408903200.

[17] Perwad, F., Azam, N., Zhang, M.Y.H., Yamashita, T., Tenenhouse, H.S., Portale, A.A., 2005. Dietary and serum phosphorus regulate fibroblast growth factor 23 expression and 1,25-dihydroxyvitamin D metabolism in mice. Endocrinology 146(12):5358—5364. https://doi.org/10.1210/en.2005-0777.

[18] Khoshniat, S., Bourgine, A., Julien, M., Weiss, P., Guicheux, J., Beck, L., 2011. The emergence of phosphate as a specific signaling molecule in bone and other cell types in mammals. Cellular and Molecular Life Sciences : CMLS 68(2):205—218. https://doi.org/10.1007/s00018-010-0527-z.

[19] Michigami, T., 2013. Extracellular phosphate as a signaling molecule. Contributions to Nephrology 180:14—24. https://doi.org/10.1159/000346776.

[20] Bergwitz, C., Jüppner, H., 2011. Phosphate sensing. Advances in Chronic Kidney Disease 18(2):132—144. https://doi.org/10.1053/j.ackd.2011.01.004.

[21] Bon, N., Couasnay, G., Bourgine, A., Sourice, S., Beck-Cormier, S., Guicheux, J., et al., 2017. Phosphate (Pi)-regulated heterodimerization of the high-affinity sodium-dependent Pi transporters PiT1/Slc20a1 and PiT2/Slc20a2 underlies extracellular Pi sensing independently of Pi uptake. The Journal of Biological Chemistry. https://doi.org/10.1074/jbc.M117.807339.

[22] Beck, L., Leroy, C., Beck-Cormier, S., Forand, A., Salaün, C., Paris, N., et al., 2010. The phosphate transporter PiT1 (Slc20a1) revealed as a new essential gene for mouse liver development. PLoS One 5(2):e9148. https://doi.org/10.1371/journal.pone.0009148.

[23] Lu, Y., Xie, Y., Zhang, S., Dusevich, V., Bonewald, L.F., Feng, J.Q., 2007. DMP1-targeted Cre expression in odontoblasts and osteocytes. Journal of Dental Research 86(4):320—325.

[24] Skarnes, W.C., Rosen, B., West, A.P., Koutsourakis, M., Bushell, W., Iyer, V., et al., 2011. A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474(7351):337—342. https://doi.org/10.1038/nature10163.

[25] Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29(9):e45.

[26] Giovannini, D., Touhami, J., Charnet, P., Sitbon, M., Battini, J.-L., 2013. Inorganic phosphate export by the retrovirus receptor XPR1 in metazoans. Cell Reports 3(6):1866–1873. https://doi.org/10.1016/j.celrep.2013.05.035.

[27] Nishida, Y., Taketani, Y., Yamanaka-Okumura, H., Imamura, F., Taniguchi, A., Sato, T., et al., 2006. Acute effect of oral phosphate loading on serum fibroblast growth factor 23 levels in healthy men. Kidney International 70(12): 2141–2147. https://doi.org/10.1038/sj.ki.5002000.

[28] Lim, J., Burclaff, J., He, G., Mills, J.C., Long, F., 2017. Unintended targeting of Dmp1-Cre reveals a critical role for Bmpr1a signaling in the gastrointestinal mesenchyme of adult mice. Bone Research 5:16049. https://doi.org/10.1038/boneres.2016.49.

[29] Smith, R.C., O'Bryan, L.M., Farrow, E.G., Summers, L.J., Clinkenbeard, E.L., Roberts, J.L., et al., 2012. Circulating αKlotho influences phosphate handling by controlling FGF23 production. Journal of Clinical Investigation 122(12): 4710–4715. https://doi.org/10.1172/JCI64986DS1.

[30] Eren, M., Place, A.T., Thomas, P.M., Flevaris, P., Miyata, T., Vaughan, D.E., 2017. PAI-1 is a critical regulator of FGF23 homeostasis. Science Advances 3(9):e1603259. https://doi.org/10.1126/sciadv.1603259.

[31] Ma, X.-X., Li, X., Yi, P., Wang, C., Weng, J., Zhang, L., et al., 2017. PiT2 regulates neuronal outgrowththrough interaction withmicrotubule-associated protein 1B. Scientific Reports, 1–13. https://doi.org/10.1038/s41598-017-17953-3.

[32] Forand, A., Koumakis, E., Rousseau, A., Sassier, Y., Journe, C., Merlin, J.-F., et al., 2016. Disruption of the phosphate transporter Pit1 in hepatocytes improves glucose metabolism and insulin signaling by modulating the USP7/IRS1 interaction. Cell Reports 17(7):1905. https://doi.org/10.1016/j.celrep.2016.10.039.

[33] Isakova, T., Barchi-Chung, A., Enfield, G., Smith, K., Vargas, G., Houston, J., et al., 2013. Effects of dietary phosphate restriction and phosphate binders on FGF23 levels in CKD. Clinical Journal of the American Society of Nephrology 8(6):1009–1018. https://doi.org/10.2215/CJN.09250912.

[34] Block, G.A., Wheeler, D.C., Persky, M.S., Kestenbaum, B., Ketteler, M., Spiegel, D.M., et al., 2012. Effects of phosphate binders in moderate CKD. Journal of the American Society of Nephrology : JASN 23(8):1407–1415. https://doi.org/10.1681/ASN.2012030223.

[35] David, V., Francis, C., Babitt, J.L., 2017. Ironing out the cross talk between FGF23 and inflammation. AJP: Renal Physiology 312(1):F1–F8. https://doi.org/10.1152/ajprenal.00359.2016.

CONCLUSION AND DISCUSSION

PiT2 and its role in bone and AT dialogue

When we first started this project, we were interested in the dialogue between bone and the adipose tissues and the impact of PiT2 on it. Especially we hypothesized that, in the absence of PiT2, the dialogue between bone and adipose tissues could be altered and impact on skeletal health, explaining the skeletal phenotype observed in the mutant mice. This thesis work has allowed to expand our knowledge on the PiT2 protein, showing for the first time its involvement in the homeostasis of adipose tissues. Indeed, we showed that PiT2 is a potential regulator of both the bone marrow and the peripheral adipose tissues, being involved in their age-related increase without altering the energetic metabolism. While the absence of PiT2 has a negative impact on bone quality and strength both in young and adult PiT2KO mice, we observed an increased bone marrow adiposity in 3-week-old PiT2KO mice together with a decreased bone marrow adiposity in adult mice. These results obtained in young PiT2KO mice were consistent with published reports describing an inverse correlation between BMAT and bone volume. However, the observed decreased BMAT in adult PiT2KO mice was unexpected. The argument of the inverse correlation between bone and BMAT volumes is the one that is very often put forward to convince of the important role of the dialogue between these two tissues in their respective physiology. However, this inverse correlation is not observed in some biological contexts. For example, obese patients who underwent vertical sleeve gastrectomy displayed both a decrease of bone and BMAT volumes and the same condition was also described for females undergoing the lactation phase (Bornstein et al., 2014; Li et al., 2019 b; Winter et al., 2020). This may actually suggest that other mechanisms are involved in the bone/BMAT relationship that cannot necessarily be traced back to an altered balance in BMSCs differentiation. Certainly, in the majority of cases where the inverse correlation between bone and BMAT is observed, the increase in BMAT has an impact on the activity of osteoblasts or osteoclasts (see table 5 in the introduction and (Rosen et al., 2017; Li et al., 2018). We have previously shown that the number and the activity of osteoblasts and osteoclast are similar between PiT2KO and WT mice (Beck-Cormier et al., 2019). Similarly, we showed in this study that the number and differentiation capacity of marrow adipocyte progenitors was identical in mutants and controls. Altogether, the results that we generated from this work do not seem to fully support our initial hypothesis, but expanded our comprehension of the role of PiT2 in the skeleton, and identified PiT2 as a new potential regulator of obesity.

Bone and adipose tissues are both endocrine organs, which can impact on each other. In the absence of PiT2 we did not observe major differences regarding the major skeletal and adipogenic secreted factors, with the exception of leptin that constantly decreased during aging, from 3 weeks to 6 months of age. While this probably reflects the decreased volume of peripheral adipose tissues, which primarily secrete

leptin, it is important to remember the direct and indirect roles of this adipokine on the bone tissue. Thus, it is possible that low level of leptin could participate to the skeletal phenotype observed in the PiT2KO mice, and would require more investigation. Osteocalcin is a protein hormone known to be solely secreted by osteoblasts and plays a role in the body's energetic metabolism, acting on, among others, fat depots. However, the levels of total osteocalcin within the serum were unchanged in the absence of PiT2 (unpublished results). Similarly, serum adiponectin was unchanged in PiT2KO mice compared to the WT. Altogether, the absence of major endocrine and metabolic defects in PiT2-deficient mice argues against a critical role of PiT2 in the dialogue between bone and adipose tissues, which in itself is not sufficient to explain the observed *in vivo* phenotype.

BMSC and adipogenic progenitors

We observed an increased BMAT volume in 3-week-old PiT2KO mice compared to the WT, both in male and female samples. These results were confirmed by qPCR analyses on the adipogenic markers FABP4 and Adiponectin, which were both significantly increased in PiT2KO whole tibias compared to controls. We hypothesized that this phenotype could come from an altered BMSCs differentiation, whereby the common progenitors between osteoblasts and adipocytes within the bone marrow would somehow be preferentially driven towards the adipogenic lineage than the osteoblastic one in the absence of PiT2. However, our cytometry analyses showed that the BMSCs population was unchanged in the absence of PiT2 and that *in vitro* BMSCs from 3-week-old PiT2KO tibias displayed an unchanged adipogenic differentiation compared to WT cells, leading to the conclusion that the effect of the absence of PiT2 on BMAT is most likely non cell-autonomous. Alternatively, we could argue that our cytometric analyses were biased and that the lack of specific markers for adipocytes in the BMSCs may prevent their detailed and correct characterization. We chose some of the classical markers described to characterize the BMSCs population and the adipogenic progenitors for FACS analyses. In addition, isolation of non-hematopoietic marrow stem cells in the bone relies on the ability of bone marrow cells to adhere to plastic plates. Because of the heterogeneity of the BM, this method may not be as reliable as we would like it to be. Therefore, an alternate possibility and, probably the most promising one, would be to perform a scRNAseq analysis. Considering the extreme cell heterogeneity of the bone marrow, this would offer the possibility to analyze the cellular transcriptome and identify potential alterations in the absence of PiT2 to create new possible directions for further investigations.This could be even more interesting if we consider that, from the main data published on scRNAseq analysis performed on BMSCs populations, PiT2 expression has been found in osteo-primed LEPR+ cells (as one of its potential partners, Fhl2) (Tikhonova et al., 2019), in CXCL12+ reticular cells (that are related to bone morphogenesis and mineralization) (Helbling et al., 2019) and in MSC-descendent osteolineage cells (OLC1) (Baryawno et al., 2019). A specific budget is however necessary to perform such experiments. Finally, and unfortunately, I did not have the opportunity to perform the

same experiments on BMSCs from adult mice. This is something that absolutely needs to be done in the near future and we are currently planning to perform those experiments as soon as we can.

The blunting of the age-induced BMAT increase in PiT2KO mice could be explained by defects in proliferation or senescence of BMSC and/or adipogenic progenitors. To test this hypothesis, we could perform immunohistochemistry analyses on tibia sections using markers of senescence (like p16 or p53) or proliferation (like Ki67 or PCNA), or check for an increased expression of those markers by qPCR analysis. Those experiments are not straightforward, mainly due to the heterogeneity of the bone marrow and a ScRNAseq approach would probably better answer those questions. Mostly if we consider the recent discovery by Zhong and colleagues of a novel adipogenic lineage cell population in bone characterized by the expression of adiponectin but without lipid droplets (Zhong et al., 2020). Indeed, in adult PiT2-deficient mice what we observe could be a decrease of mature adipocytes, but maybe there could be in parallel an increase in this lipid droplet free cell population, which would be invisible to the POM staining and consequently to CE-CT analyses. A simple IHC analysis could already give us preliminary results on the presence of adiponectin positive cells within the BM lacking the big lipid droplet we are used to see.

In vivo cellular interactions are much more complex than those observed in 2D culture. Hence, it can be assumed that not being in the tridimensional bone marrow environment would make it impossible to identify abnormal cellular interactions. One can hypothesise that the phenotype observed *in vivo* could be hardly replicated *in vitro*. The group of Michaela Reagan has developed some 3D cell culture protocols allowing to recreate the closer condition for the cells to their real environment. It would be interesting to do so using the whole BM of PiT2KO tibias to see if the outcome would change compared to the *in vitro* experiments already performed and perhaps give an outcome closer to the condition observed *in vivo*.

To conclude with this part, the increased BMAT observed in 3-week-old PiT2KO mice is quite challenging to interpret and, to date, we do not know the mechanism behind it. Could it be due to an alteration of BMAT developing process soon after birth? We know from the literature that the BMAT increases with age both in humans and mice and, in our case, this is completely lost between 3 and 16 weeks of age in the PiT2KO mice. Could PiT2 be involved in this process? Could it be somehow necessary for adipocytes development and/or maturation within the BM once weaning is complete? As often when experiments are performed, more questions rise.

Inhibition of the age-induced adipose tissues increase

We have shown that, in absence of PiT2, both peripheral and intramedullar fat depots do not increase their volume with age, as expected. This phenotype is striking and probably the most evident outcome of the absence of PiT2 after the skeletal phenotype and brain calcifications already described by our group (Jensen et al., 2013; Beck-Cormier et al., 2019). Unfortunately, we don't know yet the mechanism underlying this phenotype. We wondered if this could be the outcome of an altered energy

consumption, however nothing we considered related to this possibility seemed to confirm it. Food intake analyses did not uncover any differences between WT and PiT2KO mice and animals did not seem more stressed nor active than the WT ones enough that could explain the decreased fat depots.

Could PiT2 be involved in pathologies associated with abnormal fat mass?

