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Ana Rita Azevedo Cordeiro Gomes
Identificação de mecanismos e
biomarcadores de perda massa
óssea em pacientes com Fibrose
Quística / Identification of
mechanisms and biomarkers of
bone loss in patients with Cystic
Fibrosis

U. PORTO



**FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO**

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Trabalho efetuado sob a Orientação de:

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E sob a Coorientação de:

Professora Doutora Maria Salomé Custódio Gomes

Trabalho organizado de acordo com as normas da revista: *Journal of Cystic
Fibrosis*

FMUP

MARÇO, 2023

Eu, Ana Rita Azevedo Cordeiro Gomes, abaixo assinado, nº mecanográfico 200501814, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro ter atuado com absoluta integridade na elaboração deste projeto de opção.

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Faculdade de Medicina da Universidade do Porto, 31/01/2023

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DESIGNAÇÃO DA ÁREA DO PROJECTO

Medicina Básica

TÍTULO DISSERTAÇÃO

Identification of mechanisms and biomarkers of bone loss in patients with Cystic Fibrosis

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More than representing my medical school graduation, this thesis fuses two passions: the lab and the clinics. But I have never walked alone:

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Identification of mechanisms and biomarkers of bone loss in patients with Cystic Fibrosis

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Abstract

Background: Cystic Fibrosis (CF) Bone Disease (CFBD), accompanied by bone mass reduction, is a common but not well-understood complication in adult patients, with devastating consequences to their quality of life and longevity. Amongst the possible etiologies for CFBD, recurrent infective exacerbations correlate with increased bone degradation. We previously found in the murine model that soluble bone catabolic mediators, such as Serum Amyloid A (SAA), increase in response to infection. Therefore, we hypothesize that sustained inflammatory status induced by chronic infections substantially contributes to decreased bone mass. Here, we aimed to understand whether SAA or other immune mediators may be used as novel biomarkers to monitor bone mass in cystic fibrosis (CF) patients.

Methods: This was a transversal observational study at a single CF care center consisting of twenty-six adult CF patients. Circulating levels of several bone turnover biomarkers, cytokines and chemokines were measured in the plasma and correlated with bone mineral density (BMD).

Results: We found that SAA were increased in CF patients with normal and low BMD and did not correlate with BMD in lumbar spine and femur. Circulating SAA levels in CF patients positively correlated with pulmonary function (as measured by the percentage of predicted forced expiratory volume, %FEV1). On the other hand, BMD positively correlated with %FEV1 and IL1 β and negatively with osteoprotegerin and immune mediators such as MCP-3, M-CSF, TNF α .

Conclusions: This pilot study showed that the persistent inflammatory status of CF patients correlates with decreased bone mass. Furthermore, we identified potential candidates to monitor bone loss in these patients, laying the ground for future investigations on the mechanisms that modulate the impact of these immune mediators on bone metabolism.

1. Introduction

Cystic Fibrosis Bone Disease (CFBD) manifests by low bone density and osteoporosis in adults with cystic fibrosis (CF) [1] but also in children and adolescents [2]. Low bone mass in CF individuals is due to increased bone degradation and a decrease in bone formation [3]. The mechanisms responsible for CFBD are not fully identified. Several studies in animal and cell models recognized cell intrinsic malfunctions in bone cells and their progenitors related to the mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) and its reduced function [1]. Specifically, abnormal CFTR function in osteoblasts impairs their maturation, leading to unbalanced bone turnover in CF patients.

A cell intrinsic malfunction may predispose to the development of low bone mass, but other factors such as malnutrition, vitamin D deficiency, systemic corticosteroid therapy, and chronic lung infection might be required for the development of the disease [3]. We recently reviewed the role of the proinflammatory environment associated to CFTR dysfunction in immune cells and chronic infection in the dysregulation of bone mass in CF patients (Article in Annex III).

Early detection of bone loss in CF patients prevents its negative impact in terms of patients' quality of life. Along these lines, both the American and the European associations for CF recommend the periodic monitorization of bone mineral density (BMD) by bone densitometry (DXA) [3]. This is a useful noninvasive technique, but it is costly and exposes the patients to X radiation. Moreover, DXA does not detect alterations in the bone microarchitecture. Additionally, a recent study reported that the median DXA screening rate of adults with CF in US was only 66% [4]. These caveats of DXA highlights the risk of under diagnosis of CFBD as well as the need of better tools to stratify the patients in terms of risk of bone disease [5].

Our previous studies have shown that chronic mycobacterial infection in the mouse model decreased bone formation and increased bone degradation with increasing levels of circulating Serum Amyloid A (SAA) protein [6]. Moreover, we also found that increased SAA levels in the plasma of patients with

active tuberculosis correlated with altered markers of bone turnover [6] (for more details see Annex IV, original manuscript, under review).

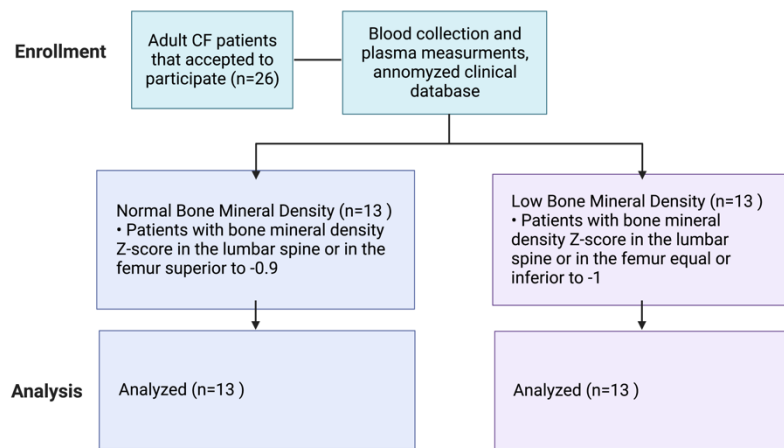
Bone turnover is determined by a balance between degradation and formation, which is orchestrated by the crosstalk of several mediators and is disturbed by proinflammatory mediators [7]. Besides being an acute phase protein, SAA has been recently involved in both arms of bone turnover. On the bone formation side, SAA inhibits osteoblast differentiation [8]. Simultaneously, SAA induces bone degradation by increasing osteoclast formation and activity [9]. Overall, SAA3 protein reduces bone formation and increases bone degradation. The dysregulation in bone turnover induced by SAA is similar to the phenotype observed in CFBD. In patients with chronic inflammatory lung diseases such as chronic obstructive pulmonary disease and sarcoidosis, the levels of SAA proteins in the serum are potential biomarkers of lung disease [10, 11]. In patients with CF and chronically infected with *Pseudomonas aeruginosa*, increased levels of SAA proteins were detected [12-14]. Based on these data, we hypothesized that the measurement of the circulating levels of SAA in CF patients is a potential tool to early detect and treat the bone loss thus, preventing the occurrence of fractures and the decline in quality of life, the increase in mortality as well as the associated economic burden. Therefore, we aimed to evaluate whether the circulating levels of SAA correlate with decreased mineral bone density. For this purpose, we studied a cohort of adult patients followed at the CF specialized center at Centro Hospitalar Universitário de São João (CHUSJ, Porto, Portugal). Firstly, commonly used circulating bone turnover markers and SAA levels were measured. Their correlation with BMD was evaluated using DXA data. Then, we aimed to identify whether different circulating immune mediators that also have a role in bone homeostasis correlated with BMD in CF patients.

2. Methods

2.1. Study population

This study was approved by the Comissão Ética para a Saúde from CHUSJ (Ref. CES CHUSJ 407/21 – CES approval documents are in Annex V). CF patients with at least 18 years of age were recruited amongst the adult CF patients followed in the CF specialized center at CHUSJ (Porto, Portugal). Patients' enrollment occurred between February and December 2022. An informative pamphlet was distributed to the patients explaining the purpose of the study, potential (even though minimal) risks related with the venipuncture procedure and the voluntary character of their participation. If they agreed to participate, an informed consent was signed. Those who consented to participate underwent a blood drawn and review of medical records. The enrolled patients and medical records were anonymized by a code. An anonymous database containing the most recent clinical information regarding the complete blood count, the circulating levels of 25-hydroxyvitamin D, calcium, phosphorus, magnesium, C-terminal telopeptide of crosslinked collagen type I (CTX-I), osteocalcin, C reactive protein, the BMD measured in the lumbar spine (LS) and femur by DXA was created. This information is usually gathered as part of the routine management of the patients and follows the current CF center protocol to monitor bone health in the patients. The patients were divided into normal and low BMD according to the most recent DXA ([Supplementary Figure 1](#)).