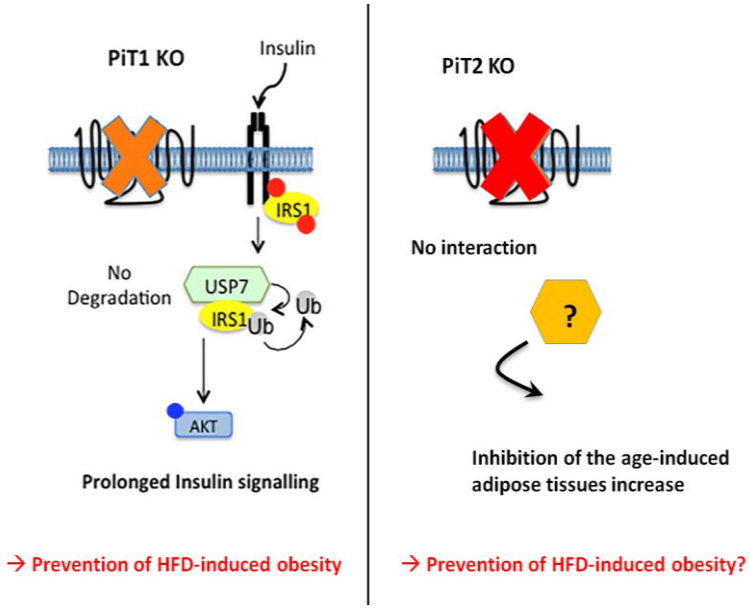
What is the contribution of BMAT to the BM niche and does its decrease have a repercussion on bone or the energetic metabolism? I asked myself many questions regarding this topic and mostly concerning our results on the peripheral fat depots. Is the decreased BMAT a consequence of the decreased peripheral adipose tissues? Is it the other way around? Are those two fat depots somehow linked to one another? We always read about how BMAT is a distinct fat depot compared to the WAT or BAT, with different location, feature and function. Both tissues are known to increase with age and also in some pathological conditions like obesity. However, this correlation is lost in other pathologies, like anorexia nervosa, where we curiously observe an increased BMAT in contrast with an almost total loss of peripheral fat depots. Importantly, the increased BMAT is reversible with recovery and so, probably, the augmented BMAT in patients with anorexia nervosa is linked with their weight loss and the chronic state of low weight. The main hypothesis to explain this paradoxical BMAT increase in this pathology is the possibility of it being a stock of nutrients in a state where there is a strong lack of them. Before the global Covid-19 pandemic striked, we planned to perform a high fat diet experiment on our mutant mice and this would have probably helped to answer this question and clarify the role of PiT2 in this context. The idea of challenging both BMAT and pAT with a protocol of induced obesity would have given us the possibility to investigate how those two tissues would have reacted in a context of caloric abundance and, moreover, to test whether the absence of PiT2 would have protected against the onset of obesity. In the same context, another challenge that would have been interesting to perform is the one coming from caloric restriction. In this way, we would have still challenged the two tissues, but with an opposite result: caloric restriction is known to increase the BMAT volume and to decrease peripheral fat mass. We will hopefully be able to plan HFD and caloric restriction protocols next year.

Phosphate, phosphate transport and adipose tissues

Few papers in the literature described a link between phosphate and the adipose tissues. In humans, an inverse correlation between serum Pi levels and obesity has been observed, and an association between high phosphate diet and the suppression of lipogenesis in white adipose tissue has been described both in humans and rodents (Park et al., 2009; Eller et al., 2011; Obeid, 2013; Ayoub et al., 2015; Abuduli et al., 2016; Bassil et al., 2016; Billington et al., 2017; Imi et al., 2018). Importantly, PiT2KO mice display no differences in serum phosphate levels (Jensen et al., 2016; Wallingford et al., 2016; Yamada et al., 2017; Beck-Cormier et al., 2019). Does this exclude the possibility of an altered phosphate transport in adipose

cells due to the absence of PiT2? We are currently performing phosphate transport experiments on 3T3-L1 mature adipocytes to test whether the observed *in vivo* phenotype may be due to the absence of PiT2 as a phosphate transporter. The outcome could be either a compensation of Pi transport carried out by PiT1, (even though we did not observe an increased expression of PiT1 in the absence of PiT2 within the whole tibia or the peripheral adipose tissue), or a non-compensated decrease in phosphate transport due solely to the absence of PiT2. In this way, we could finally answer the question if the effect of the absence of PiT2 is depending or not to its function as a phosphate transporter? A new genetically-modified mouse model with a version of PiT2 mutated in order to block the transport of phosphate could also help us to finally answer that question.

Since recent data suggest that PiT2 is a multifunctional protein, we can hypothesize that the phenotype could be due to the absence of an interaction between PiT2 and a potential protein partner like already described for its paralog PiT1 (Forand et al., 2016; Couasnay et al., 2019) and for PiT2 during neuronal growth via MAP1B (Ma et al., 2017). Above all, it is interesting to consider the work of Forand and colleagues linking PiT1 to energetic metabolism and insulin signalling through its protein partner USP7 (Forand et al, 2016). Similar to PiT1, which is able to modulate insulin signalling and glucose metabolism leading to the inhibition of HFD-induced obesity, we could imagine a comparable condition for PiT2.



The interaction of PiT2 with a protein partner involved in the homeostasis of adipose tissues or in a related function could explain its contribution to the natural increase of fat depots with age. In the absence of PiT2, this interaction would be disrupted, preventing the increased adipose tissues with age. Among the potential partners of PiT2, we focused on the ones with a high probability to interact with its 4th intracellular loop and known to be somehow involved in bone or adipose tissues homeostasis. We first focused on Fhl2 because of its potential interaction with both PiT1 and PiT2, which is all the more

interesting as we recently identified the function of the PiT1-PiT2 heterodimer as Pi sensor (Bon et al, 2018). Fhl2 is known to be localized both in the cytoplasm and in the nucleus, and to interact with Runx2, a key regulator of osteoblast function, operating as a transcriptional coactivator and actually increasing its transcriptional activity (Günther et al., 2005). Interestingly, the iLoop2 contains a nuclear localization signal (NLS) and, upon a potential cleavage, it could be translocated to the nucleus in complex with Fhl2 and have a transcriptional effect. Unpublished data from our group indeed described a nuclear localization for transfected iLoop2 (Figure 25).

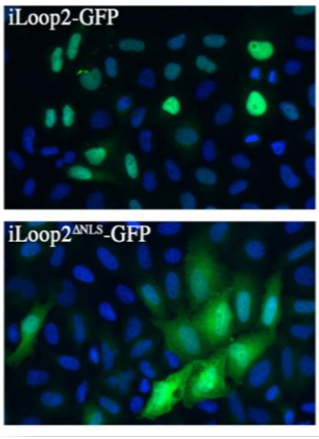


Figure 25. iLoop2 localization within the nucleus.
Unpublished data from our lab showing iLoop2 localization within the nucleus of HEK293T cells.

For now, we have confirmed the molecular interaction between PiT2 and Fhl2 by over-expression in HEK293T cells and co-immunoprecipitation. Further analyses need to be performed to validate this interaction in a physiological context. However, knowing that Fhl2KO mice display a decreased BMAT and a skeletal phenotype (osteopenia), in the biological context of the PiT2KO mice it is tempting to speculate a potential role for this protein interacting with PiT2 and potentially being involved in the phenotype we observe. We could also imagine a role for the heterodimer PiT1/PiT2 in this context, all the more considering the implication of PiT1, or better its absence, in the prevention of HFD-induced obesity described by Forand and colleagues (Forand et al., 2016).

Another promising potential partner is Pappalysin (PAPP-A), for which we also confirmed the interaction *via* co-immunoprecipitation. Studies in mice indicate a role for PAPP-A in regulating fat depots and PAPP-A KO mice are smaller than their WT littermates, showing signs of dwarfism. The PAPP-A KO mice have delayed appearance of ossification centers and reduced calcified bone mass (Conover et al., 2004). In addition, studies in mice suggest an important role for PAPP-A in regulating visceral fat depots (Conover et al. 2013; Bale et al. 2018). Conover's lab has shown that inhibition of PAPP-A through gene deletion in mice has many beneficial effects, including a remarkable extension of lifespan by 30–40%, suppression of atherosclerotic plaque progression, and prevention of visceral obesity and fatty liver (Harrington et al. 2007; Conover et al. 2010, 2013). The PAPP-A protein is usually secreted and localized at the cell surface to control IGF signaling, making it hard to imagine an interaction with the intracellular iLoop2. It could actually

be an artefact of the Y2H screen, mostly considering that PAPP-A is highly expressed in pregnant women and we performed the analysis on a placental library. However potential truncated version of this protein could be found in the cytoplasm and likely interact with the iLoop2 (Boldt et al., 2004).

What could explain the bone phenotype of PiT2KO mice?

We recently described the skeletal phenotype of the PiT2KO mice, which display altered biomechanical parameters and decreased bone quality (Beck-Cormier et al, 2019). Bone quality is characterized by both structural and material properties. We are currently investigating the material properties of PiT2-deficient bones thanks to our collaborators (MabLab in Boulogne-sur-Mer and GEROM Lab in Angers). Our preliminary data indicate that PiT2 seems dispensable for the calcium-phosphate crystal maturation in bones, while the crystal structure analysed by Fourier-transform infrared microscopy (FTIRM) appears to be altered. In addition, in the absence of PiT2, we observed an alteration of the collagen maturation and structure. These results suggest that the skeletal phenotype of the PiT2KO mice might actually be partly caused by an altered maturation of the organic component of the bone matrix, in particular of its collagen fibers. I personally find this possibility very promising. The prolyl 4-hydroxylase (P4H) catalyzes the formation of 4-hydroxyproline in collagens and this is needed to stabilize the collagen triple helices under physiological conditions (Figure 26 for the biosynthetic pathway of collagen).

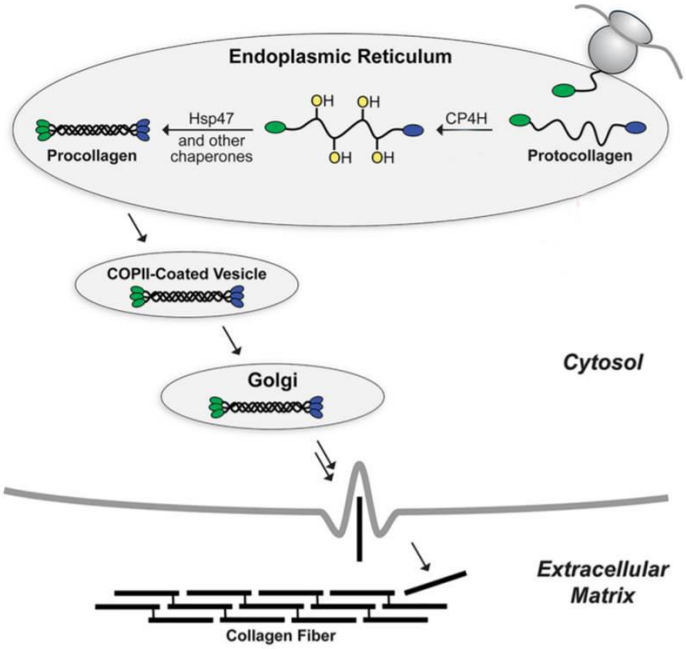


Figure 26. Biosynthetic pathway of collagen.
Collagen synthesis begins in the endoplasmic reticulum, after extensive post-translational modification by CP4H and other enzymes, protocollagens form triple helices, a process facilitated by chaperones such as Hsp47. Procollagen triple helices are then transported to the Golgi. Ultimately, processed fibrils are incorporated into growing collagen fibers, which are stabilized by covalent cross-links between triple helices. CP4H = P4H complex. Figure adapted from (Vasta et al., 2018).

Preliminary results obtained by Raman spectroscopy showed that the hydroxylation of collagen proline residues within PiT2KO mice is reduced, which implies less thermostable collagen trimers. Interestingly, a protein partner of PiT1, PDI/P4HB (Couasnay et al., 2019), is a subunit of the P4H complex. In the absence of PiT2 or, better, in the absence of PiT1/PiT2 heterodimers there could be an abnormal activity of the P4H complex, leading to an abnormal maturation of collagen. Another interesting finding is that the existence of another potential partner of PiT2, EFEMP1, known to physically interact with the subunit beta of P4H (P4HB) (Hulleman et al., 2016) and may also could play a role in this context. These results need to be confirmed, but they already represent a promising starting point for further investigations.

Personal conclusion

We knew from the beginning that there was more than one chance this project would not go as planned. But which research project really goes as we expect? Once, during class, a professor told us that rarely a PhD is a straight line. When we start, we hope to go from a point A directly to a point B without hesitation and demonstrate our hypothesis, but it actually quite never happens. He said: “a real PhD never goes from A to B. It brings you from A into an entangled line and you end up eventually at point C, without even seeing from far away the point B you so much wanted to reach. And this is the beauty of research: you think you know the answer and you go straight where you assume you will find it, while if you listen carefully to your data, they will actually bring you where you really need to go”. At the time I thought that just considering doing a PhD was a crazy idea. Instead, here I am, writing the conclusion to three years of entangled lines. I reached my point C and it is nothing like I imagined it to be.

LIST OF PUBLICATIONS AND COMMUNICATIONS

PUBLICATIONS

G. Frangi, M. Guicheteau, G. Pyka, G. Kerckhofs, M. Feyeux, J. Veziers, B. Halgand, S. Sourice, J. Guicheux, X. Prieur, L. Beck and S. Beck-Cormier. **"PiT2 deficiency protects against age-induced fat gain"**. *Journal of Bone and Mineral Research (under submission).*

N. Bon, G. Frangi, S. Sourice, J Guicheux, S. Beck-Cormier and L. Beck. **"Phosphate-dependent FGF23 secretion is modulated by PiT2/Slc20a2".** *Molecular Metabolism, 2018.*

O. De Lageneste, A. Julien, R. Abou-Khalil, G. Frangi, C. Carvalho, N. Cagnard, C. Cordier, J.S. Conway, C. Colnot **"Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin".** *Nature Communications, 2018.*

ORAL COMMUNICATIONS

G. Frangi, G. Pyka, G. Kerckhofs, M. Feyeux, J. Veziers, B. Halgand, S. Sourice, J. Guicheux, X. Prieur, L. Beck and S. Beck-Cormier. **"PiT2: a new regulator of the bone marrow adipose tissue homeostasis?"**
National Day of the French Society of Biology and Mineral Research (SFBTM) 2020 – ECTS@home

G.Frangi, X. Prieur, L. Beck and S. Beck-Cormier. "**Etude du rôle de PiT2 dans la communication entre le squelette et les tissus adipeux"**
Les folles souris nantaises 2019 (Nantes, France)

G. Frangi, G. Kerckhofs, J. Boulestreau, F. Autrusseau , J. Veziers, B. Halgand, J. Guicheux, G. Penel, C. Chauveau, X. Prieur, L. Beck and S. Beck-Cormier. "**Slc20a2/PiT2 : nouveau régulateur de l'homéostasie du tissu adipeux médullaire?"**
Congrès français de Rhumatologie 2019 (Paris, France)

G. Frangi, G. Kerckhofs, J. Boulestreau, F. Autrusseau , J. Veziers, B. Halgand, J. Guicheux, G. Penel, C. Chauveau, X. Prieur, L. Beck and S. Beck-Cormier. "**Altered bone marrow adiposity in PiT2-deficient mice**"
Les journées françaises de biologie des tissus minéralisés 2019 (Boulogne sur Mer, France)
Award as one of the best oral communications.

G. Frangi, G. Kerckhofs, J. Boulestreau, F. Autrusseau , J. Veziers, B. Halgand, J. Guicheux, G. Penel, C. Chauveau, X. Prieur, L. Beck and S. Beck-Cormier. "**Role of PiT2 in bone and adipose tissues inter-communication?"**
The 4th international meeting on Bone Marrow Adiposity 2018 (Lille, France)

G. Frangi, G. Kerckhofs, J. Boulestreau, F. Autrusseau , J. Veziers, B. Halgand, J. Guicheux, G. Penel, C. Chauveau, X. Prieur, L. Beck and S. Beck-Cormier. "**PiT2 deficiency results in skeletal phenotype associated with alteration of Bone Marrow Adipose Tissue"**
Les journées françaises de biologie des tissus minéralisés 2018 (Monaco, Principality of Monaco)
Award as one of the best oral communications.

ORAL POSTER PRESENTATIONS

G. Frangi, G. Pyka, G. Kerckhofs, M. Feyeux, J. Veziers, B. Halgand, S. Sourice, J. Guicheux, X. Prieur, L. Beck and S. Beck-Cormier. **"PiT2: a new regulator of the bone marrow adipose tissue homeostasis?"**
The European Calcified Tissue Society Congress 2020 (digital live prime time congress)

G. Frangi, G. Kerckhofs, J. Boulestreau, F. Autrusseau , J. Veziers, B. Halgand, J. Guicheux, G. Penel, C. Chauveau, X. Prieur, L. Beck and S. Beck-Cormier. ***"PiT2 deficiency results in skeletal phenotype associated with alteration of bone marrow adipose tissue"***
The European Calcified Tissue Society Congress 2019 (Budapest, Hungary)

POSTERS

G. Frangi, G. Pyka, G. Kerckhofs, M. Feyeux, J. Veziers, B. Halgand, S. Sourice, J. Guicheux, X. Prieur, L. Beck and S. Beck-Cormier. **"PiT2: a new regulator of the bone marrow adipose tissue homeostasis?"**
The European Calcified Tissue Society Congress 2020 (digital live prime time congress)

G. Frangi, G. Kerckhofs, J. Boulestreau, F. Autrusseau , J. Veziers, B. Halgand, J. Guicheux, G. Penel, C. Chauveau, X. Prieur, L. Beck and S. Beck-Cormier. ***"PiT2 deficiency results in skeletal phenotype associated with alteration of bone marrow adipose tissue"***
The European Calcified Tissue Society Congress 2019 (Budapest, Hungary)

BIBLIOGRAPHY

Abuduli, M.; Ohminami, H.; Otani, T.; Kubo, H.; Ueda, H.; Kawai, Y.; Masuda, M.; Yamanaka-Okumura, H.; Sakaue, H.; Yamamoto, H.; Takeda, E.; Taketani, Y., 2016: Effects of dietary phosphate on glucose and lipid metabolism. *American Journal of Physiology - Endocrinology and Metabolism*., **310**, E526–E538.

Ali, A. T.; Hochfeld, W. E.; Myburgh, R.; Pepper, M. S., 2013: Adipocyte and adipogenesis. *European Journal of Cell Biology*.

Arita, Y.; Kihara, S.; Ouchi, N.; Takahashi, M.; Maeda, K.; Miyagawa, J. ichiro; Hotta, K.; Shimomura, I.; Nakamura, T.; Miyaoka, K.; Kuriyama, H.; Nishida, M.; Yamashita, S.; Okubo, K.; Matsubara, K.; Muraguchi, M.; Ohmoto, Y.; Funahashi, T.; Matsuzawa, 1999: Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity. *Biochemical and Biophysical Research Communications*.

Ayoub, J. J.; Samra, M. J. A.; Hlais, S. A.; Bassil, M. S.; Obeid, O. A., 2015: Effect of phosphorus supplementation on weight gain and waist circumference of overweight/obese adults: A randomized clinical trial. *Nutrition and Diabetes*., **5**.

Badr, S.; Legroux-gérot, I.; Vignau, J.; Chauveau, C.; Ruschke, S.; Karampinos, D. C.; Budzik, J. françois; Cortet, B.; Cotten, A., 2019: Comparison of regional bone marrow adiposity characteristics at the hip of underweight and weight-recovered women with anorexia nervosa using magnetic resonance spectroscopy. *Bone*., **127**, 135–145.

Bai, L.; Collins, J. F.; Ghishan, F. K., 2000: Cloning and characterization of a type III Na-dependent phosphate cotransporter from mouse intestine. *Am J Physiol Cell Physiol*.

Baryawno, N.; Przybylski, D.; Kowalczyk, M. S.; Kfoury, Y.; Severe, N.; Gustafsson, K.; Kokkaliaris, K. D.; Tabaka, M.; Hofree, M.; Dionne, D.; Papazian, A.; Ashenberg, O.; Subramanian, A.; Vaishnav, E. D.; Regev, A.; Scadden, D. T., 2019: A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. *Cell*., **177**, 1915–1932.

Bassil, M. S.; Obeid, O. A., 2016: Phosphorus supplementation recovers the blunted diet-induced thermogenesis of overweight and obese adults: A pilot study. *Nutrients*., **8**.

Baum, T.; Yap, S. P.; Karampinos, D. C.; Nardo, L.; Kuo, D.; Burghardt, A. J.; Masharani, U. B.; Schwartz, A. V.; Li, X.; Link, T. M., 2012: Does vertebral bone marrow fat content correlate with abdominal adipose tissue, lumbar spine bone mineral density, and blood biomarkers in women with type 2 diabetes mellitus? *Journal of Magnetic Resonance Imaging*., **35**, 117–124.

Baum, T.; Rohrmeier, A.; Syväri, J.; Diefenbach, M. N.; Franz, D.; Dieckmeyer, M.; Scharr, A.; Hauner, H.; Ruschke, S.; Kirschke, J. S.; Karampinos, D. C., 2018: Anatomical variation of age-related changes in vertebral bone marrow composition using chemical shift encoding-based water-fat magnetic resonance imaging. *Frontiers in Endocrinology*., **9**.

Beck-Cormier, S.; Lelliott, C. J.; Logan, J. G.; Lafont, D. T.; Merametdjian, L.; Leitch, V. D.; Butterfield, N. C.; Protheroe, H. J.; Croucher, P. I.; Baldock, P. A.; Gaultier-Lintia, A.; Maugars, Y.; Nicolas, G.; Banse, C.; Normant, S.; Magne, N.; Gérardin, E.; Bon, N.; Sourice, S. et al., 2019: Slc20a2 , encoding the phosphate transporter PiT2, is an important genetic determinant of bone quality and strength . *Journal of Bone and Mineral Research*.

Beck, G. R.; Zerler, B.; Moran, E., 2000: Phosphate is a specific signal for induction of osteopontin gene expression. *Proceedings of the National Academy of Sciences of the United States of America*., **97**, 8352–8357.

Beck, G. R., 2003: Inorganic phosphate as a signaling molecule in osteoblast differentiation. *Journal of Cellular Biochemistry*., **90**, 234–243.

Beck, L.; Leroy, C.; Salaün, C.; Margall-Ducos, G.; Desdouets, C.; Friedlander, G., 2009: Identification of a novel function of PiT1 critical for cell proliferation and independent of its phosphate transport activity. *Journal of Biological Chemistry*., **284**, 31363–31374.

Beck, L.; Leroy, C.; Beck-cormier, S.; Forand, A.; Salau, C.; Ollero, M.; Prie, D.; Bernier, A.; Uren, P., 2010: The Phosphate Transporter PiT1 (Slc20a1) Revealed As a New Essential Gene for Mouse Liver Development. *PLoS ONE*., **5**.

Beck, L.; Beck-Cormier, S., 2020: Extracellular phosphate sensing in mammals: What do we know? *Journal of Molecular Endocrinology*., **65**, R53–R63.

Benova, A.; Tencerova, M., 2020: Obesity-Induced Changes in Bone Marrow Homeostasis. *Frontiers in Endocrinology*., **11**, 1–15.

Berendsen, A. D.; Olsen, B. R., 2015: Bone development. *Bone*., **80**, 14–18.

Berg, A. H.; Combs, T. P.; Du, X.; Brownlee, M.; Scherer, P. E., 2001: The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nature Medicine*., **7**, 947–953.

Bergwitz, C.; Juppner, H., 2010: Regulation of Phosphate Homeostasis by PTH, Vitamin D, and FGF23. *Annu Rev Med*.

Berner, H. S.; Lyngstadaas, S. P.; Spahr, A.; Monjo, M.; Thommesen, L.; Drevon, C. A.; Syversen, U.; Reseland, J. E., 2004: Adiponectin and its receptors are expressed in bone-forming cells. *Bone*., **35**, 842–849.

Bianco, P.; Robey, P. G., 2015: Skeletal Stem Cells. *Development*.

Billington, E. O.; Gamble, G. D.; Bristow, S.; Reid, I. R., 2017: Serum phosphate is related to adiposity in healthy adults, 486–493.

Biver, E.; Salliot, C.; Combescure, C.; Gossec, L.; Hardouin, P.; Legroux-gerot, I.; Cortet, B., (n.d.) Influence of Adipokines and Ghrelin on Bone Mineral Density and Fracture Risk : A Systematic Review and, **96**, 2703–2713.

Blebea, J. S.; Houseni, M.; Torigian, D. A.; Fan, C.; Mavi, A.; Zhuge, Y.; Iwanaga, T.; Mishra, S.; Udupa, J.; Zhuang, J.; Gopal, R.; Alavi, A., 2007: Structural and Functional Imaging of Normal Bone Marrow and Evaluation of Its Age-Related Changes. *Seminars in Nuclear Medicine*., **37**, 185–194.

Boldt, H. B.; Kjaer-Sorensen, K.; Overgaard, M. T.; Weyer, K.; Poulsen, C. B.; Sottrup-Jensen, L.; Conover, C. A.; Giudice, L. C.; Oxvig, C., 2004: The Lin12-Notch repeats of pregnancy-associated plasma protein-A bind calcium and determine its proteolytic specificity. *Journal of Biological Chemistry*., **279**, 38525–38531.

Bolsoni-Lopes, A.; Alonso-Vale, M. I. C., 2015: Lipolysis and lipases in white adipose tissue – An update. *Archives of Endocrinology and Metabolism*.

Bon, N.; Couasnay, G.; Bourgine, A.; Sourice, S.; Beck-Cormier, S.; Guicheux, J.; Beck, L., 2018a: Phosphate (Pi)-regulated heterodimerization of the high affinity sodium-dependent pitransporters pit1/slc20a1 and pit2/slc20a2 underlies extracellular Pi sensing independently of Pi uptake. *Journal of Biological Chemistry*., **293**, 2102–2114.

Bon, N.; Frangi, G.; Sourice, S.; Guicheux, J.; Beck-Cormier, S.; Beck, L., 2018b: Phosphate-dependent FGF23 secretion is modulated by PiT2/Slc20a2. *Molecular Metabolism*., **11**, 197–204.

Bornstein, S.; Brown, S. A.; Le, P. T.; Wang, X.; DeMambro, V.; Horowitz, M. C.; MacDougald, O.; Baron, R.; Lotinun, S.; Karsenty, G.; Wei, W.; Ferron, M.; Kovacs, C. S.; Clemmons, D.; Wan, Y.; Rosen, C. J., 2014: FGF-21 and skeletal remodeling during and after lactation in C57BL/6J mice. *Endocrinology*., **155**, 3516–3526.

Botolin, S.; Mccabe, L. R., 2007: Bone Loss and Increased Bone Adiposity in Spontaneous and Pharmacologically Induced Diabetic Mice, **148**, 198–205.

Bøttger, P.; Pedersen, L., 2002: Two Highly Conserved Glutamate Residues Critical for Type III Sodium-dependent Phosphate Transport Revealed by Uncoupling Transport Function from Retroviral Receptor Function * □. *The Journal of Biological Chemistry*.

Bøttger, P.; Pedersen, L., 2004: The Central Half of Pit2 Is Not Required for Its Function as a Retroviral Receptor, **78**, 9564–9567.

Bøttger, P.; Pedersen, L., 2005: Evolutionary and experimental analyses of inorganic phosphate transporter PiT family reveals two related signature sequences harboring highly conserved aspartic acids critical for sodium-dependent phosphate transport function of human PiT2. *The FEBS Journal*.

Bøttger, P.; Pedersen, L., 2011: Mapping of the minimal inorganic phosphate transporting unit of human PiT2 suggests a structure universal to PiT-related proteins from all kingdoms of life. *BMC Biochemistry*., **12**, 21.

Bourgine, A.; Pilet, P.; Diouani, S.; Sourice, S.; Lesoeur, J.; Beck-Cormier, S.; Khoshniat, S.; Weiss, P.; Friedlander, G.; Guicheux, J.; Beck, L., 2013: Mice with Hypomorphic Expression of the Sodium-Phosphate Cotransporter PiT1/Slc20a1 Have an Unexpected Normal Bone Mineralization. *PLoS ONE*., **8**, 1–10.

Bredella, M. A.; Fazeli, P. K.; Miller, K. K.; Misra, M.; Torriani, M.; Thomas, B. J.; Ghomi, R. H.; Rosen, C. J.; Klibanski, A., 2009: Increased bone marrow fat in anorexia nervosa. *Journal of Clinical Endocrinology and Metabolism*., **94**, 2129–2136.