Additionally, 15 plasma samples (from 8 males and 7 females) were obtained from blood donors at the Clinic Hematology Service of Centro Hospitalar Universitário do Porto (Porto, Portugal) in the same metropolitan area as CHUSJ. The median age of blood donors was 47 (percentile 25: 34; percentile 75: 51) years old. These samples were used as healthy controls for determination of SAA levels.



Supplementary Figure 1. Flow diagram of patient enrollment and data analysis.

2.2 Measurement of circulating levels of SAA, cytokines/ chemokines, and bone markers

Peripheral blood was collected by venous puncture into EDTA-coated tubes (Becton Dickson, San Jose, CA). Plasma and peripheral blood cells were separated by density gradient (HistoPaque-1077, Sigma) and SepMate tubes (Stem Cell Technologies). Plasma was stored at -80°C until further use. SAA levels were measured using the human SAA Enzyme-linked immunosorbent assay (ELISA, Sigma-Aldrich), following the manufacturer's instruction. Circulating levels of cytokines/ chemokines and bone biomarkers were determined using the Human Cytokine/Chemokine Panel A 48-Plex Discovery Assay® Array (HD48A) and Human Bone 13-Plex Discovery Assay® Array (HDBN13), respectively (Eve Technologies, Calgary, Canada). TRAP5B activity was determined using BoneTRAP (TRAcP 5b) ELISA from ImmunoDiagnostics Systems, following the manufacture's protocol. All measurements were performed by a blinded operator.

2.3. Statistical analysis

For quantitative variables, data are presented as median, P25 and P75. When comparing two groups, Mann-Whitney test was performed for continuous variables and Chi-squared test for categorical variables. Correlation coefficients were obtained using Spearman's rho. Statistical significance was reached when

p value was lower than 0.05. Statistical analysis was performed using SPSS from IBM and Prism GraphPad.

3. Results

3.1. Subject demographics

We enrolled twenty-six adults (thirteen males and thirteen females) with CF with a median age of twenty-six years old ([Table 1](#)). According to the most recent DXA, thirteen patients (nine males and four females) had bone mineral density (BMD) Z-score in the LS or in the femur equal or inferior to -1 and were identified as low BMD [3]. The remaining thirteen patients had a Z-score superior to -0.9 and were grouped as normal BMD. The difference in BMD between the two groups was statistically significant. Three patients (10.7%) were under anti-osteoporotic treatment with alendronate for at least two years. Median levels of 25-hydroxyvitamin D were 22 ng/mL despite of twenty-three patients (82.1%) being supplemented with cholecalciferol. The two groups of patients were comparable in terms of the other parameters analyzed ([Table 1](#)).

3.2. Circulating bone turnover markers are not good indicators of low bone mass

Biochemical markers of bone turnover are useful tools to study alterations in bone remodeling [15]. As an initial characterization of bone turnover in our cohort of CF patients, several biochemical markers were measured in the plasma. The circulating levels of CTX-I and osteopontin (byproducts of bone degradation), DKK1 (a bone catabolic and antianabolic factor) and osteoprotegerin (OPG, a decoy receptor for RANKL) were similar between CF patients with normal and low bone mass ([Figure 1A-D](#)). Similarly, the activity of bone specific TRAP (an enzyme secreted by osteoclast during bone resorption) did not differ between the two groups ([Figure 1E](#)). On the bone formation side, the median levels of circulating osteocalcin did also not differ between CF patients with normal and low bone mass ([Figure 1F](#)). Then, we calculate the rho Spearman's correlation rank between the different bone turnover markers and BMD in the LS and in the femur. OPG strong and negatively correlated with BMD in the LS ([Figure 1G](#)).

Using linear regression, circulating levels of OPG did not predict the variation in LS BMD ($R^2=0.016$, $p=0.535$). Therefore, none of the biochemical markers analyzed appeared particularly useful to predict the variations in BMD and the risk of Low BMD.

Table 1.
Clinical and Biochemical Characteristics of CF patients

	All CF patients N=26	Normal BMD N=13 (50%)	Low BMD N=13 (50%)	p value
Age	26 (20; 35)	25 (20; 33)	34 (26; 40)	0.149
Sex				0.115
Male, N (%)	13 (50%)	4 (30.8%)	9 (69.2%)	
Female, N (%)	13 (50%)	9 (69.2%)	4 (30.8%)	
BMD Z score				
Lumbar Spine	-0.8 (-1.2; -0.3)	-0.3 (-0.7; 0.2)	-1.2 (-1.4; -1.0)	0.0001
Femur	-0.5 (-1.0; 0.4)	-0.5 (-0.8; 0.8)	-1.0 (-1.3; -0.4)	0.003
BMD (g/cm ²)				
Lumbar Spine	1.086 (1.047;1.161)	1.156 (1.086;1.237)	1.048 (1.030;1.083)	0.0002
Femur	0.970 (0.909;1.048)	0.998 (0.945;1.127)	0.913 (0.856;0.999)	0.018
BMI (kg/m ²)	22.68 (20.98; 24.54)	22.62 (20.88; 24.45)	22.74 (21.21; 24.68)	1.000
CFTR Genotype				0.776
F508del homo.	11 (42.3%)	6 (46.2%)	5 (38.5%)	
F508del hetero.	10 (38.5%)	4 (30.8%)	6 (46.2%)	
no F508del	5 (19.2%)	3 (23.0%)	2 (15.4%)	
IVA/TEX/ELX treatm.	16 (61.5%)	6 (46.2%)	10 (77.0%)	0.226
Chronic Lung Infection	25 (96.2%)	13 (100%)	12 (92.3%)	1.000
Exocrine Panc. Insuf.	23 (88.5%)	12 (92.3.0%)	11 (84.6%)	1.000
CF related diabetes	6 (23.1%)	3 (23.1%)	3 (23.1%)	1.000
%FEV1	71.2 (41.6; 92.7)	71.6 (58.4; 94.4)	45.5 (37.2; 94.0)	0.448
%FVC	84.0 (66.7; 100.8)	84.8 (69.2; 99.8)	77.5 (61.4; 104.4)	0.697
%FEV1/FVC ratio	72.5 (58.6-78.3)	74.4 (62.8-78.3)	71.7 (54.0; 79.1)	0.479
Bisphos. treatment	3 (11.5%)	1 (0.0.8%)	2 (15.4%)	1.000
25-OHvit D (ng/mL)	22 (16; 32)	21 (16; 30)	29 (16;35)	0.256
Vit. D suppl.	21 (80.8%)	11 (64.4%)	10 (76.9%)	1.000
Calcium suppl.	6 (23.1%)	2 (15.4%)	4 (30.8%)	0.645
C Reactive protein	4.8 (0.5; 8.0)	5.9 (1.0; 12.0)	4.4 (0.4; 6.4)	0.269

Data are expressed as median with percentiles 25 and 75, respectively, in parentheses. BMI, body mass index; %FEV1, forced expiratory volume in 1 s % predicted; %FVC, forced vital capacity % predicted.