Bredella, M. A.; Torriani, M.; Ghomi, R. H.; Thomas, B. J.; Brick, D. J.; Gerweck, A. V.; Rosen, C. J.; Klibanski, A.; Miller, K. K., 2011: Vertebral bone marrow fat is positively associated with visceral fat and inversely associated with IGF-1 in obese women. *Obesity*., **19**, 49–53.

Breusegem, S. Y.; Takahashi, H.; Giral-Arnal, H.; Wang, X.; Jiang, T.; Verlander, J. W.; Wilson, P.; Miyazaki-Anzai, S.; Sutherland, E.; Caldas, Y.; Blaine, J. T.; Segawa, H.; Miyamoto, K. I.; Barry, N. P.; Levi, M., 2009: Differential regulation of the renal sodium-phosphate cotransporters NaPi-IIa, NaPi-IIc, and PiT-2 in dietary potassium deficiency. *American Journal of Physiology - Renal Physiology*., **297**, 350–361.

Campfield, L. A.; Smith, F. J.; Guisez, Y.; Devos, R.; Burn, P., 1995: Recombinant Mouse OB Protein : Evidence for a Peripheral Signal Linking Adiposity and Central Neural Networks, **269**, 546–550.

Cawthorn, W. P.; Scheller, E. L.; Learman, B. S.; Parlee, S. D.; Simon, B. R.; Mori, H.; Ning, X.; Bree, A. J.; Schell, B.; Broome, T.; Soliman, S. S.; Delproposto, J. L.; Lumeng, C. N.; Mitra, A.; Pandit, S. V; Gallagher, K. A.; Miller, J. D.; Krishnan, V.; Hui, S. K. et al., 2014: Bone marrow adipose tissue is an endocrine organ that contributes to increased circulating adiponectin during caloric restriction. *Cell Metab*., **20**, 368–375.

Cen, Z.; Chen, Y.; Chen, S.; Wang, H.; Yang, D.; Zhang, H.; Wu, H.; Wang, L.; Tang, S.; Ye, J.; Shen, J.; Wang, H.; Fu, F.; Chen, X.; Xie, F.; Liu, P.; Xu, X.; Cao, J.; Cai, P. et al., 2020: Biallelic loss-of-function mutations in JAM2 cause primary familial brain calcification. *Brain*., **143**, 491–502.

Chait, A.; den Hartigh, L. J., 2020: Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease. *Frontiers in Cardiovascular Medicine*., **7**, 1–41.

Chande, S.; Bergwitz, C., 2018: Role of phosphate sensing in bone and mineral metabolism. *Nature Reviews Endocrinology*.

Chande, S.; Ho, B.; Fetene, J.; Bergwitz, C., 2019: Transgenic mouse model for conditional expression of influenza hemagglutinin-tagged human SLC20A1/PIT1. *PLoS ONE*., **14**, 1–13.

Chande, S.; Caballero, D.; Ho, B. B.; Fetene, J.; Serna, J.; Pesta, D.; Nasiri, A.; Jurczak, M.; Chavkin, N. W.; Hernando, N.; Giachelli, C. M.; Wagner, C. A.; Zeiss, C.; Shulman, G. I.; Bergwitz, C., 2020: Slc20a1/Pit1 and Slc20a2/Pit2 are essential for normal skeletal myofiber function and survival. *Scientific Reports*.

Chavkin, N. W., Chia, J. J., Crouthamel, M. H. & Giachelli, C. M., 2015: Phosphate uptake-independent signaling functions of the type III sodium-dependent phosphate transporter, PiT-1, in vascular smooth muscle cells. *Exp Cell Res*., **333**, 39–48.

Chien, M. ling; Foster, J. L.; Douglas, J. L.; Garcia, J. V., 1997: The Amphotropic Murine Leukemia Virus Receptor Gene Encodes a 71-Kilodalton Protein That Is Induced by Phosphate Depletion. *Journal of Virology*., **71**, 4564–4570.

Chiou, T. J.; Lin, S. I., 2011: Signaling network in sensing phosphate availability in plants. *Annual Review of Plant Biology*., **62**, 185–206.

Clarke, B., 2008: Normal bone anatomy and physiology. *Clinical journal of the American Society of Nephrology : CJASN*.

Cohen, A.; Dempster, D. W.; Stein, E. M.; Nickolas, T. L.; Zhou, H.; McMahon, D. J.; Müller, R.; Kohler, T.; Zwahlen, A.; Lappe, J. M.; Young, P.; Recker, R. R.; Shane, E., 2012: Increased marrow adiposity in premenopausal women with idiopathic osteoporosis. *Journal of Clinical Endocrinology and Metabolism*., **97**, 2782–2791.

Combs, T. P.; Marliss, E. B., 2014: Adiponectin signaling in the liver. *Reviews in Endocrine and Metabolic Disorders*.

Conover, C. A.; Bale, L. K.; Overgaard, M. T.; Johnstone, E. W.; Laursen, U. L.; Füchtbauer, E. M.; Oxvig, C.; van Deursen, J., 2004: Metalloproteinase pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development. *Development*., **131**, 1187–1194.

Conover, C. A.; Harstad, S. L.; Tchkonia, T.; Kirkland, J. L., 2013: Preferential impact of pregnancy-associated plasma protein-A deficiency on visceral fat in mice on high-fat diet. *American Journal of Physiology - Endocrinology and Metabolism*., **305**, 1–20.

Considine, R. V.; Sinha, M. K.; Heiman, M. L.; Kriauciunas, A.; Stephens, T. W.; Nyce, M. R.; Ohannesian, J. P.; Marco, C. C.; Mckee, L. J.; Bauer, T. L.; Caro, J. F., 1996: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine*., **334**, 292–295.

Couasnay, G.; Bon, N.; Devignes, C. S.; Sourice, S.; Bianchi, A.; Véziers, J.; Weiss, P.; Elefteriou, F.; Provot, S.; Guicheux, J.; Beck-Cormier, S.; Beck, L., 2019: PiT1/Slc20a1 Is Required for Endoplasmic Reticulum Homeostasis, Chondrocyte Survival, and Skeletal Development. *Journal of Bone and Mineral Research*., **34**, 387–398.

Craft, C. S.; Robles, H.; Lorenz, M. R.; Hilker, E. D.; Magee, K. L.; Andersen, T. L.; Cawthorn, W. P.;

MacDougald, O. A.; Harris, C. A.; Scheller, E. L., 2019: Bone marrow adipose tissue does not express UCP1 during development or adrenergic-induced remodeling. *Scientific Reports*., **9**, 1–14.

Crouthamel, M. H.; Lau, W. L.; Leaf, E. M.; Chavkin, N.; Chin, T.; Levi, M.; Giachelli, C. M., 2013: Sodium-dependent phosphate cotransporters and phosphate- induced calcification of vascular smooth muscle cells: Redundant roles for PiT-1 and PiT-2. *Arterioscler Thromb Vasc Biol*., **33**, 2625–2632.

de Bournonville, S.; Vangrunderbeeck, S.; Ly, H. G. T.; Geeroms, C.; De Borggraeve, W. M.; Parac-Vogt, T. N.; Kerckhofs, G., 2020: Exploring polyoxometalates as non-destructive staining agents for contrast-enhanced microfocus computed tomography of biological tissues. *Acta Biomaterialia*., **105**, 253–262.

Devlin, M. J.; Cloutier, A. M.; Thomas, N. A.; Panus, D. A.; Lotinun, S.; Pinz, I.; Baron, R.; Rosen, C. J.; Bouxsein, M. L., 2010: Caloric Restriction Leads to High Marrow Adiposity and, **25**, 2078–2088.

Di Iorgi, N.; Rosol, M.; Mittelman, S. D.; Gilsanz, V., 2008: Reciprocal relation between marrow adiposity and the amount of bone in the axial and appendicular skeleton of young adults. *Journal of Clinical Endocrinology and Metabolism*., **93**, 2281–2286.

Diegel, C. R.; Hann, S.; Ayturk, U. M.; Hu, J. C. W.; Lim, K. E.; Droscha, C. J.; Madaj, Z. B.; Foxa, G. E.; Izaguirre, I.; Paracha, N.; Pidhaynyy, B.; Dowd, T. L.; Robling, A. G.; Warman, M. L.; Williams, B. O., 2020: An osteocalcin-deficient mouse strain without endocrine abnormalities. *PLoS Genetics*., **16**, 1–18.

Doucette, C. R.; Horowitz, M. C.; Berry, R.; Macdougald, O. A.; Anunciado-Koza, R.; Koza, R. A.; Rosen, C. J., 2015: A High Fat Diet Increases Bone Marrow Adipose Tissue (MAT) But Does Not Alter Trabecular or Cortical Bone Mass in C57BL/6J Mice. *Journal of Cellular Physiology*., **230**, 2032–2037.

Dreyer, K.; Pedersen, F. S., 2000: A 13-Amino-Acid Pit1-Specific Loop 4 Sequence Confers Feline Leukemia Virus Subgroup B Receptor Function upon Pit2, **74**, 2926–2929.

Ducy, P., 2011: The role of osteocalcin in the endocrine cross-talk between bone remodelling and energy metabolism. *Diabetologia*., **54**, 1291–1297.

Eller, P.; Eller, K.; Kirsch, A. H.; Patsch, J. J.; Wolf, A. M.; Tagwerker, A.; Stanzl, U.; Kaindl, R.; Kahlenberg, V.; Mayer, G.; Patsch, J. R.; Rosenkranz, A. R., 2011: A murine model of phosphate nephropathy. *American Journal of Pathology*., **178**, 1999–2006.

Emont, M. P.; Yu, H.; Jun, H.; Hong, X.; Maganti, N.; Stegemann, J. P.; Wu, J., 2015: Using a 3D Culture System to Differentiate Visceral Adipocytes in vitro, **156**, 4761–4768.

Ermetici, F.; Briganti, S.; Delnevo, A.; Cannaò, P.; Leo, G. Di; Benedini, S.; Terruzzi, I.; Sardanelli, F.; Luzi, L., 2018: Bone marrow fat contributes to insulin sensitivity and adiponectin secretion in premenopausal women. *Endocrine*., **59**, 410–418.

Fairfield, H.; Falank, C.; Farrell, M.; Vary, C.; Boucher, J. M.; Driscoll, H.; Liaw, L.; Rosen, C. J.; Reagan, M. R., 2018: Development of a 3D bone marrow adipose tissue model. *Bone*., **118**, 77–88.

Fan, Y.; Hanai, J. ichi; Le, P. T.; Bi, R.; Maridas, D.; DeMambro, V.; Figueroa, C. A.; Kir, S.; Zhou, X.; Mannstadt, M.; Baron, R.; Bronson, R. T.; Horowitz, M. C.; Wu, J. Y.; Bilezikian, J. P.; Dempster, D. W.; Rosen, C. J.; Lanske, B., 2017: Parathyroid Hormone Directs Bone Marrow Mesenchymal Cell Fate. *Cell Metabolism*., **25**, 661–672.

Farmer, S. R., 2006: Transcriptional control of adipocyte formation. *Cell Metabolism*.

Farrell, K. B.; Tusnady, G. E.; Eiden, M. V., 2009: New structural arrangement of the extracellular regions of

the phosphate transporter SLC20A1, the receptor for gibbon ape leukemia virus. *Journal of Biological Chemistry*., **284**, 29979–29987.

Fasshauer, M.; Blüher, M., 2015: Adipokines in health and disease. *Trends in Pharmacological Sciences*., **36**, 461–470.

Feldman, S. A.; Farrell, K. B.; Murthy, R. K.; Russ, J. L.; Eiden, M. V, 2004: Identification of an Extracellular Domain within the Human PiT2 Receptor That Is Required for Amphotropic Murine Leukemia Virus Binding, **78**, 595–602.

Felsenberg, D.; Boonen, S., 2005: The bone quality framework: Determinants of bone strength and their interrelationships, and implications for osteoporosis management. *Clinical Therapeutics*., **27**, 1–11.

Fenzl, A.; Kiefer, F. W., 2014: Brown adipose tissue and thermogenesis. *Hormone Molecular Biology and Clinical Investigation*.

Ferland-McCollough, D.; Maselli, D.; Spinetti, G.; Sambataro, M.; Sullivan, N.; Blom, A.; Madeddu, P., 2018: MCP-1 feedback loop between adipocytes and mesenchymal stromal cells causes fat accumulation and contributes to hematopoietic stem cell rarefaction in the bone marrow of patients with diabetes. *Diabetes*., **67**, 1380–1394.

Festing, M. H.; Speer, M. Y.; Yang, H. Y.; Giachelli, C. M., 2009: Generation of mouse conditional and null alleles of the type III sodium-dependent phosphate cotransporter PiT-1. *Genesis*.

Forand, A.; Beck, L.; Leroy, C.; Rousseau, A.; Boitez, V.; Cohen, I.; Courtois, G.; Hermine, O.; Friedlander, G., 2013: EKLF-driven PIT1 expression is critical for mouse erythroid maturation in vivo and in vitro. *Blood*., **121**, 666–678.

Forand, A.; Koumakis, E.; Rousseau, A.; Sassier, Y.; Journe, C.; Merlin, J. F.; Leroy, C.; Boitez, V.; Codogno, P.; Friedlander, G.; Cohen, I., 2016: Disruption of the Phosphate Transporter Pit1 in Hepatocytes Improves Glucose Metabolism and Insulin Signaling by Modulating the USP7/IRS1 Interaction. *Cell Reports*., **16**, 2736–2748.

Forster, I.; Hernando, N.; Sorribas, V.; Werner, A., 2011: Phosphate Transporters in Renal, Gastrointestinal, and Other Tissues. *Advances in Chronic Kidney Disease*.

Forster, I. C.; Hernando, N.; Biber, J.; Murer, H., 2012: *Phosphate Transport Kinetics and Structure-Function Relationships of SLC34 and SLC20 Proteins*. *Current Topics in Membranes*. Elsevier, Vol. 70.

Frederich, R. C.; Hamann, A.; Anderson, S.; Lollmann, B.; Lowell, B. B.; Flier, J. S., 1995: Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nature Medicine*., **1**.

Friedman, J.; Halaas, D., 1998: Leptin and the regulation of body composition. *Nature*., **395**, 763–770.

Friedman, J. M., 2002: The function of leptin in nutrition, weight, and physiology. *Nutrition Reviews*., Vol. 60pp. 1–14.