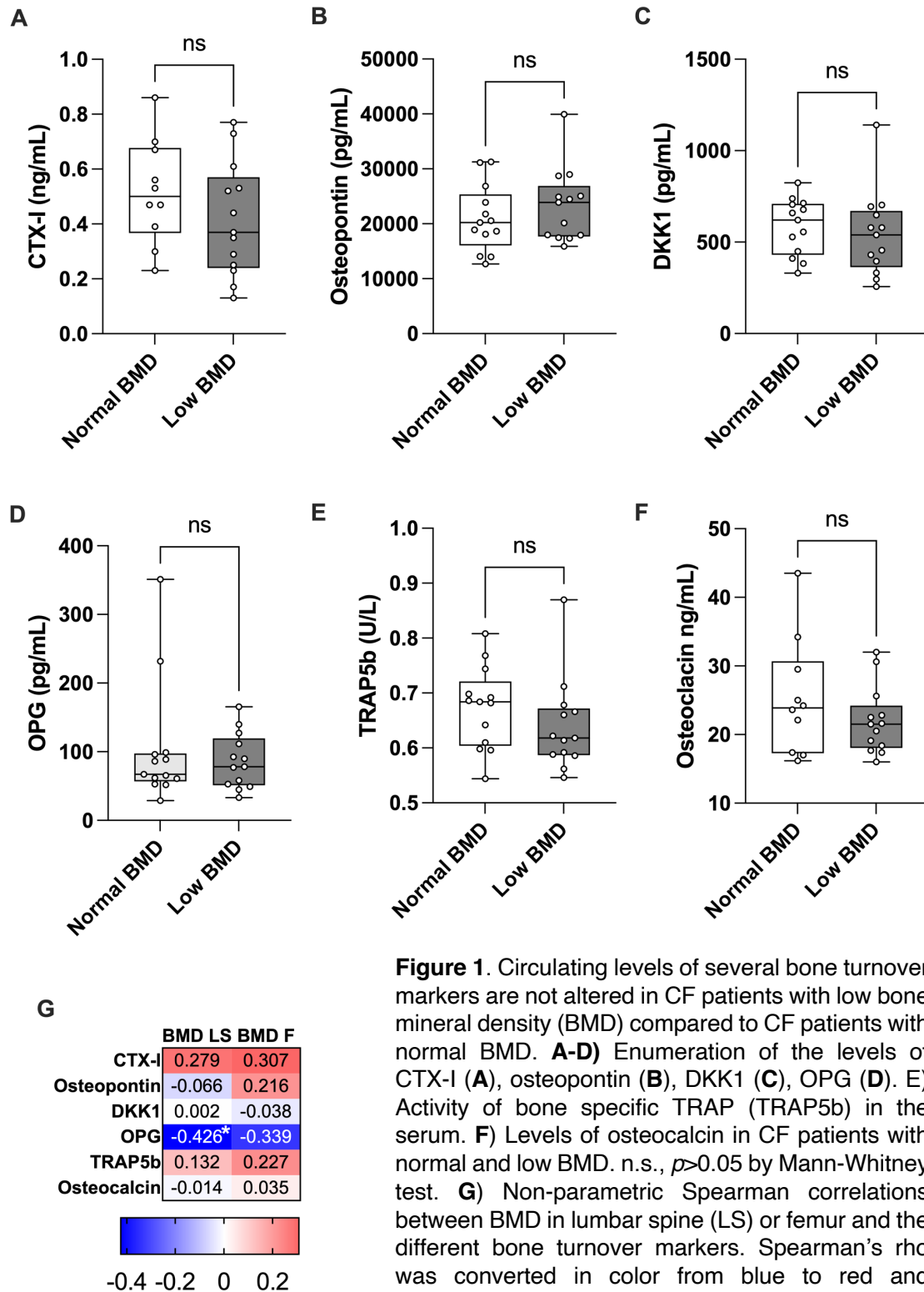


Figure 1. Circulating levels of several bone turnover markers are not altered in CF patients with low bone mineral density (BMD) compared to CF patients with normal BMD. **A-D)** Enumeration of the levels of CTX-I (**A**), osteopontin (**B**), DKK1 (**C**), OPG (**D**). **E)** Activity of bone specific TRAP (TRAP5b) in the serum. **F)** Levels of osteocalcin in CF patients with normal and low BMD. n.s., $p > 0.05$ by Mann-Whitney test. **G)** Non-parametric Spearman correlations between BMD in lumbar spine (LS) or femur and the different bone turnover markers. Spearman's rho was converted in color from blue to red and significance within positive or negative correlations were shown as *, $p < 0.05$.

3.3. Increased circulating levels of SAA in CF patients are associated with poor lung function

Our previous work in animal models of infection showed that SAA proteins may play an important role in bone homeostasis and serve as markers of bone loss. We thus decided to investigate their relevance in CF patients. First, we compared the circulating levels of SAA between healthy controls and CF patients and found that CF patients have increased amounts of SAA (**Figure 2A**). To test whether systemic SAA levels were associated with low bone mass, we compared the levels of SAA in CF patients with normal and low BMD. The median levels of SAA did not differ between the two groups of patients (**Figure 2B**). Then, to gain some insight on the association of SAA with bone health of CF patients, we performed association tests between SAA and other specific parameters (**Figure 2C**). We failed to detect a correlation between SAA and BMD (both in the LS and femur). However, the circulating levels of SAA strongly, and negatively correlated with %FEV1. On the other hand, there was a strong and positive association between SAA and the amounts of C reactive protein, granulocyte-colony stimulating factor (G-CSF), interleukin (IL) 6, and OPG in circulation, indicating that inflammation and poor lung function correlate with increased levels of SAA in CF patients.

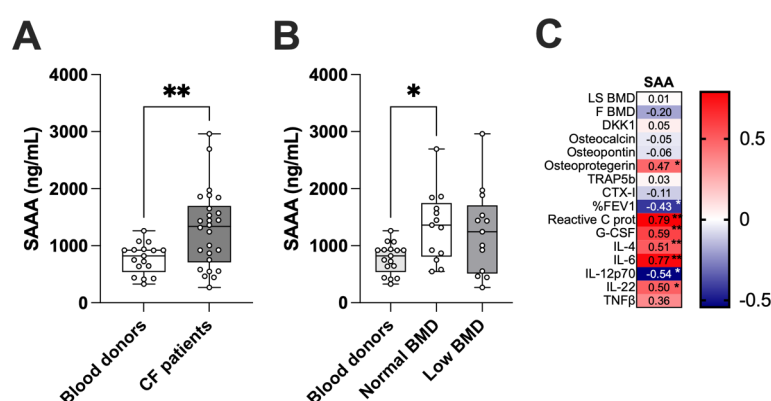


Figure 2. CF patients have higher levels of SAA in circulation, **A**) Quantification of SAA levels in the plasma of CF patients compared to blood donors. ** $p < 0.001$ by Mann-Whitney test. **B**) Circulating SAA levels in blood donors and CF patients with normal or low BMD. *, $p < 0.05$ by Kruskal-Wallis test. **C**) Non-parametric Spearman correlations between circulating levels of SAA and bone mineral density (BMD) in lumbar spine (LS) or femur (F), different bone turnover markers and cytokines. Spearman's rho was converted in color from blue to red and significance within positive or negative correlations were shown as *, $p < 0.05$, ** $p < 0.001$.

3.4. Bone Mineral Density in CF patients correlates with M-CSF, $TNF\alpha$ and MCP-3

To gain some insight in the mechanisms of bone mass dysregulation in CF patients, several cytokines and chemokines related with bone metabolism were measured in the plasma of CF patients (**Table 2**). IL1 β and IL2 were significantly decreased in the patients with low BMD. Of note, the median levels of macrophage-colony stimulating factor (M-CSF) were 5.6 times increased in CF patients with low BMD.

Then, we performed association tests between LS and femur BMD, circulating levels of bone turnover markers, immune mediators, and clinical parameters (**Figure 3**). BMD in LS and femur strongly and significantly associated with each other. Amongst the measured bone turnover markers, LS BMD negatively correlated with OPG. Furthermore, lung function as measured by %FEV1 also positively and significantly associated with BMD but no relationship was found between BMD and 25-hydroxyvitamin D or age. A moderate association was found between LS BMD and BMI. Regarding the measured cytokines/chemokines, moderate negative correlations were found between BMD and CX3CL1, CXCL1, granulocyte/macrophage-colony stimulating factor (GM-CSF), M-CSF, IL8, IL13, macrophage inflammatory protein (MIP) 1a, tumor necrosis factor ($TNF\alpha$). Strong negative correlation was found between LS BMD and monocyte chemotactic protein (MCP) 3, which was recently involved in the recruitment of osteoclast progenitors [16]. Of note, strong positive associations were found between BMD and CCL22, IL1 α , IL1 β .