Frühbeck, G., 2008: Overview of Adipose Tissue and Its Role. *Methods in molecular biology*., **456**, 1–22.

Ge, C.; Cawthorn, W. P.; Li, Y.; Zhao, G.; Macdougald, O. A.; Franceschi, R. T., 2016: Reciprocal Control of Osteogenic and Adipogenic Differentiation by ERK/MAP Kinase Phosphorylation of Runx2 and PPARγ Transcription Factors. *Journal of Cellular Physiology*., **231**, 587–596.

Genoux, A.; Bastard, J. P., 2020: Effects of leptin and adiponectin on the cardiovascular system. *Annales de*

Biologie Clinique., **78**, 253–260.

Ghali, O.; Broux, O.; Falgayrac, G.; Haren, N.; van Leeuwen, J. P. T. M.; Penel, G.; Hardouin, P.; Chauveau, C., 2015: Dexamethasone in osteogenic medium strongly induces adipocyte differentiation of mouse bone marrow stromal cells and increases osteoblast differentiation. *BMC cell biology*., **16**, 9.

Giral, H.; Caldas, Y.; Sutherland, E.; Wilson, P.; Breusegem, S.; Barry, N.; Blaine, J.; Jiang, T.; Wang, X. X.; Levi, M., 2009: Regulation of rat intestinal Na-dependent phosphate transporters by dietary phosphate. *Am J Physiol Renal Physiol*.

Goldberg, I. J., 1996: Lipoprotein lipase and lipolysis: Central roles in lipoprotein metabolism and atherogenesis. *Journal of Lipid Research*.

Guerreiro, P. M.; Bataille, A. M.; Parker, S. L.; Renfro, J. L., 2014: Active removal of inorganic phosphate from cerebrospinal fluid by the choroid plexus. *Am J Physiol Renal Physiol*.

Günther, T.; Poli, C.; Müller, J. M.; Catala-Lehnen, P.; Schinke, T.; Yin, N.; Vomstein, S.; Amling, M.; Schüle, R., 2005: Fhl2 deficiency results in osteopenia due to decreased activity of osteoblasts. *EMBO Journal*., **24**, 3049–3056.

Halaas, J. L.; Gajiwala, K. S.; Maffei, M.; Cohen, S. L.; Chait, B. T.; Rabinowitz, D.; Lallone, R. L.; Burley, S. K.; Friedman, J. M., 1995: Encoded by the obese Gene, **269**.

Ham, J.; Lever, L.; Fox, M.; Reagan, M. R., 2019: In Vitro 3D Cultures to Reproduce the Bone Marrow Niche. *JBMR Plus*., **3**, e10228.

Han, Y.; You, X.; Xing, W.; Zhang, Z.; Zou, W., 2018: Paracrine and endocrine actions of bone - The functions of secretory proteins from osteoblasts, osteocytes, and osteoclasts. *Bone Research*., **6**, 1–11.

Harms, M.; Seale, P., 2013: Brown and beige fat: Development, function and therapeutic potential. *Nature Medicine*.

Helbling, P. M.; Piñeiro-Yáñez, E.; Gerosa, R.; Boettcher, S.; Al-Shahrour, F.; Manz, M. G.; Nombela-Arrieta, C., 2019: Global Transcriptomic Profiling of the Bone Marrow Stromal Microenvironment during Postnatal Development, Aging, and Inflammation. *Cell Reports*., **29**, 3313-3330.e4.

Hoag, H. M.; Martel, J.; Gauthier, C.; Tenenhouse, H. S., 1999: Effects of Npt2 gene ablation and low-phosphate diet on renal Na+/phosphate cotransport and cotransporter gene expression. *Journal of Clinical Investigation*., **104**, 679–686.

Houschyar, K. S.; Tapking, C.; Borrelli, M. R.; Popp, D.; Duscher, D.; Maan, Z. N.; Chelliah, M. P.; Li, J.; Harati, K.; Wallner, C.; Rein, S.; Pförringer, D.; Reumuth, G.; Grieb, G.; Mouraret, S.; Dadras, M.; Wagner, J. M.; Cha, J. Y.; Siemers, F. et al., 2019: Wnt Pathway in Bone Repair and Regeneration – What Do We Know So Far. *Frontiers in Cell and Developmental Biology*., **6**, 1–13.

Hsu, S. C.; Sears, R. L.; Lemos, R. R.; Quintáns, B.; Huang, A.; Spiteri, E.; Nevarez, L.; Mamah, C.; Zatz, M.; Pierce, K. D.; Fullerton, J. M.; Adair, J. C.; Berner, J. E.; Bower, M.; Brodaty, H.; Carmona, O.; Dobricić, V.; Fogel, B. L.; García-Estevez, D. et al., 2013: Mutations in SLC20A2 are a major cause of familial idiopathic basal ganglia calcification. *Neurogenetics*.

Hu, E.; Liang, P.; Spiegelman, B. M., 1996: AdipoQ is a novel adipose-specific gene dysregulated in obesity. *Journal of Biological Chemistry*., **271**, 10697–10703.

Hulleman, J. D.; Generaux, J. C.; Nguyen, A., 2016: MAPPING WILD-TYPE AND R345W FIBULIN-3

INTRACELLULAR INTERACTOMES. *Exp Eye Res.*, **153**, 165–169.

Ikeda, K.; Maretich, P.; Kajimura, S., 2018: The common and distinct features of brown and beige adipocytes. *Trends Endocrinol Metab.*

Imi, Y.; Yabiki, N.; Abuduli, M.; Masuda, M.; Yamanaka-Okumura, H.; Taketani, Y., 2018: High phosphate diet supresses lipogenesis in white adipose tissue. *J. Clin. Biochem. Nutr. |*., **63**, 181–191.

Inden, M.; Iriyama, M.; Takagi, M.; Kaneko, M.; Hozumi, I., 2013: Localization of type-III sodium-dependent phosphate transporter 2 in the mouse brain. *Brain Research*., **1531**, 75–83.

Inden, M.; Iriyama, M.; Zennami, M.; Sekine, S. I.; Hara, A.; Yamada, M.; Hozumi, I., 2016: The type III transporters (PiT-1 and PiT-2) are the major sodium-dependent phosphate transporters in the mice and human brains. *Brain Research*., **1637**, 128–136.

Jensen, N.; Schrøder, H. D.; Hejbøl, E. K.; Füchtbauer, E. M.; De Oliveira, J. R. M.; Pedersen, L., 2013: Loss of function of Slc20a2 associated with familial idiopathic basal ganglia calcification in humans causes brain calcifications in mice. *Journal of Molecular Neuroscience*., **51**, 994–999.

Jensen, N.; Autzen, J. K.; Pedersen, L., 2016: Slc20a2 is critical for maintaining a physiologic inorganic phosphate level in cerebrospinal fluid. *Neurogenetics*., **17**, 125–130.

Jensen, N.; Schrøder, H. D.; Hejbøl, E. K.; Thomsen, J. S.; Brüel, A.; Larsen, F. T.; Vinding, M. C.; Orlowski, D.; Füchtbauer, E. M.; Oliveira, J. R. M.; Pedersen, L., 2018: Mice Knocked Out for the Primary Brain Calcification–Associated Gene Slc20a2 Show Unimpaired Prenatal Survival but Retarded Growth and Nodules in the Brain that Grow and Calcify Over Time. *American Journal of Pathology*., **188**, 1865–1881.

Jiang, X.; Song, D.; Ye, B.; Wang, X.; Song, G.; Yang, S.; Hu, J., 2011: Effect of intermittent administration of adiponectin on bone regeneration following mandibular osteodistraction in rabbits. *Journal of Orthopaedic Research*., **29**, 1081–1085.

Johann, S. V; Gibbons, J. J.; O'Hara, B., 1992: GLVR1, a receptor for gibbon ape leukemia virus, is homologous to a phosphate permease of Neurospora crassa and is expressed at high levels in the brain and thymus. *J Virol*.

Johann, S. V; van Zeijl, M.; Cekleniak, J.; O'Hara, B., 1993: Definition of a Domain of GLVR1 Which Is Necessary for Infection by Gibbon Ape Leukemia Virus and Which Is Highly Polymorphic between Species. *Journal of Virology*.

Jono, S.; McKee, M. D.; Murry, C. E.; Shioi, A.; Nishizawa, Y.; Mori, K.; Morii, H.; Giachelli, C. M., 2000: Phosphate regulation of vascular smooth muscle cell calcification. *Circulation research*., **87**.

Justesen, J.; Stenderup, K.; Ebbesen, E. N.; Mosekilde, L.; Steiniche, T.; Kassem, M., 2001: Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. *Biogerontology*., **2**, 165–171.

Kahn, S. E.; Haffner, S. M.; Heise, M. A.; Herman, W. H.; Holman, R. R.; Jones, N. P.; Kravitz, B. G.; Lachin, J. M.; O'Neill, M. C.; Zinman, B.; Viberti, G., 2006: Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *New England Journal of Medicine*., **355**, 2427–2443.

Kaneko, I.; Segawa, H.; Furutani, J.; Kuwahara, S.; Aranami, F.; Hanabusa, E.; Tominaga, R.; Giral, H.; Caldas, Y.; Levi, M.; Kato, S.; Miyamoto, K. I., 2011: Hypophosphatemia in vitamin D receptor null mice: Effect of rescue diet on the developmental changes in renal Na+-dependent phosphate cotransporters. *Pflugers Archiv European Journal of Physiology*., **461**, 77–90.

Karampinos, D. C.; Ruschke, S.; Dieckmeyer, M.; Diefenbach, M.; Franz, D.; Gersing, A. S.; Krug, R.; Baum, T., 2018: Quantitative MRI and spectroscopy of bone marrow. *Journal of Magnetic Resonance Imaging*., **47**, 332–353.

Karsenty, G.; Kronenberg, H. M.; Settembre, C., 2009: Genetic control of bone formation. *Annual Review of Cell and Developmental Biology*., **25**, 629–648.

Karsenty, G.; Oury, F., 2012: Biology without walls: The novel endocrinology of bone. *Annual Review of Physiology*., **74**, 87–105.

Katai, K.; Miyamoto, K. ichi; Kishida, S.; Segawa, H.; Nii, T.; Tanaka, H.; Tani, Y.; Arai, H.; Tatsumi, S.; Morita, K.; Taketani, Y.; Takeda, E., 1999: Regulation of intestinal Na+-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D3. *Biochem J*.

Kavanaugh, M. P.; Miller, D. G.; Zhang, W.; Law, W.; Kozak, S. L.; Kabat, D.; Miller, A. D., 1994: Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. *Proceedings of the National Academy of Sciences of the United States of America*., **91**, 7071–7075.

Kavanaugh, M. P.; Kabat, D., 1996: Identification and characterization of a widely expressed phosphate transporter/retrovirus receptor family. *Kidney International*., **49**, 959–963.

Kerckhofs, G.; Stegen, S.; van Gastel, N.; Sap, A.; Falgayrac, G.; Penel, G.; Durand, M.; Luyten, F. P.; Geris, L.; Vandamme, K.; Parac-Vogt, T.; Carmeliet, G., 2018: Simultaneous three-dimensional visualization of mineralized and soft skeletal tissues by a novel microCT contrast agent with polyoxometalate structure. *Biomaterials*., **159**, 1–12.

Khoshniat, S.; Bourgine, A.; Julien, M.; Weiss, P.; Guicheux, J.; Beck, L., 2011: The emergence of phosphate as a specific signaling molecule in bone and other cell types in mammals. *Cellular and Molecular Life Sciences*., **68**, 205–218.

Kido, S.; Miyamoto, K. I.; Mizobuchi, H.; Taketani, Y.; Ohkido, I.; Ogawa, N.; Kaneko, Y.; Harashima, S.; Takeda, E., 1999: Identification of regulatory sequences and binding proteins in the type II sodium/phosphate cotransporter NPT2 gene responsive to dietary phosphate. *Journal of Biological Chemistry*., **274**, 28256–28263.

Kimura, T.; Miura, T.; Aoki, K.; Saito, S.; Hondo, H.; Konno, T.; Uchiyama, A.; Ikeuchi, T.; Takahashi, H.; Kakita, A., 2016: Familial idiopathic basal ganglia calcification : Histopathologic features of an autopsied patient with an SLC20A2 mutation. *Neuropathology*.

Kritmetapak, K.; Kumar, R., 2019: Phosphate as a Signaling Molecule. *Calcified Tissue International*.

Kwok, K. H. M.; Lam, K. S. L.; Xu, A., 2016: Heterogeneity of white adipose tissue: Molecular basis and clinical implications. *Experimental and Molecular Medicine*.

L Newton, A.; J Hanks, L.; Davis, M.; Casazza, K., 2013: The relationships among total body fat, bone mineral content and bone marrow adipose tissue in early-pubertal girls. *BoneKEy Reports*., **2**, 1–7.

L, T.; M, D.; X, W.; N, D.; J, C.; R, D.; GJ, R.; LA, C.; FT, C.; J, D.; C, M.; S, S.; A, M.; KJ, M.; JS, S.; GG, M.; EA, W.; CA, M.; RI., T., 1995: Identification and expression cloning of a leptin receptor, OB-R. *Cell.*, **83**, 1263–1271.

Lagrue, E.; Abe, H.; Lavanya, M.; Touhami, J.; Bodard, S.; Chalon, S.; Battini, J. luc; Sitbon, M.; Castelnau, P., 2010: Regional characterization of energy metabolism in the brain of normal and MPTP-intoxicated mice using new markers of glucose and phosphate transport. *Journal of Biomedical Science*.

Laporte, M. H.; Chatellard, C.; Vauchez, V.; Hemming, F. J.; Deloulme, J. C.; Vossier, F.; Blot, B.; Fraboulet, S.; Sadoul, R., 2017: Alix is required during development for normal growth of the mouse brain. *Scientific Reports*., **7**, 1–16.

Larsen, F. T.; Jensen, N.; Autzen, J. K.; Kongsfelt, I. B.; Pedersen, L., 2017: Primary Brain Calcification Causal PiT2 Transport-Knockout Variants can Exert Dominant Negative Effects on Wild-Type PiT2 Transport Function in Mammalian Cells. *Journal of Molecular Neuroscience*., **61**, 215–220.