Table 2.
Levels of circulating cytokines/chemokines in CF patients

pg/mL	All CF patients N=26	Normal BMD N=13 (50%)	Low BMD N=13 (50%)	p value
sCD40L	2114 (1123; 3420)	2871 (1803; 3420)	1947 (1123; 4084)	0.687
EGF	157.2 (105.2; 229.2)	164.2 (118.2; 222.8)	130.7 (112.2; 232.4)	0.687
Eotaxin	38.1 (32.9; 53.6)	34.7 (31.2; 46.5)	47.1 (34.7; 57.6)	0.186
FGF-2	212.4 (154.0; 265.0)	226.8 (186.6; 312.3)	171.1 (141.7; 291.2)	0.186
FLT3L	14.24 (9.12; 17.27)	13.8 (8.8; 16.4)	14.7 (9.7; 19.2)	0.448
CX3CL1	208.3 (187.9; 227.1)	202.6 (168.8; 224.5)	213.8 (202.6; 298.9)	0.113
G-CSF	73.3 (60.8; 99.7)	94.0 (67.1; 115.7)	70.2 (57.6; 106.6)	0.204
GM-CSF	172.9 (66.21; 270.1)	146.5 (69.4; 196.1)	216.1 (96.6; 276.9)	0.186
CXCL1	37.9 (22.8; 43.8)	36.6 (25.0; 40.0)	42.7 (22.8; 55.4)	0.390
IFN α 2	194.0 (151.2; 233.0)	194.0 (157.8; 253.5)	182.5 (126.4; 228.3)	0.503
IFN γ	2.20 (1.58; 3.51)	2.79 (1.77; 3.99)	1.96 (1.16; 2.56)	0.186
IL1 α	166.5 (136.5; 235.8)	219.7 (131.9; 358.6)	148.5 (127.4; 213.8)	0.223
IL1 β	22.9 (17.3; 28.0)	24.8 (21.1; 43.6)	17.3 (15.4; 21.1)	0.005
IL1RA	13.3 (9.6; 17.5)	13.9 (10.3-18.5)	13.0 (8.2; 14.0)	0.243
IL2	4.01 (3.52; 4.62)	4.21 (3.79; 4.89)	3.66 (2.88; 4.14)	0.022
IL3	3.02 (2.36; 3.84)	3.02 (2.36; 4.17)	3.68 (2.52; 4.01)	0.724
IL4	3.28 (2.24; 4.40)	3.51 (2.67; 5.03)	3.62 (2.22; 4.18)	0.287
IL5	5.75 (3.06; 7.75)	3.21 (2.63; 6.22)	6.51 (4.69; 7.75)	0.064
IL6	1.85 (0.83; 3.18)	2.64 (1.24; 4.33)	2.36 (0.45; 3.90)	0.650
IL7	4.56 (3.16; 6.96)	4.56 (1.07; 10.04)	4.74 (3.96; 8.16)	0.418
IL8	4.52 (3.50; 6.64)	4.52 (3.46; 7.13)	5.82 (3.31; 6.81)	0.920
IL9	20.7 (13.9; 23.8)	20.7 (13.9; 23.6)	15.3 (0.0; 22.9)	0.448
IL10	3.77 (2.52; 6.94)	3.42 (1.89; 5.54)	5.63 (3.42; 7.44)	0.264
IL12p40	94.1 (81.0; 110.0)	94.1 (82.4; 123.1)	94.1 (78.0; 104.2)	0.614
IL12p70	17.5 (14.9; 21.2)	17.5 (16.2; 20.3)	16.7 (13.3; 20.6)	0.511
IL13	185.6 (143.8; 205.9)	177.1 (139.0; 209.8)	185.6 (140.8; 207.9)	0.579
IL15	0.00 (0.00; 0.89)	0.00 (0.00; 1.76)	0.00 (0.00; 0.66)	0.511
IL17A	14.5 (12.8; 19.6)	17.0 (12.8; 21.8)	13.7 (11.9; 14.9)	0.064
IL17E/IL25	1321 (922; 1873)	1151 (886.3; 1823)	1410 (1193; 1822)	0.390
IL17F	0.00 (0.00; 34.0)	0.00 (0.00; 43.93)	0.00 (0.00; 12.27)	0.362
IL-18	283.4 (154.6; 207.2)	275.8 (239.4; 375.9)	313.9 (247.7; 380.9)	0.545
IL22	185.0 (154.6; 207.2)	183.7 (149.5; 205.9)	176.7 (145.8; 212.4)	1.000
IL27	658.5 (570.3; 821.4)	778.3 (562.0; 939.8)	653.8 (579.7; 804.0)	0.511
CXCL10	76.0 (52.8; 106.1)	70.7 (50.3; 131.6)	79.6 (57.8; 94.0)	1.000
MCP-1	329.2 (253.0; 460.3)	262.7 (185.1; 467.6)	329.2 (301.4; 432.1)	0.264
MCP-3	30.9 (0.0; 37.7)	29.5 (25.4; 32.6)	35.0 (27.9; 41.2)	0.101
M-CSF	30.4 (0.0; 78.1)	7.99 (0.00; 66.2)	44.5 (18.1; 97.2)	0.081
CCL22	691.8 (442.8; 904.5)	745.0 (529.3; 904.5)	648.8 (440.6; 807.8)	0.362
CXCL9	2014 (1236; 2716)	2239 (1152; 3065)	2233 (1273; 2812)	0.960
MIP-1 α	82.1 (73.8; 94.4)	78.1 (67.9; 87.6)	84.0 (76.0; 92.8)	0.113
MIP-1 β	34.9 (27.0; 43.1)	34.9 (32.6; 40.3)	34.5 (27.0; 51.8)	0.840
PDGF-AA	249.7 (189.6; 403.7)	396.3 (201.9; 469.6)	249.7 (194.5; 399.3)	0.390
PDGF-AB/BB	47349 (31519; 80648)	65874 (41668; 86562)	47349 (31021; 91352)	0.724
RANTES	69138 (44870; 100486)	87113 (38597; 102780)	63292 (44870; 91398)	0.511
TGF α	2.65 (1.05; 7.80)	1.91 (0.91; 4.84)	2.06 (1.26; 4.74)	0.687
TNF α	65.0 (50.2; 78.8)	58.7 (53.6; 72.0)	67.7 (54.8; 79.7)	0.287
TNF β	6.27 (0.80; 11.12)	7.06 (2.14; 10.20)	4.58 (0.00; 13.45)	0.852
VEGF-A	131.3 (90.9; 156.9)	144.9 (90.9; 193.6)	120.1 (101.2; 136.6)	0.186

Data are expressed as median with percentiles 25 and 75, respectively, in parentheses.

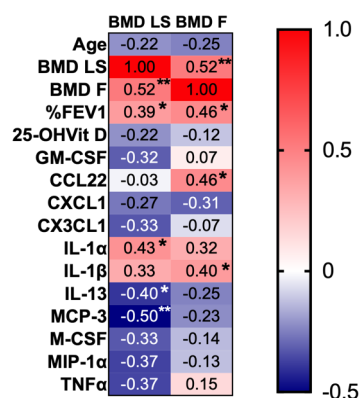


Figure 3. Non-parametric Spearman correlations between circulating levels of SAA and bone mineral density (BMD) in lumbar spine (LS) or femur (F) and cytokines/chemokines. Spearman's rho was converted in color from blue to red and significance within positive or negative correlations were shown as *, $p < 0.05$, ** $p < 0.001$.

4. Discussion

In this pilot study, we aimed to find new circulating markers to predict bone loss in adults CF patients. Overall, we found that the systemic levels of commonly used bone turnover markers did not significantly differ between CF patients with low and normal BMD, indicating that these markers are not useful to monitor bone mass in these patients. The serum marker with the strongest correlation with BMD was OPG, which was higher in patients with low BMD. In pediatric CF patients, OPG was reduced compared to healthy controls [17]. These results may be explained by CFTR defects in osteoblasts. These cells in patients with the delF508 mutation in CFTR produced reduced amounts of OPG [18-20]. Decreased expression of OPG leads to a higher amount of RANKL (an osteoclast activating and bone catabolic cytokine) available to induce osteoclastogenesis and bone loss. Interestingly, in vitro studies restoring CFTR function with CFTR modulators in osteoblasts reduced the ratio of RANKL/OPG [20]. The role of OPG in bone homeostasis and its potential as bone turnover marker deserves further investigation.

Our previous work showed an association between SAA and bone loss (Annex IV). In the search for better tools to monitor bone mass in CF patients, we hypothesized that SAA, produced in response to the ongoing chronic inflammation observed in these patients, is such marker. Compared to healthy controls, CF patients had increased levels of circulating SAA, which agrees with previous studies [12-14]. However, we didn't find significant differences in SAA levels between patients with low or normal BMD. Our results indicate a strong and negative correlation between SAA and lung function and a positive and strong correlation with C reactive protein, G-CSF, and IL6 levels, which are known pro-inflammatory mediators. Even though the amount of SAA in circulation did not differ between CF patients with normal and low BMD, it will be useful to understand the impact of elevated levels of SAA in these patients and its relationship with bone health. Interestingly, in our cohort of CF patients, there was

a positive and strong association between SAA and OPG. Besides the previously reported intrinsic defect in OPG production of osteoblast of CF patients with F508del-CFTR, it would be expected that SAA signaling in osteoblast progenitors may further reduce new bone formation and OPG synthesis. Future studies are required to understand the impact of SAA on OPG production in CF patients.

To further characterize the alterations in the inflammatory status that could contribute to bone loss in CF patients, we measured a large panel of cytokines in the sera of our cohort.