Lecka-Czernik, B.; Stechschulte, L. A.; Czernik, P. J.; Sherman, S. B.; Huang, S.; Krings, A., 2017: Marrow adipose Tissue: Skeletal location, sexual dimorphism, and response to sex steroid deficiency. *Frontiers in Endocrinology*., **8**, 1–12.

Lee, B.; Shao, J., 2012: Adiponectin and lipid metabolism in skeletal muscle. *Acta Pharmaceutica Sinica B*., **2**, 335–340.

Lee, G. H.; Pratley, R. .; Fei, H.; Friedman, J. .; Halaas, J.; Ravussin, E.; Kern, P. .; Kim, S.; Zhang, Y.; Lallone, R.; Ranganathan, S.; Maffei, M., 1997: Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nature Medicine*., **1**, 1155–1161.

Lee, N. K.; Sowa, H.; Hinoi, E.; Ferron, M.; Ahn, J. D.; Confavreux, C.; Dacquin, R.; Mee, P. J.; Mckee, M. D.; Jung, D. Y.; Zhang, Z.; Kim, J. K.; Mauvais-jarvis, F.; Ducy, P.; Karsenty, G., 2007: Endocrine Regulation of Energy Metabolism by the Skeleton. *Cell*., **130**, 456–469.

Lefterova, M. I.; Lazar, M. A., 2009: New developments in adipogenesis. *Trends in Endocrinology and Metabolism*.

Lemos, R. R.; Ramos, E. M.; Legati, A.; Nicolas, G.; Jenkinson, E. M.; Livingston, J. H.; Crow, Y. J.; Campion, D.; Coppola, G.; Oliveira, J. R. M., 2015: Update and mutational analysis of SLC20A2: A major cause of primary familial brain calcification. *Human Mutation*., **36**, 489–495.

Li, G. W.; Xu, Z.; Chen, Q. W.; Tian, Y. N.; Wang, X. Y.; Zhou, L.; Chang, S. X., 2014: Quantitative evaluation of vertebral marrow adipose tissue in postmenopausal female using MRI chemical shift-based water-fat separation. *Clinical Radiology*., **69**, 254–262.

Li, Y.; Meng, Y.; Yu, X., 2019a: The Unique Metabolic Characteristics of Bone Marrow Adipose Tissue. *Frontiers in Endocrinology*., **10**, 1–13.

Li, Z.; Hardij, J.; Bagchi, D. P.; Scheller, E. L.; MacDougald, O. A., 2018: Development, regulation, metabolism and function of bone marrow adipose tissues. *Bone*., **110**, 134–140.

Li, Z.; Hardij, J.; Evers, S. S.; Hutch, C. R.; Choi, S. M.; Shao, Y.; Learman, B. S.; Lewis, K. T.; Schill, R. L.; Mori, H.; Bagchi, D. P.; Romanelli, S. M.; Kim, K. S.; Bowers, E.; Griffin, C.; Seeley, R. J.; Singer, K.; Sandoval, D. A.; Rosen, C. J. et al., 2019b: G-CSF partially mediates effects of sleeve gastrectomy on the bone marrow niche. *Journal of Clinical Investigation*., **129**, 2404–2416.

Liu, L.; Sanchez-Bonilla, M.; Crouthamel, M. H.; Giachelli, C. M.; Keel, S., 2013: Mice lacking the sodium-dependent phosphate import protein, PiT1 (SLC20A1), have a severe defect in terminal erythroid differentiation and early B cell development. *Exp Hematol*.

Ma, X. X.; Li, X.; Yi, P.; Wang, C.; Weng, J.; Zhang, L.; Xu, X.; Sun, H.; Feng, S.; Liu, K.; Chen, R.; Du, S.; Mao, X.; Zeng, X.; Zhang, L. Y.; Liu, M.; Tang, B. S.; Zhu, X.; Jin, S. et al., 2017: PiT2 regulates neuronal outgrowth through interaction with microtubule-associated protein. *Scientific Reports*., **7**, 1–13.

Makarovsky, I.; Markel, G.; Hoffman, A.; Schein, O.; Finkelstien, A.; Brosh-Nissimov, T.; Tashma, Z.;

Dushnitsky, T.; Eisenkraft, A., 2007: Osmium tetroxide: A new kind of weapon. *Israel Medical Association Journal*., **9**, 750–752.

Matsumoto, Y.; La Rose, J.; Lim, M.; Adissu, H. A.; Law, N.; Mao, X.; Cong, F.; Mera, P.; Karsenty, G.; Goltzman, D.; Changoor, A.; Zhang, L.; Stajkowski, M.; Grynpas, M. D.; Bergmann, C.; Rottapel, R., 2017: Ubiquitin ligase RNF146 coordinates bone dynamics and energy metabolism. *Journal of Clinical Investigation*., **127**, 2612–2625.

McCabe, L. R., 2007: Understanding the pathology and mechanisms of type I diabetic bone loss. *Journal of Cellular Biochemistry*.

McLaughlin, P. J.; Bakall, B.; Choi, J.; Liu, Z.; Sasaki, T.; Davis, E. C.; Marmorstein, A. D.; Marmorstein, L. Y., 2007: Lack of fibulin-3 causes early aging and herniation, but not macular degeneration in mice. *Human Molecular Genetics*., **16**, 3059–3070.

Merametdjian, L.; Beck-Cormier, S.; Bon, N.; Couasnay, G.; Sourice, S.; Guicheux, J.; Gaucher, C.; Beck, L., 2018: Expression of Phosphate Transporters during Dental Mineralization. *Journal of Dental Research*., **97**, 209–217.

Meunier, P.; Aaron, J.; Edouard, C.; Vignon, G., 1971: Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. *Clinical orthopaedics and related research*.

Millán, J. L., 2013: The role of phosphatases in the initiation of skeletal mineralization. *Calcified Tissue International*., **93**, 299–306.

Miller, D. G.; Edwards, R. H.; Miller, A. D., 1994a: Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. *Proc. Natl. Acad. Sci. USA*., **91**, 78–82.

Miller, D. G.; Miller, A. D., 1994b: A family of retroviruses that utilize related phosphate transporters for cell entry. *Journal of Virology*., **68**, 8270–8276.

Moriishi, T.; Ozasa, R.; Ishimoto, T.; Nakano, T.; Hasegawa, T.; Miyazaki, T.; Liu, W.; Fukuyama, R.; Wang, Y.; Komori, H.; Qin, X.; Amizuka, N.; Komori, T., 2020: Osteocalcin is necessary for the alignment of apatite crystallites, but not glucose metabolism, testosterone synthesis, or muscle mass. *PLoS Genetics*., **16**, 1–29.

Murphy, C. S.; Liaw, L.; Reagan, M. R., 2019: In vitro tissue-engineered adipose constructs for modeling disease. *BMC Biomed Eng.*

Naot, D.; Watson, M.; Callon, K. E.; Tuari, D.; Musson, D. S.; Choi, A. J.; Sreenivasan, D.; Fernandez, J.; Tu, P. T.; Dickinson, M.; Gamble, G. D.; Grey, A.; Cornish, J., 2016: Reduced bone density and cortical bone indices in female adiponectin-knockout mice. *Endocrinology*., **157**, 3550–3561.

Naveiras, O.; Nardi, V.; Wenzel, P. L.; Fahey, F.; Daley, G. Q., 2009: Bone marrow adipocytes as negative regulators of the hematopoietic microenvironment. *Nature*.

Nielsen, L. B.; Pedersen, F. S.; Pedersen, L., 2001: Expression of type III sodium-dependent phosphate transporters/retroviral receptors mRNAs during osteoblast differentiation. *Bone*., **28**, 160–166.

Nishimura, M.; Aito, S. N., 2008: Tissue-specific mRNA Expression Profiles of Human Solute Carrier Transporter Superfamilies. *Drug Metab. Pharmacokinet.*

O'Hara, B.; Johann, S. V; Klinger, H. P.; Blair, D. G.; Rubinson, H.; Dunn, K. J.; Sass, P.; Vitek, S. M.; Robins, T., 1990: Characterization of a Human Gene Conferring Sensitivity to Infection by Gibbon Ape Leukemia Virus. *Cell Growth and Differentiation*.

Obeid, O. A., 2013: Low phosphorus status might contribute to the onset of obesity. *Obesity Reviews*., **14**, 659–664.

Olah, Z.; Lehel, C.; Anderson, W. B.; Eiden, M. V.; Wilson, C. A., 1994: The cellular receptor for gibbon ape leukemia virus is a novel high affinity sodium-dependent phosphate transporter. *Journal of Biological Chemistry*., **269**, 25426–25431.

Oshima, K.; Nampei, A.; Matsuda, M.; Iwaki, M.; Fukuhara, A.; Hashimoto, J.; Yoshikawa, H.; Shimomura, I., 2005: Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast. *Biochemical and Biophysical Research Communications*., **331**, 520–526.

Oury, F.; Sumara, G.; Sumara, O.; Ferron, M.; Chang, H.; Smith, C. E.; Hermo, L.; Suarez, S.; Roth, B. L.; Ducy, P.; Karsenty, G., 2011: Endocrine regulation of male fertility by the skeleton. *Cell*., **144**, 796–809.

Paccou, J.; Penel, G.; Chauveau, C.; Cortet, B.; Hardouin, P., 2019: Marrow adiposity and bone: Review of clinical implications. *Bone*., **118**, 8–15.

Pajvani, U. B.; Hawkins, M.; Combs, T. P.; Rajala, M. W.; Doebber, T.; Berger, J. P.; Wagner, J. A.; Wu, M.; Knopps, A.; Xiang, A. H.; Utzschneider, K. M.; Kahn, S. E.; Olefsky, J. M.; Buchanan, T. A.; Scherer, P. E., 2004: Complex Distribution, Not Absolute Amount of Adiponectin, Correlates with Thiazolidinedione-mediated Improvement in Insulin Sensitivity. *Journal of Biological Chemistry*., **279**, 12152–12162.

Pal China, S.; Sanyal, S.; Chattopadhyay, N., 2018: Adiponectin signaling and its role in bone metabolism. *Cytokine*., **112**, 116–131.

Palmer, G.; Zhao, J.; Bonjour, J.; Hofstetter, W.; Caverzasio, J., 1999: In vivo expression of transcripts encoding the glvr-1 phosphate transporter/retrovirus receptor during bone development. *Bone*., **24**, 1–7.

Palmer, G.; Guicheux, J.; Bonjour, J. P.; Caverzasio, J., 2000: Transforming growth factor-β stimulates inorganic phosphate transport and expression of the type III phosphate transporter Glvr-1 in chondrogenic ATDC5 cells. *Endocrinology*., **141**, 2236–2243.

Papaioannou, A.; Kennedy, C., 2015: Diagnostic criteria for osteoporosis should be expanded. *The Lancet Diabetes and Endocrinology*., **3**, 234–236.

Park, W.; Kim, B. S.; Lee, J. E.; Huh, J. K.; Kim, B. J.; Sung, K. C.; Kang, J. H.; Lee, M. H.; Park, J. R.; Rhee, E. J.; Oh, K. W.; Lee, W. Y.; Park, C. Y.; Park, S. W.; Kim, S. W., 2009: Serum phosphate levels and the risk of cardiovascular disease and metabolic syndrome: A double-edged sword. *Diabetes Research and Clinical Practice*., **83**, 119–125.

Paula, F. J. A. De; Rosen, C. J., 2020: Marrow Adipocytes : Origin , Structure , and Function.

Pavy-Le Traon, A.; Heer, M.; Narici, M. V.; Rittweger, J.; Vernikos, J., 2007: *From space to Earth: Advances in human physiology from 20 years of bed rest studies (1986-2006). European Journal of Applied Physiology*., Vol. 101.

Peacock, M., 2020: Phosphate Metabolism in Health and Disease. *Calcified Tissue International*.

Pedersen, L.; Johann, S. V; van Zeijl, M.; Pedersen, F. S.; O'Hara, B., 1995: Chimeras of receptors for gibbon ape leukemia virus/feline leukemia virus B and amphotropic murine leukemia virus reveal different modes of receptor recognition by retrovirus. *Journal of virology*., **69**, 2401–2405.

Pedersen, L.; van Zeijl, M.; Johann, S. V; O'Hara, B., 1997: Fungal phosphate transporter serves as a receptor backbone for gibbon ape leukemia virus. *Journal of virology*., **71**, 7619–7622.

Pelleymounter, M. A.; Cullen, M. J.; Baker, M. B.; Hecht, R.; Winters, D.; Boone, T.; Collins, F., 1995: Effects of the obese gene product on body weight regulation in ob/ob mice. *Science*., **269**, 540–543.

Petrovic, N.; Walden, T. B.; Shabalina, I. G.; Timmons, J. A.; Cannon, B.; Nedergaard, J., 2010: Chronic peroxisome proliferator-activated receptor γ (PPARγ) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *Journal of Biological Chemistry*., **285**, 7153–7164.

Piney, A., 1922: The Anatomy Of The Bone Marrow : With Special Reference To The Distribution Of The Red Marrow. *The British Medical Journal*.

Ravera, S.; Murer, H.; Forster, I. C., 2013: An Externally Accessible Linker Region in the Sodium-Coupled Phosphate Transporter PiT-1 (SLC20A1) is Important for Transport Function. *Cellular physiology and biochemistry*., **41**, 187–199.

Reagan, M. R.; Rosen, C. J., 2016: Navigating the bone marrow niche: translational insights and cancer-driven dysfunction. *Nat Rev Rheumatol*.

Reid, I. R.; Baldock, P. A.; Cornish, J., 2018: Effects of Leptin on the Skeleton. *Endocrine Reviews*.

Rendina-Ruedy, E.; Rosen, C. J., 2020: Lipids in the Bone Marrow: An Evolving Perspective. *Cell Metabolism*., **31**, 219–231.

Rharass, T.; Lucas, S., 2018: Bone marrow adiposity and bone, a bad romance? *European Journal of Endocrinology*., **179**, R165–R182.

Rosen, C. J.; Ackert-Bicknell, C.; Rodriguez, J. P.; Pino, A. M., 2009: Marrow fat and the bone microenvironment: Developmental, functional, and pathological implications. *Critical Reviews in Eukaryotic Gene Expression*., **19**, 109–124.

Rosen, C. J.; Rendina-Ruedy, E., 2017: Bone-fat interaction. *Endocrinol Metab Clin North Am*., 1–12.