IL1 β and IL2 were significantly reduced in CF patients with low bone mass compared to those with normal bone mass. Furthermore, IL1 β positively correlated with BMD in the LS. These results are unexpected as IL1 β has been shown to be involved in CF lung disease severity, response to chronic infection and inflammatory bone loss [21-23]. Furthermore, IL1 β has been well-described as a bone catabolic cytokine [24-26]. However, most studies focus on the local production of IL1 β using sputum or bronchoalveolar lavage fluid from CF patients or even animal models. One recent study found that compared to monocytes from healthy controls, monocytes of CF patients downregulated the expression of IL1 β and IL1 α in response to *Pseudomonas aeruginosa* infection [27]. One could speculate that the reduced systemic levels of IL1 β in patients with low BMD is due to a protective mechanism to maintain bone mass. These reduced IL1 β levels may also protect for other CF related comorbidities as higher IL1 β levels were found in the pancreas of CF patients [28]. Therefore, to further understand the systemic influence of inflammation in bone disease it would be important to obtain the transcriptome of peripheral blood mononuclear cells from CF patients with normal and reduced bone mass to understand whether circulating leukocytes had different signatures and how those correlates with bone metabolism and immune function.

Although not statistically significant, the median levels of M-CSF in patients with low BMD were 5.6x higher than in patients with normal BMD. M-CSF is a bone catabolic cytokine that together with TNF α , activates bone resorption [29, 30]. Moreover, we observed that M-CSF and TNF α , which induce osteoclast formation and activity, negatively correlated with BMD in CF patients. Furthermore, TNF α stimulated bone loss was reverted by treatment with CFTR modulators [31, 32]. Based on the insightful observations that CFTR modulators improve osteoblast maturation and bone mineralization [20, 32, 33] and reduce inflammatory response [34, 35], it will be fundamental to understand the impact of CFTR-modulators on CFBD and their potential to ameliorate and/or prevent bone loss in CF patients. The current therapeutic tools available for CFBD are the preventive vitamin D and calcium supplementation, and maintenance of lung health and nutrition [36]. Yet, without evidence of benefit in clinical trials [37]. Pharmacological intervention with bisphosphonates or denosumab should be considered on an individual basis [38].

Overall, our results suggest that the BMD in CF patients negatively correlates with higher levels of bone catabolic cytokines. Previous studies have shown that during periods of infective exacerbations, osteoclast activity and bone resorption was increased (as discussed in the review article in Annex III). Our preliminary data suggest that outside infective exacerbations, there is also a correlation between increasing circulating levels of bone catabolic cytokines and reduced bone mass.

One major caveat of our study is the reduced number of CF patients enrolled. Additionally, we did not study individuals with established CFBD, which is defined by a BMD Z-score of -2.0 or lower [3]. To overcome this, we aim to increase the number of observations, by extending our study to other CF reference centers and to pediatric ages. Our findings rely mostly on statistical correlations between the systemic levels of immune mediators and BMD. Being aware of the key role of myeloid cells in both CF and bone disease, we are currently studying whether deregulated monocyte/macrophage's response to infecting bacteria in CF patients rewires osteoclastogenesis, leading to reduced bone mass. These studies, which were funded by a Pilot Research Grant from the CF Foundation, will be important to complement the observations presented in this manuscript and will enhance our understanding of the mechanisms underlying osteopenia in CF patients. This way, we will contribute to the identification of a biomarker to detect those at a higher risk of developing reduced bone mass, allowing an earlier detection and intervention. In the long run, we will also contribute to the development of new preventive and therapeutic strategies to control this pathology.

5. Conclusion

This pilot study shows that the persistent inflammatory status of CF patients correlates with decreased bone mass. Furthermore, we identified potential candidates to monitor bone loss in these patients, laying the ground for future investigations on the mechanisms that modulate the impact of these immune mediators on bone metabolism.

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Annex I

STROB Statement

STROBE Statement—Checklist of items that should be included in reports of *cross-sectional studies*

Checklist of items that should be included in reports of cross-sectional studies			
	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	2
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	3, 4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	3
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants	3
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	3, 4
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	3, 4
Bias	9	Describe any efforts to address potential sources of bias	3, 4
Study size	10	Explain how the study size was arrived at	3
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	4
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	4
		(b) Describe any methods used to examine subgroups and interactions	4
		(c) Explain how missing data were addressed	4
		(d) If applicable, describe analytical methods taking account of sampling strategy	NA
		(e) Describe any sensitivity analyses	NA, no sensitivity analyses were performed
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	3, 4

		(b) Give reasons for non-participation at each stage	NA, because no participants were excluded
		(c) Consider use of a flow diagram	4
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4, 5
		(b) Indicate number of participants with missing data for each variable of interest	NA because all the participants had the variables of interest measured
Outcome data	15*	Report numbers of outcome events or summary measures	5
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	4 to 9
		(b) Report category boundaries when continuous variables were categorized	4 and 5
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA because no estimates of risk were involved in the study
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	4
Discussion			
Key results	18	Summarise key results with reference to study objectives	9, 10
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	9
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9, 10
Generalisability	21	Discuss the generalisability (external validity) of the study results	9, 10
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	11

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

Annex II

Journal of Cystic Fibrosis author's guidelines



JOURNAL OF CYSTIC FIBROSIS

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AUTHOR INFORMATION PACK

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DESCRIPTION

The *Journal of Cystic Fibrosis* is the official journal of the [European Cystic Fibrosis Society](#). The journal is devoted to promoting the **research** and **treatment** of **cystic fibrosis**. To this end the journal publishes original scientific articles, editorials, case reports, short communications and other information relevant to cystic fibrosis. The journal also publishes news and articles concerning the activities and policies of the ECFS as well as those of other societies related the ECFS.

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Annex III

Cystic Fibrosis Bone Disease: more than the dysregulation in the CFTR function

Manuscript published in *Biomolecules*

Review

Cystic Fibrosis Bone Disease: The Interplay between CFTR Dysfunction and Chronic Inflammation

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Abstract: Cystic fibrosis is a monogenic disease with a multisystemic phenotype, ranging from pre-disposition to chronic lung infection and inflammation to reduced bone mass. The exact mechanisms unbalancing the maintenance of an optimal bone mass in cystic fibrosis patients remain unknown. Multiple factors may contribute to severe bone mass reduction that, in turn, have devastating consequences in the patients' quality of life and longevity. Here, we will review the existing evidence linking the CFTR dysfunction and cell-intrinsic bone defects. Additionally, we will also address how the proinflammatory environment due to CFTR dysfunction in immune cells and chronic infection impairs the maintenance of an adequate bone mass in CF patients.

Keywords: cystic fibrosis related bone disease; osteoporosis; CFTR; bone

1. Introduction

Cystic fibrosis is a monogenetic autosomal recessive disorder with systemic and heterogeneous involvement [1]. The disease was first identified in 1938 [2]; then, the median life expectancy was a few months [3]. However, the implementation of newborn screening programs, the creation of reference centers to follow and treat cystic fibrosis patients, the elaboration of protocols to treat and eradicate chronic infection, and the improvement in the nutritional support, amongst other factors, increased the lifespan of cystic fibrosis patients to ~50 years old [1]. The improved longevity of cystic fibrosis patients creates new challenges, namely the understanding and treatment of several comorbidities such as cystic fibrosis bone disease [4].

Cystic fibrosis is most common in Northern Europe descendants but has been diagnosed worldwide [5], with increasing incidence in Asia and Africa [6,7]. According to the most recent patients registry reports, there are 52,246 patients in Europe and 31,411 in North America [8,9]. The disease is caused by mutations in the cystic fibrosis transmembrane conductance receptor (CFTR) [1,10,11], an ATP-binding cassette that also functions as a chloride and bicarbonate channel [12,13]. To date, over 2000 CFTR variations have been identified, but most of them are either very rare or have uncertain clinical significance [14,15]. The most frequent disease-causing mutation in European cystic fibrosis patients is the Phe508del (F508del). Individuals of other ethnicities are less likely to have this mutation, which has important therapeutic implications [16]. Mutations in CFTR with clinical phenotypes have a wide range of functional consequences and conceptually have been categorized in six classes (Figure 1). Whilst the severity of the clinical presentation correlates with the severity of the functional defect in CFTR (ranging from folding errors to complete lack of CFTR), several mutations associate with several functional deficiencies, exhibiting features of more than one mutation class [1,15]. Besides the particular CFTR mutation, the presence of different modifier genes and complex alleles in the same individual may also account for the differences in the disease severity and clinical manifestations [10].



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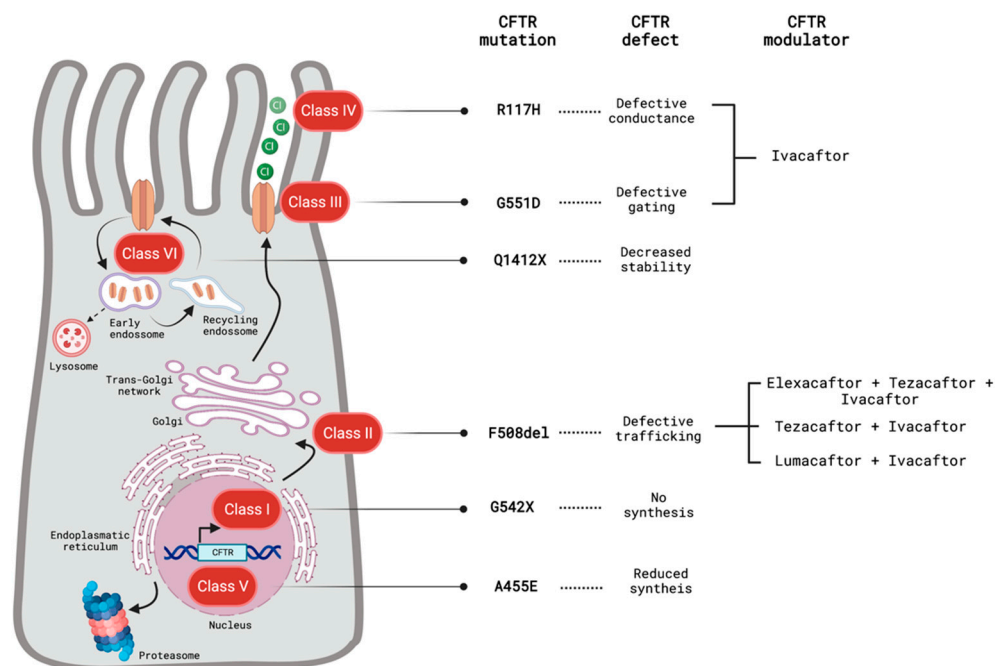


Figure 1. Classification and effects of CFTR mutations and approved CFTR function-restoring pharmacological interventions. Class I mutations result in the premature termination of CFTR transcription and in the absence of a functional CFTR. Class II mutations lead to the misfolding of CFTR protein and aberrant trafficking of the protein. Class III mutations are gating mutations causing ineffective chloride/bicarbonate transport. Mutations belonging to class IV decrease the conduction of ions through the channel. Class III and IV mutations lead to normal levels of CFTR expressed at the cell surface but a reduced CFTR function. Splicing and missense mutations belong to class V and cause reduced CFTR synthesis. Class VI mutations reduce CFTR stability of the cell membrane due to increased CFTR recycling. Thus far, the CFTR modulators in the clinic target class II, class III and IV mutations. Image created using Biorender.com (www.biorender.com; access on 22 November 2022).

In the lung and upper airways, dysfunction of CFTR leads to the formation of thick mucopurulent secretions and impaired mucociliary clearance, predisposing individuals to the establishment of chronic lung infections, mostly by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [17]. The vicious cycle of infection and inflammation causes structural lung disease, such as the development of bronchiectasis, and in the latter and more severe stages of disease, respiratory failure [18]. The implications of chronic inflammation/infection in bone homeostasis will be discussed in Section 5.1.

Besides the effects on respiratory function, cystic fibrosis presents with endocrine alterations such as exocrine pancreatic insufficiency and cystic fibrosis-related diabetes. In pancreatic ducts, CFTR secretes chloride and bicarbonate, alkalinizing the ductal fluid, neutralizing peptic acid and optimizing pH for enzymatic digestion [19]. In the absence of CFTR function, ductal obstruction and epithelial damage, inflammation, fibrosis and fatty infiltration lead to an increase in pancreatic destruction [20]. In the most severe CFTR mutations, namely class I–IV and VI, these alterations begin in the uterus. Thus, pancreatic exocrine disease is a condition present at birth in almost all cystic fibrosis newborns. The small percentage of babies with cystic fibrosis that are pancreatic sufficient transition to insufficiency over time [20]. Pancreatic exocrine insufficiency is associated with malabsorption and liposoluble vitamin deficiency. Thus, lifelong pancreatic enzyme replacement and vitamins A, D, E and K supplementations are implemented in cystic fibrosis individuals with pancreatic exocrine disease [20]. The deficiency in vitamin D and its relationship with low bone mass is discussed below. Overtime, the pancreatic destruction leads to the loss of islet cells and the development of insulin deficiency and glucose intolerance. This condition is referred to as cystic fibrosis-related diabetes, and

shares features with type 1 and type 2 diabetes mellitus [21]). The development of cystic fibrosis-related diabetes and insufficient insulin production might be relevant for reduced bone mass and will be discussed in Section 5.2.

The sequencing of CFTR in 1989 [22] was a turning point for the understanding of the disease pathophysiology, and for the search of a potential full-cure therapy. Gene therapy and heterologous or gene-corrected autologous stem cell transplantation have been proposed but with little clinical success [23,24]. Yet, a dramatic change in the therapeutical approach and in the hope of a full cure came with the development of CFTR modulators. These enclose several classes of small molecules that bind to CFTR, enhancing or even restoring the function of specific cystic fibrosis-causing mutants [25–30]. CFTR modulators were fast tracked for drug development and regulatory approval, with a timeframe of six years since the ivacaftor discovery and approval for treatment [26]. The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have thus far licensed four products: ivacaftor (Kalydeco[®], Boston, MA, USA) in monotherapy; the combination of ivacaftor with lumacaftor (Orkambi[®], Boston, MA, USA) or with texacaftor (Symdeko[®], Boston, MA, USA; Symkevi[®], Dublin, Ireland); and the triple combination of ivacaftor with elexacaftor and tezacaftor (Trikafta[®], Boston, MA, USA; Kaftrio[®], Dublin, Ireland) [31]. These therapeutical options have improved the respiratory function and nutritional status of cystic fibrosis patients, overall enhancing quality of life without major safety concerns [32,33]. Of note, a recent study followed thirteen cystic fibrosis patients on ivacaftor/lumacaftor treatment and eight patients on ivacaftor/tezacaftor treatment and found that the circulating levels of pro-inflammatory cytokines such as TNF α decreased significantly with the treatment, indicating that combinations of CFTR modulators have potent anti-inflammatory properties [34]. Furthermore, some studies have demonstrated that ivacaftor enhanced the antimicrobial capacity of immune cells in cystic fibrosis patients treated with this CFTR modulator [35–37]). The understanding of how CFTR modulator therapy impacts extrapulmonary manifestations of cystic fibrosis will have the upmost importance for better understanding the pathophysiology of these manifestations and the search for directed and effective therapeutics against them [38]. The potential impact of CFTR modulators in cystic fibrosis-related bone disease will be discussed in Section 6.

2. Cystic Fibrosis-Related Bone Disease

Cystic fibrosis bone disease, accompanied by a severe bone mass reduction, is a common but not well understood complication in these patients, with devastating consequences to their quality of life and longevity. Bone loss in cystic fibrosis is multifactorial, and not very well understood. Indeed, the genetically determined CFTR malfunction coupled with the pro-inflammatory status, steroid therapy, amongst others factors, may all contribute to the dysregulation in the bone tissue (Figure 2).

The first report of reduced bone mass in patients with cystic fibrosis was in 1979 [39]. From then on, the prevalence of low bone mass in several cohorts of cystic fibrosis patients has been thoroughly studied, ranging from 20 to 35% [40–45]. Yet, the pathophysiology of the disease continues to be mostly unknown. Despite improved clinical management in terms of pulmonary function and vitamin D status, a significant and constant number of cystic fibrosis patients have reduced mineral density [46], emphasizing the importance of dedicated cystic fibrosis bone disease management.

Physical activity, good exercise tolerance and nutritional status correlate with increased bone mineral density [47,48]. Early studies associated suboptimal vitamin D levels with cystic fibrosis bone disease [39,49], namely in individuals with end-stage lung disease [50]. However, the correction of vitamin D did not improve abnormalities in calcium, bone turnover markers [51], and bone mineral density [52]. Moreover, cystic fibrosis patients that underwent a lung transplant remained at risk of skeletal fragility [53], perhaps due to prolonged treatment with oral corticosteroids in the post-transplantation period.