Rosen, E. D.; Spiegelman, B. M., 2014: What we talk about when we talk about fat. *Cell*.

Rylander, E.; Pribylova, H.; Lind, J., 1972: A thermpgenic study of infants exposed to cold. *Acta Pædiatrica Scand*., **61**, 42–48.

Rzonca, S. O.; Suva, L. J.; Gaddy, D.; Montague, D. C.; Lecka-Czernik, B., 2004: Bone Is a Target for the Antidiabetic Compound Rosiglitazone. *Endocrinology*., **145**, 401–406.

Sáinz, N.; Barrenetxe, J.; Moreno-aliaga, M. J.; Martínez, J. A., 2015: Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. *Metabolism*., **64**, 35–46.

Salaün, C.; Rodrigues, P.; Heard, J. M., 2001: Transmembrane Topology of PiT-2, a Phosphate Transporter-Retrovirus Receptor. *Journal of Virology*., **75**, 5584–5592.

Salaün, C.; Leroy, C.; Rousseau, A.; Boitez, V.; Beck, L.; Friedlander, G., 2010: Identification of a novel transport-independent function of PiT1/SLC20A1 in the regulation of TNF-induced apoptosis. *Journal of Biological Chemistry*., **285**, 34408–34418.

Scheller, E. L.; Troiano, N.; Vanhoutan, J. N.; Bouxsein, M. A.; Fretz, J. A.; Xi, Y.; Nelson, T.; Katz, G.; Berry, R.; Church, C. D.; Doucette, C. R.; Rodeheffer, M. S.; MacDougald, O. A.; Rosen, C. J.; Horowitz, M. C., 2014: Use of osmium tetroxide staining with microcomputerized tomography to visualize and quantify bone marrow adipose tissue in vivo. *Methods in Enzymology*. 1st edn. Elsevier Inc., Vol. 537pp. 123–139.

Scheller, E. L.; Doucette, C. R.; Learman, B. S.; Cawthorn, W. P.; Khandaker, S.; Schell, B.; Wu, B.; Ding, S. Y.; Bredella, M. A.; Fazeli, P. K.; Khoury, B.; Jepsen, K. J.; Pilch, P. F.; Klibanski, A.; Rosen, C. J.; MacDougald, O. A., 2015: Region-specific variation in the properties of skeletal adipocytes reveals regulated and constitutive marrow adipose tissues. *Nature Communications*., **6**, 1–13.

Scheller, E. L.; Khoury, B.; Moller, K. L.; Wee, N. K. Y.; Khandaker, S.; Kozloff, K. M.; Abrishami, S. H.; Zamarron, B. F.; Singer, K., 2016: Changes in skeletal integrity and marrow adiposity during high-fat diet and after weight loss. *Frontiers in Endocrinology*., **7**, 1–13.

Scherer, P. E.; Williams, S.; Fogliano, M.; Baldini, G.; Lodish, H. F., 1995: A novel serum protein similar to C1q, produced exclusively in adipocytes. *Journal of Biological Chemistry*., **270**, 26746–26749.

Schorr, M.; Klibanski, A., 2018: Anorexia nervosa and bone. *Curr Opin Endocr Metab Res.*

Schwartz, A. V.; Sigurdsson, S.; Hue, T. F.; Lang, T. F.; Harris, T. B.; Rosen, C. J.; Vittinghoff, E.; Siggeirsdottir, K.; Sigurdsson, G.; Oskarsdottir, D.; Shet, K.; Palermo, L.; Gudnason, V.; Li, X., 2013: Vertebral bone marrow fat associated with lower trabecular BMD and prevalent vertebral fracture in older adults. *Journal of Clinical Endocrinology and Metabolism*., **98**, 2294–2300.

Schwartz, A. V., 2016: Epidemiology of fractures in type 2 diabetes. *Bone*., **82**, 2–8.

Seale, P.; Bjork, B.; Yang, W.; Kajimura, S.; Kuang, S.; Scime, A.; Devarakonda, S.; Chin, S.; Conroe, H. M.; Erdjument-Bromage, H.; Tempst, P.; Rudnicki, M. A.; Beier, D. R.; Spiegelman, B. M., 2008: PRDM16 Controls a Brown Fat/Skeletal Muscle Switch. *Nature*.

Sebo, Z. L.; Rendina-Ruedy, E.; Ables, G. P.; Lindskog, D. M.; Rodeheffer, M. S.; Fazeli, P. K.; Horowitz, M. C., 2019: Bone Marrow Adiposity: Basic and Clinical Implications. *Endocrine Reviews*.

Sethi, J. K.; Vidal-Puig, A. J., 2007: Thematic review series: Adipocyte Biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. *Journal of Lipid Research*.

Shapiro, L.; Scherer, P. E., 1998: The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Current Biology*., **8**, 335–340.

Shen, W.; Chen, J.; Punyanitya, M.; Shapses, S.; Heshka, S.; Heymsfield, S. B., 2007: MRI-measured bone marrow adipose tissue is inversely related to DXA-measured bone mineral in Caucasian women. *Osteoporosis International*., **18**, 641–647.

Shen, W.; Chen, J.; Gantz, M.; Punyanitya, M.; Heymsfield, S. B.; Gallagher, D.; Albu, J.; Engelson, E.; Kotler, D.; Pi-Sunyer, X.; Shapses, S., 2012: Ethnic and sex differences in bone marrow adipose tissue and bone mineral density relationship. *Osteoporosis International*., **23**, 2293–2301.

Slade, J. M.; Coe, L. M.; Meyer, R. A.; Mccabe, L. R., 2012: Journal of Diabetes and Its Complications Human bone marrow adiposity is linked with serum lipid levels not T1-diabetes. *Journal of Diabetes and Its Complications*., **26**, 1–9.

Starup-Linde, J.; Hygum, K.; Harsløf, T.; Langdahl, B., 2019: Type 1 diabetes and bone fragility: Links and risks. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*.

Straub, L. G.; Scherer, P. E., 2019: Metabolic Messengers: adiponectin. *Nature Metabolism*., **1**, 334–339.

Suchacki, K. J.; Cawthorn, W. P.; Rosen, C. J., 2016: Bone marrow adipose tissue: Formation, function and regulation. *Current Opinion in Pharmacology*., **28**, 50–56.

Suchacki, K. J.; Tavares, A. A. S.; Mattiucci, D.; Scheller, E. L.; Papanastasiou, G.; Gray, C.; Sinton, M. C.; Ramage, L. E.; McDougald, W. A.; Lovdel, A.; Sulston, R. J.; Thomas, B. J.; Nicholson, B. M.; Drake, A. J.; Alcaide-Corral, C. J.; Said, D.; Poloni, A.; Cinti, S.; Macpherson, G. J. et al., 2020: Bone marrow adipose tissue is a unique adipose subtype with distinct roles in glucose homeostasis. *Nature Communications*., **11**.

Sulston, R. J.; Cawthorn, W. P., 2016: Bone marrow adipose tissue as an endocrine organ: Close to the bone? *Hormone Molecular Biology and Clinical Investigation*., **28**, 21–38.

Sun, H.; Kim, J. K.; Mortensen, R. M.; Mutyaba, P. L.; Hankenson, K. D.; Krebsbach, P. H., 2013: Osteoblast-targeted Suppression of PPARγ Increases Osteogenesis through Activation of mTOR Signaling. *Stem Cells*.

Sun, W.; Dong, H.; Balaz, M.; Slyper, M.; Drokhlyansky, E.; Colleluori, G.; Giordano, A.; Kovanicova, Z.; Stefanicka, P.; Balazova, L.; Ding, L.; Husted, A. S.; Rudofsky, G.; Ukropec, J.; Cinti, S.; Schwartz, T. W.; Regev, A.; Wolfrum, C., 2020: snRNA-seq reveals a subpopulation of adipocytes that regulates thermogenesis. *Nature*., **587**.

Suzuki, A.; Palmer, G.; Bonjour, J. P.; Caverzasio, J., 2001: Stimulation of sodium-dependent inorganic phosphate transport by activation of Gi/o-protein-coupled receptors by epinephrine in MC3T3-E1 osteoblast-like cells. *Bone*., **28**, 589–594.

Suzuki, A.; Ghayor, C.; Guicheux, J.; Magne, D.; Quillard, S.; Kakita, A.; Ono, Y.; Miura, Y.; Oiso, Y.; Itoh, M.; Caverzasio, J., 2006: Enhanced expression of the inorganic phosphate transporter Pit-1 is involved in BMP-2-induced matrix mineralization in osteoblast-like cells. *Journal of Bone and Mineral Research*., **21**, 674–683.

Suzuki, A.; Ammann, P.; Nishiwaki-Yasuda, K.; Sekiguchi, S.; Asano, S.; Nagao, S.; Kaneko, R.; Hirabayashi, M.; Oiso, Y.; Itoh, M.; Caverzasio, J., 2010: Effects of transgenic Pit-1 overexpression on calcium phosphate and bone metabolism. *Journal of Bone and Mineral Metabolism*., **28**, 139–148.

Takeda, S.; Elefteriou, F.; Levasseur, R.; Liu, X.; Zhao, L.; Parker, K. L.; Armstrong, D.; Ducy, P.; Karsenty, G., 2002: Leptin regulates bone formation via the sympathetic nervous system. *Cell*., **111**, 305–317.

Tanaka, T.; Yoshida, N.; Kishimoto, T.; Akira, S., 1997: Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. *EMBO Journal*., **16**, 7432–7443.

Tatsumi, S.; Segawa, H.; Morita, K.; Haga, H.; Kouda, T.; Yamamoto, H.; Inoue, Y.; Nii, T.; Katai, K.; Taketani, Y.; Miyamoto, K. ichi; Takeda, E., 1998: Molecular Cloning and Hormonal Regulation of PiT-1 , a Sodium-Dependent Phosphate Cotransporter from Rat Parathyroid Glands *. *Endocrinology*.

Temple, J. P.; Hutton, D. L.; Hung, B. P.; Huri, P. Y.; Cook, C. A.; Kondragunta, R.; Jia, X.; Grayson, W. L., 2014: Engineering anatomically shaped vascularized bone grafts with hASCs and 3D-printed PCL scaffolds. *Journal of Biomedical Materials Research - Part A*., **102**, 4317–4325.

Tencerova, M.; Kassem, M., 2016: The bone marrow-derived stromal cells: Commitment and regulation of adipogenesis. *Frontiers in Endocrinology*., **7**.

Tencerova, M.; Figeac, F.; Ditzel, N.; Taipaleenmäki, H.; Nielsen, T. K.; Kassem, M., 2018: High-Fat Diet–Induced Obesity Promotes Expansion of Bone Marrow Adipose Tissue and Impairs Skeletal Stem Cell Functions in Mice. *Journal of Bone and Mineral Research*., **33**, 1154–1165.

Tenenhouse, H. S.; Roy, S.; Martel, J.; Gauthier, C., 1998: Differential expression , abundance , and regulation of Na + -phosphate cotransporter genes in murine kidney. *Journal of Physiology*.

Tikhonova, A. N.; Dolgalev, I.; Hu, H.; Sivaraj, K. K.; Hoxha, E.; Pinho, S.; Akhmetzyanova, I.; Gao, J.;

Guillamot, M. R.; Gutkin, M. C.; Zhang, Y.; Marier, C.; Diefenbach, C.; Kousteni, S.; Heguy, A.; Zhong, H.; David, R.; Butler, J. M.; Economides, A. et al., 2019: The bone marrow microenvironment at single-cell resolution. *Nature*., **569**, 222–228.

Tosa, I.; Yamada, D.; Yasumatsu, M.; Hinoi, E.; Ono, M.; Oohashi, T.; Kuboki, T.; Takarada, T., 2019: Postnatal Runx2 deletion leads to low bone mass and adipocyte accumulation in mice bone tissues. *Biochemical and Biophysical Research Communications*.

Tran, T. T.; Yamamoto, Y.; Gesta, S.; Kahn, C. R., 2008: Beneficial Effects of Subcutaneous Fat Transplantation on Metabolism. *Cell Metabolism*., **7**, 410–420.

Tratwal, J.; Labella, R.; Bravenboer, N.; Kerckhofs, G.; Douni, E.; Scheller, E. L.; Badr, S.; Karampinos, D. C.; Beck-Cormier, S.; Palmisano, B.; Poloni, A.; Moreno-Aliaga, M. J.; Fretz, J.; Rodeheffer, M. S.; Boroumand, P.; Rosen, C. J.; Horowitz, M. C.; van der Eerden, B. C. J.; Veldhuis-Vlug, A. G. et al., 2020: Reporting Guidelines, Review of Methodological Standards, and Challenges Toward Harmonization in Bone Marrow Adiposity Research. Report of the Methodologies Working Group of the International Bone Marrow Adiposity Society. *Frontiers in Endocrinology*., **11**, 1–36.

Trudel, G.; Payne, M.; Mädler, B.; Ramachandran, N.; Lecompte, M.; Wade, C.; Biolo, G.; Blanc, S.; Hughson, R.; Bear, L.; Uhthoff, H. K., 2009: Bone marrow fat accumulation after 60 days of bed rest persisted 1 year after activities were resumed along with hemopoietic stimulation: The Women International Space Simulation for Exploration study. *Journal of Applied Physiology*., **107**, 540–548.

van Zeijl, M.; Johann, S. V; Closst, E.; Cunningham, J.; Eddy, R.; Shows, T. B.; O'Hara, B., 1994: A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. *Proc. Natl. Acad. Sci. USA*., **91**, 1168–1172.

Vasta, J. D.; Raines, R. T., 2018: Collagen Prolyl 4-Hydroxylase as a Therapeutic Target. *Journal of Medicinal Chemistry*., **61**, 10403–10411.

Veldhuis-Vlug, A. G.; Rosen, C. J., 2017: Mechanisms of marrow adiposity and its implications for skeletal health. *Metabolism: Clinical and Experimental*., **67**, 106–114.

Verma, S.; Hussain, M. E., 2017: Obesity and diabetes: An update. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*., **11**, 73–79.

Villa-bellosta, R.; Ravera, S.; Sorribas, V.; Stange, G.; Levi, M.; Murer, H.; Biber, J.; Forster, I. C., 2009: The Na + -P i cotransporter PiT-2 (SLC20A2) is expressed in the apical membrane of rat renal proximal tubules and regulated by dietary Pi. *Am J Physiol Renal Physiol*.