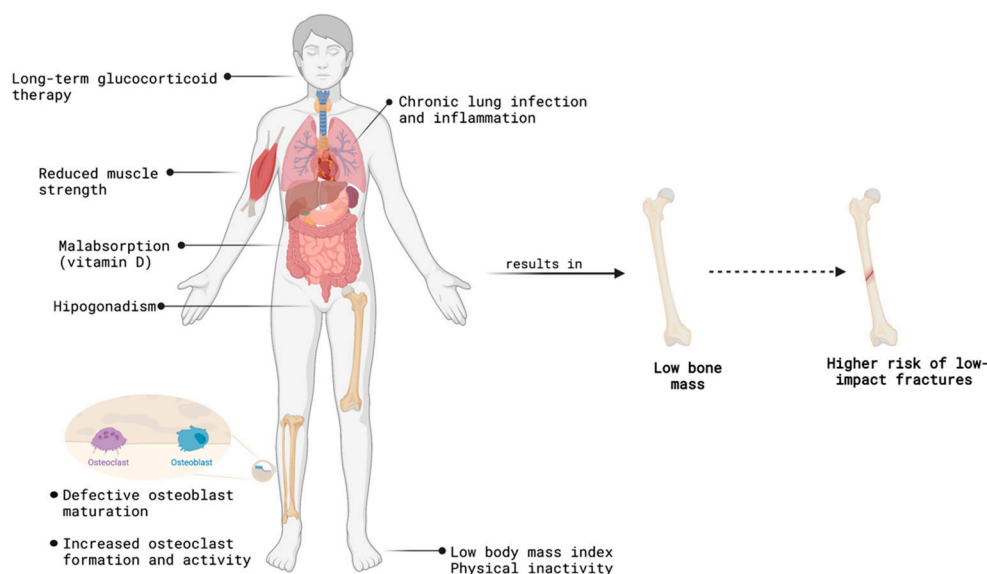


Figure 2. Cystic fibrosis bone disease is a multifactorial disease due to chronic organ involvement, the direct impact of CFTR dysfunction and long-term therapeutical approaches. Scheme was created using Biorender.com (www.biorender.com; access on 22 November 2022).

Age and Bone Mass in Cystic Fibrosis

Cystic fibrosis bone disease has been mostly diagnosed in adult patients, but several studies indicate that bone defects appear early in life. Indeed, children with cystic fibrosis already have bone alterations [54–58] which increase with age [59]. The progressive bone loss [48] in cystic fibrosis patients correlates with reduced lung function [60–62], recurrent pulmonary exacerbations [63], inflammation [64] and reduced muscle strength [65]. Yet, these correlations and a reduced bone mineral density are not always identified in all cystic fibrosis populations [45], suggesting that other factors may also play a role in the development of bone loss in these patients [66]. Importantly, the development of osteoporosis in cystic fibrosis patients occurs with a deteriorating clinical status [67].

Cystic fibrosis bone disease predisposes individuals to low-impact fractures and skeletal abnormalities [68,69], decreasing the patients' quality of life and even increasing the probability of a fatal outcome [70]. Moreover, cystic fibrosis bone disease is in some centers a counterindication for lung transplantation [71].

3. Cystic Fibrosis-Induced Alterations in Bone Architecture and Turnover

Bone is a specialized type of connective tissue that is responsible for the locomotion and protection of noble organs such as the brain, heart and lungs. Moreover, the skeletal system also functions as a mineral and hormone reservoir. Skeletogenesis starts during embryonic life through intramembranous or endochondral ossification (as reviewed in [72]). After birth and throughout life, bone mass and shape are regenerated due to bone modeling and remodeling. Throughout growth and aging, the bone shape adapts to variable mechanical demands through bone resorption and formation on opposing cortical and trabecular surfaces. This process refers to bone modeling, whereas the replacement of old or damaged bone by new bone in the same surface is called bone remodeling [73]. Bone remodeling or turnover is essential for an adequate maintenance of the bone mass throughout life and depends on a tight balance between the activity of bone-resorbing osteoclasts and bone-forming osteoblasts [74]. The imbalance in bone turnover is a natural consequence of aging, but it is often aggravated by several pathological conditions, namely those with an immunological etiology, such as HIV infection and rheumatoid arthritis [75]. Altered bone turnover may cause alterations in the bone architecture and strength, leading to bone microdamage that will be later translated into bone fragility [76]. Of note, reduced bone mineral density is not always the functional readout of an altered bone turnover.

Cystic fibrosis bone disease is not restricted to a reduced bone mineral density. Structure and strength deficits in the bone of cystic fibrosis patients have been reported, namely at the cortical and trabecular levels [77], independently of the body mass index and size [78]. The bones of patients with cystic fibrosis with end-stage lung disease show reduced mass, altered microarchitecture, imbalanced remodeling and increased microdamage [79]. A histomorphometry analysis of the iliac bone of cystic fibrosis adults demonstrated an altered bone turnover with reduced mineral apposition, indicating reduced osteoblast activity [80]. Another study relying on histomorphometry analysis of postmortem vertebral bodies biopsy specimens demonstrated severe cortical and trabecular osteopenia with reduced osteoblasts and increased osteoclasts numbers [81]. This phenotype was also observed in mice deficient in CFTR [82]. Along these lines, an altered bone turnover with increased bone resorption and decreased new bone formation has been detected in patients with normal nutritional status and without acute pulmonary disease [83]. Indeed, reduced levels of osteocalcin (a biomarker of osteoblast activity) were found in cystic fibrosis patients with vertebral fractures [84]. In another study, besides reduced osteocalcin levels, an increased receptor activator of the nuclear factor kappa-B ligand (RANKL)/Osteoprotegerin (OPG) ratio was found [54]. In bone metabolism, RANKL is a cytokine produced mostly by osteoblasts that binds to its receptor RANK expressed at the surface of osteoclasts and their precursors, thus inducing osteoclast formation and activity. Simultaneously, osteoblasts produce OPG, a decoy receptor for RANKL to control osteoclast differentiation and activity. An increased RANKL/OPG ratio indicates that an increased amount of RANKL is available for osteoclasts and consequently bone resorption is potentiated. Furthermore, the observations of reduced bone mineral density in young cystic fibrosis children with mild disease and normal nutritional status [85], as well as a reduction to half of the bone density in cystic fibrosis individuals compared to their healthy counterparts during childhood and puberty [86], point out that cystic fibrosis bone disease results from a primary defect in bone homeostasis. Furthermore, the dysregulation in bone homeostasis may be worsened by several periods of increased bone turnover and resorption during infective exacerbations [87].

4. CFTR Dysfunction in Bone Cells and Their Progenitors

CFTR is expressed by several cells in the organism, namely by hematopoietic progenitors in the bone marrow and osteoclasts, as well as osteoblasts, osteocytes and chondrocytes and their progenitors [88–91]. Therefore, altered CFTR function may compromise osteoblast and osteoclast development and bone remodeling. The development of mouse models with *Cftr* deletion or with the F508del mutation have been fundamental for experimentally studying the role of CFTR in bone biology without confounders such as chronic lung infection and/or chronic malnutrition [82,92–96]. Furthermore, there is a correlation between the F508del mutation and low bone mass in mice [94,97], in agreement with previous observations in humans [92,98,99]. The absence of CFTR in newborn pigs led to an altered bone microstructure and chemical composition [100], and alterations in bone mineral density and architecture were found in *Cftr*-deficient mice and rats in the absence of other overt disease symptoms [93,101].

4.1. CFTR Dysfunction on Osteoblasts and Bone Formation

Osteoblasts are responsible for the secretion of the organic matrix composing the bone. These cells have a mesenchymal origin and mature into osteocytes when they become entrapped in the bone matrix. *Cftr* deletion hampers osteoblast differentiation [82,92–94]. Osteoblasts carrying the F508del mutation in CFTR exhibited a reduced expression of pro-osteoblastogenic factors such as the mothers against decapentaplegic homolog 2 (SMAD2), cyclooxygenase-2 (COX-2) as well as of OPG, but produced increased amounts of RANKL compared with osteoblasts with a fully functional CFTR [94,102,103]. These results indicate a possible defect in the activation of the Wnt/ β -catenin signaling pathway that is fundamental for osteoblast maturation. Murine osteoblasts with F508del-CFTR demonstrated

overactive nuclear factor kappa B (NF- κ B) transcriptional activity, resulting in increased β -catenin phosphorylation and reduced β -catenin expression, as well as an altered expression of Wnt/ β -catenin target genes [104]. Besides the Wnt/ β -catenin signaling pathway, Wnt3a and parathyroid hormone-stimulated canonical Wnt signaling was also defective in mice lacking CFTR [96], as the lack of direct interaction between CFTR and dishevelled (Dsh) proteins prevents the stabilization of Dsh and its further interaction with Dpr1 [105,106]. Reduced Wnt/ β -catenin signaling in F508del-CFTR osteoblasts was corrected via genetic or pharmacologic targeting of Keratin 8 [107], reinforcing that cystic fibrosis severity and associated comorbidities depend on the presence of different modifier genes and complex alleles [10]. Besides these molecular alterations, osteoblasts expressing F508del-CFTR secreted lower amounts of prostaglandin E₂ (PGE₂) and OPG [102,108]. Likewise, in lung cells, the absence of a functional CFTR chloride channel activity reduced the production of PGE₂ [109]. PGE₂ modulates Wnt/ β -catenin signaling and induces osteoblast differentiation, maturation and activity [110–112]. Therefore, the reduced levels of PGE₂ may account for the reduced bone formation. Besides the decreased OPG production, Tumor Necrosis Factor α (TNF α) stimulation and pharmacological inhibition of CFTR function together in osteoblasts in vitro decreased interleukin (IL) 8 secretion [108]. Higher circulating levels of IL-8 correlate with decreased femoral bone mineral density [113]. Additionally, TNF α and IL-17 further stimulate the production of RANKL via osteoblasts from cystic fibrosis patients [114]. RANKL is crucial for osteoclasts survival and differentiation [74].