Villa-bellosta, R.; Sorribas, V., 2010: Compensatory regulation of the sodium / phosphate (SLC20A2) during Pi deprivation and acidosis. *Pflugers Arch - Eur J Physiol*.

Villa-Bellosta, R.; Bogaert, Y. E.; Levi, M.; Sorribas, V., 2007: Characterization of phosphate transport in rat vascular smooth muscle cells: implications for vascular calcification. *Arteriosclerosis, Thrombosis, and Vascular Biology*., **27**, 1030–1036.

Virkki, L. V.; Biber, J.; Murer, H.; Forster, I. C., 2007: Phosphate transporters: a tale of two solute carrier families. *AJP: Renal Physiology*., **293**, F643–F654.

Wajchenberg, B. L., 2000: Subcutaneous and Visceral Adipose Tissue : Their Relation to the Metabolic Syndrome. *Endocrine Reviews*., **21**, 697–738.

Waki, H.; Yamauchi, T.; Kamon, J.; Ito, Y.; Uchida, S.; Kita, S.; Hara, K.; Hada, Y.; Vasseur, F.; Froguel, P.;

Kimura, S.; Nagai, R.; Kadowaki, T., 2003: Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. *Journal of Biological Chemistry*., **278**, 40352–40363.

Wallingford, M. C.; Gammill, H. S.; Giachelli, C. M., 2016: Slc20a2 deficiency results in fetal growth restriction and placental calcification associated with thickened basement membranes and novel CD13 and lamininα1 expressing cells. *Reproductive Biology*., **16**, 13–26.

Wallingford, M. C.; Leaf, E. M.; Borgeia, S.; Chavkin, N. W.; Sawangmake, C.; Marro, K.; Cox, T. C.; Speer, M. Y.; Giachelli, C. M., 2017: Slc20a2 deficiency in mice leads to elevated phosphate levels in cerebrospinal fluid and glymphatic pathway-associated arteriolar calcification, and recapitulates human idiopathic basal ganglia calcification. *Brain Pathol*., **27**.

Walsh, J. S.; Vilaca, T., 2017: Obesity, Type 2 Diabetes and Bone in Adults. *Calcified Tissue International*., **100**, 528–535.

Wang, C.; Li, Y.; Shi, L.; Ren, J.; Patti, M.; Wang, T.; Oliveira, J. R. M. De; Sobrido, M. jesús; Quintáns, B.; Baquero, M.; Cui, X.; Zhang, X. yang; Wang, L.; Xu, H.; Wang, J.; Yao, J.; Dai, X.; Liu, J.; Zhang, L. et al., 2012: Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. *Nature Publishing Group*., **44**, 254–256.

Wang, F.; Wang, P. xia; Wu, X. lin; Dang, S. ying; Chen, Y.; Ni, Y. yin; Gao, L. hong; Lu, S. yuan; Kuang, Y.; Huang, L.; Fei, J.; Wang, Z. gang; Pang, X. fen, 2013: Deficiency of Adiponectin Protects against Ovariectomy-Induced Osteoporosis in Mice. *PLoS ONE*., **8**.

Wang, N. D.; Finegold, M. J.; Bradley, A.; Ou, C. N.; Abdelsayed, S. V; Wilde, M. D.; Taylor, L. R.; Wilson, D. R.; Darlington, G. J., 1995: Impaired energy homeostasis in C/EBPα knockout mice. *Science*., **269**, 1108–1112.

Wang, Z. V.; Scherer, P. E., 2016: Adiponectin, the past two decades. *Journal of Molecular Cell Biology*., **8**, 93–100.

Westenberger, A.; Balck, A.; Klein, C., 2019: Primary familial brain calcifications: Genetic and clinical update. *Current Opinion in Neurology*.

Weyer, C.; Funahashi, T.; Tanaka, S.; Hotta, K.; Matsuzawa, Y.; Pratley, R. E.; Tataranni, P. A., 2001: Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. *Journal of Clinical Endocrinology and Metabolism*., **86**, 1930–1935.

Winter, E. M.; Ireland, A.; Butterfield, N. C.; Haffner-Luntzer, M.; Horcajada, M. N.; Veldhuis-Vlug, A. G.; Oei, L.; Colaianni, G.; Bonnet, N., 2020: Pregnancy and lactation, a challenge for the skeleton. *Endocrine Connections*.

Woods, G. N.; Ewing, S. K.; Sigurdsson, S.; Kado, D. M.; Eiriksdottir, G.; Gudnason, V.; Hue, T. F.; Lang, T. F.; Vittinghoff, E.; Harris, T. B.; Rosen, C.; Xu, K.; Li, X.; Schwartz, A. V., 2020: Greater Bone Marrow Adiposity Predicts Bone Loss in Older Women. *Journal of Bone and Mineral Research*., **35**, 326–332.

Wren, T. A. L.; Chung, S. A.; Dorey, F. J.; Bluml, S.; Adams, G. B.; Gilsanz, V., 2011: Bone Marrow Fat Is Inversely Related to Cortical Bone in Young and Old Subjects. *J Clin Endocrinol Metab*., **96**, 782–786.

Wu, J.; Bostrom, P.; Sparks, L. M.; Ye, L.; Choi, J. H.; Giang, A. H.; Khandekar, M.; Nuutila, P.; Schaart, G.; Huang, K.; Tu, H.; van Marken Lichtenbelt, W. D.; Hoeks, J.; Enerback, S.; Schrauwen, P.; Spiegelman, B. M., 2012: Beige Adipocytes are a Distinct Type of Thermogenic Fat Cell in Mouse and Human. *Cell*.

Wu, J.; Cohen, P.; Spiegelman, B. M., 2013: Adaptive thermogenesis in adipocytes: Is beige the new brown? *Genes and Development*.

Wubuli, A.; Reyer, H.; Muráni, E.; Ponsuksili, S.; Wolf, P.; Oster, M.; Wimmers, K., 2019: Tissue-wide gene expression analysis of sodium/phosphate co-transporters in pigs. *International Journal of Molecular Sciences*., **20**, 1–12.

Xiao, Z.; Zhang, S.; Cao, L.; Qiu, N.; David, V.; Quarles, L. D., 2010: Conditional disruption of Pkd1 in osteoblasts results in osteopenia due to direct impairment of bone formation. *Journal of Biological Chemistry*., **285**, 1177–1187.

Xue, B.; Rim, J. S.; Hogan, J. C.; Coulter, A. A.; Koza, R. A.; Kozak, L. P., 2007: Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. *Journal of Lipid Research*., **48**, 41–51.

Yadav, M. C.; Bottini, M.; Cory, E.; Bhattacharya, K.; Kuss, P.; Narisawa, S.; Sah, R. L.; Beck, L.; Fadeel, B.; Farquharson, C.; Millán, J. L., 2016: Skeletal Mineralization Deficits and Impaired Biogenesis and Function of Chondrocyte-Derived Matrix Vesicles in Phospho1-/- and Phospho1/Pit1 Double-Knockout Mice. *Journal of Bone and Mineral Research*., **31**, 1275–1286.

Yamada, S.; Wallingford, M. C.; Borgeia, S.; Cox, T. C.; Giachelli, C. M., 2017: Loss of PiT-2 results in abnormal bone development and decreased bone mineral density and length in mice. *Biochemical and Biophysical Research Communications*.

Yamauchi, T.; Kamon, J.; Waki, H.; Terauchi, Y.; Kubota, N.; Hara, K.; Mori, Y.; Ide, T.; Murakami, K.; Tsuboyama-Kasaoka, N.; Ezaki, O.; Akanuma, Y.; Gavrilova, O.; Vinson, C.; Reitman, M. L.; Kagechika, H.; Shudo, K.; Yoda, M.; Nakano, Y. et al., 2001: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine*., **7**, 941–946.

Yamauchi, T.; Kadowaki, T., 2013: Review Adiponectin Receptor as a Key Player in Healthy Longevity and Obesity-Related Diseases. *Cell Metabolism*., **17**, 185–196.

Yang, J.; Croniger, C. M.; Lekstrom-Himes, J.; Zhang, P.; Fenyus, M.; Tenen, D. G.; Darlington, G. J.; Hanson, R. W., 2005: Metabolic response of mice to a postnatal ablation of CCAAT/enhancer- binding protein α. *Journal of Biological Chemistry*., **280**, 38689–38699.

Yoshiko, Y.; Candeliere, G. A.; Maeda, N.; Aubin, J. E., 2007: Osteoblast Autonomous Pi Regulation via Pit1 Plays a Role in Bone Mineralization. *Molecular and Cellular Biology*., **27**, 4465–4474.

Yu, B.; Huo, L.; Liu, Y.; Deng, P.; Szymanski, J.; Li, J.; Luo, X.; Hong, C.; Lin, J.; Wang, C. Y., 2018: PGC-1α Controls Skeletal Stem Cell Fate and Bone-Fat Balance in Osteoporosis and Skeletal Aging by Inducing TAZ. *Cell Stem Cell*.

Zhang Y; Proenca R; Maffei M; Barone M; Leopold L; Friedman JM, 1994: Positional cloning of the mouse obese gene and its human homologue. *Nature*., **372**, 425–432.

Zhao, D.; Sani, F. V.; Nilsson, J.; Rodenburg, M.; Stocking, C.; Linde, A.; Gritli-Linde, A., 2006: Expression of Pit2 sodium-phosphate cotransporter during murine odontogenesis is developmentally regulated. *European Journal of Oral Sciences*., **114**, 517–523.

Zhong, L.; Yao, L.; Tower, R. J.; Wei, Y.; Miao, Z.; Park, J.; Shrestha, R.; Wang, L.; Yu, W.; Holdreith, N.; Huang, X.; Zhang, Y.; Tong, W.; Gong, Y.; Ahn, J.; Susztak, K.; Dyment, N.; Li, M.; Long, F. et al., 2020: Single cell transcriptomics identifies a unique adipose lineage cell population that regulates bone marrow environment. *eLife*., **9**, 1–28.

Zhou, B. O.; Yu, H.; Yue, R.; Zhao, Z.; Rios, J. J.; Naveiras, O.; Morrison, S. J., 2017: Bone marrow adipocytes promote the regeneration of stem cells and hematopoiesis by secreting SCF. *Nat Cell Biol.*

Zou, W.; Rohatgi, N.; Brestoff, J. R.; Li, Y.; Barve, R. A.; Tycksen, E.; Kim, Y.; Silva, M. J.; Teitelbaum, S. L., 2020: Ablation of Fat Cells in Adult Mice Induces Massive Article Ablation of Fat Cells in Adult Mice Induces Massive Bone Gain. *Cell Metabolism*., 1–13.

Zwick, R. K.; Guerrero-Juarez, C. F.; Horsley, V.; Plikus, M. V, 2018: Anatomical, Physiological, and Functional Diversity of Adipose Tissue. *Cell Metabolism*.

DOCTORAT BIOLOGIE BRETAGNE SANTE LOIRE

UNIVERSITÉ DE NANTES

Titre : **Étude du rôle de la PiT2 à l'interface entre l'os et les tissus adipeux**

Mots clés : PiT2, Phosphate, Transporteur, Métabolisme, Os, Cellules stromales de la moelle, Tissu adipeux médullaire, Tissu adipeux périphérique

Résumé : Le dialogue entre l'os et les tissus adipeux est indispensable pour la physiologie de ces deux organes. En effet, ils exercent des fonctions endocrines l'un vers l'autre et partagent des cellules progénitrices communes. Récemment, nos travaux ont montré que la protéine PiT2, connue pour permettre l'entrée du Pi extracellulaire à partir de la membrane plasmique, contrôle la qualité et la solidité du tissu osseux. Or, les anomalies squelettiques des souris déficientes pour PiT2 (PiT2KO) ne sont pas associées à un effet direct de l'absence de PiT2 dans les cellules du squelette. Nous faisons l'hypothèse que l'absence de PiT2 altère la fonction des tissus adipeux, affectant la qualité et la solidité des os. Nous avons caractérisé le phénotype des tissus adipeux des souris PiT2KO. Chez les jeunes souris PiT2KO, nous montrons une augmentation significative du volume de tissu adipeux intramédullaire qui n'est dû ni à une augmentation du pool de cellules progénitrices ni à une augmentation de leur potentiel adipogénique. Au cours du vieillissement, l'absence de PiT2 empêche l'augmentation du volume des tissus adipeux normalement induite par l'âge, sans altération métabolique majeure. La seconde partie de ce travail a permis d'identifier plusieurs protéines interagissant physiquement avec la 4ème boucle intracellulaire de PiT2, suggérant que les mécanismes moléculaires d'action mis en jeu soient de nature intracellulaire et indépendants de la fonction de transport de phosphate par PiT2. En conclusion, nos travaux apportent de nouveaux arguments définissant PiT2 comme une protéine multifonctionnelle et l'identifiant comme un régulateur potentiel de l'obésité.

Title : **Study of the role of PiT2 at the interface between bone and adipose tissues**

Keywords : PiT2, Phosphate, Transporter, Metabolism, Bone, Bone Marrow Mesenchymal Stromal Cells, Bone Marrow Adipose Tissue, Peripheral Adipose Tissue

Abstract : The dialogue between bone and the adipose tissues is essential for the physiology of these two tissues. Indeed, they perform endocrine functions towards each other and share common progenitor cells. Recently, our work has shown that the PiT2 protein, known to allow the entry of extracellular Pi from the plasma membrane, controls the quality and strength of the bone tissue. However, skeletal abnormalities in PiT2-deficient mice (PiT2KO) are not associated with a direct effect of the absence of PiT2 in skeletal cells. We hypothesize that the absence of PiT2 alters the function of adipose tissues, affecting bone quality and strength. We have characterized the phenotype of the adipose tissues of the PiT2KO mice. In young PiT2KO mice, we show a significant increase in the volume of intramedullary adipose tissue that is neither due to an increase in the pool of progenitor cells nor to an increase in their adipogenic potential. In adult mice, the absence of PiT2 prevents the increase in adipose tissues volume normally induced by age, without however major metabolic alteration. The second part of this work identified several proteins interacting physically with the 4th intracellular loop of PiT2, suggesting that the molecular mechanisms of action of PiT2 are intracellular and independent of its phosphate transport function. In conclusion, our work brings new elements defining PiT2 as a multifunctional protein and identifying PiT2 as a potential regulator of obesity.