Overall, CFTR dysfunction leads to decreased osteoblast formation and maturation (Figure 3).

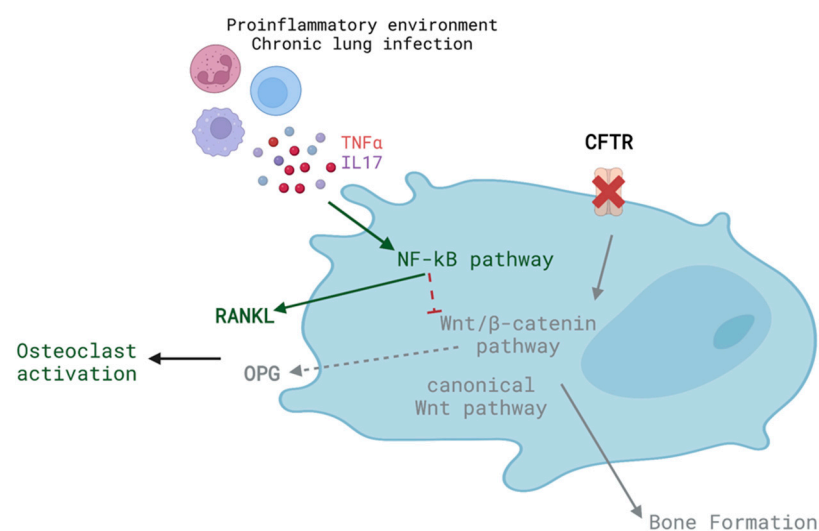


Figure 3. Defects in CFTR function hamper osteoblast maturation. Scheme was created using Biorender.com (www.biorender.com; access on 6 December 2022).

4.2. CFTR Dysfunction and Osteoclastogenesis

Osteoclasts are giant multinucleated cells that originate from hematopoietic stem cells [74]. CFTR deficiency in mice was associated with increased numbers of osteoclasts and consequently to increased bone degradation [96]. Increased numbers of osteoclast progenitors were found in the peripheral blood of cystic fibrosis patients at the beginning of infective exacerbations and decreased after antibiotic treatment and resolution of the episode [115]. There are some conflicting results regarding the capacity for the osteoclast differentiation of peripheral mononuclear cells from cystic fibrosis patients. In one study, the formation of osteoclasts from circulating mononuclear cells was reduced [116], whereas in another study, peripheral blood mononuclear cells from cystic fibrosis individuals originated higher numbers of osteoclasts and bone resorption events when stimulated in vitro with osteoclast lineage-instructing cytokines [115]. These contradictory results could not be explained by the type of CFTR mutation present as both studies used cells

from patients carrying at least one F508del allele, but perhaps differences in the patients age, inflammatory status and/or genetic background may explain these discrepancies.

An alteration in CFTR function results in the activation of the NF- κ B pathway and the secretion of several cytokines such as chemokine (C-C motif) ligand 2 (CCL2) upon bacterial infection or lipopolysaccharide stimulation [117–122]. Increased production of CCL2 induces osteoclast differentiation and bone resorption [123]. Indeed, osteoclast formation significantly correlated with the serum levels of TNF α , OPG, osteocalcin and N-telopeptide (NTx), and osteoclast activity significantly correlated with serum IL-6 and NTx [124]. Furthermore, monocytes from cystic fibrosis adults and pediatric patients express higher levels of macrophage colony-stimulating factor (M-CSF) receptors and show an increased propensity to differentiate into pro-inflammatory macrophages [125], suggesting that the enhanced osteoclastogenesis in these patients may be potentiated by recurrent inflammation or infection (Figure 4).

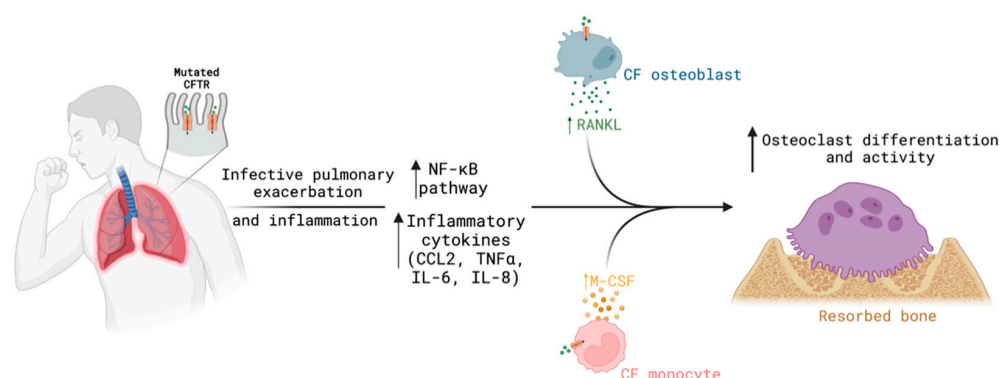


Figure 4. CFTR dysfunction and recurrent pro-inflammatory environment potentiates osteoclast formation and activity, contributing to bone loss and higher risk of fractures. Scheme was created using Biorender.com (www.biorender.com; access on 9 December 2022).

5. CFTR-Indirect Effects on Bone Health

5.1. Implications of Chronic Infection

Cystic fibrosis is associated with a hyperinflammatory state due to the conjugation of chronic lung infection, epithelial dysfunction and innate and adaptive immune dysregulation [126,127]. Alveolar macrophages and neutrophils accumulate in the lungs of cystic fibrosis patients [126]. Increased numbers of macrophages are found in the airways of cystic fibrosis newborns [128,129], and the majority of these cells are derived from circulating monocytes [130], suggesting an inflammatory bias in the lungs of these individuals. This basal inflammation may trigger alterations in bone homeostasis. Chronic infection of cystic fibrosis lungs perpetuates pro-inflammatory cytokine production, leading to elevated levels of TNF α , IL-6 and IL-1 β and reduced levels of IL-10 [131–133]. Indeed, increased numbers of neutrophils and alveolar macrophages in the BAL fluid of *Cftr*-deficient mice at basal conditions impaired the control of *Pseudomonas aeruginosa* infection due to increased proinflammatory cytokine production such as TNF α , IL-1 β and IL-6 [134]. Further supporting the role of the immune response in cystic fibrosis bone disease is the observation that the patients with a higher production of TNF α due to polymorphisms in *TNFA* and/or TNF α and TNF β (LT- α) genes showed a decreased bone density [135]. TNF α induces osteoclast formation and bone resorption and impairs osteoblast formation [74,136,137].

The dysregulated pro-inflammatory response in the absence of CFTR is partially corrected via the transplantation of hematopoietic cells with normal CFTR function [138], suggesting that the pharmacological correction of CFTR functions may have several benefits in both lung health and other cystic fibrosis-associated diseases.

5.2. Dysregulation of Glucose Homeostasis

Cystic fibrosis-related diabetes is a manifestation that usually appears around the second decade of life due to insulin deficiency [139]. Osteoblasts express insulin receptors and in response to insulin signaling, downregulate the expression of OPG and upregulate the production of osteocalcin [140,141]. Whereas the decrease in OPG increases the amount of RANKL available to bind to and activate osteoclasts and bone resorption, the production of osteocalcin has effects on whole-body glucose metabolism [142]. Undercarboxylated osteocalcin is an osteoblast-specific osteocalcin isoform that stimulates insulin secretion by pancreatic beta cells as well as increases insulin sensitivity in the liver, muscle and adipose tissue [143]. Besides osteocalcin, osteoblasts also produce the neuropeptide Y (NPY) that controls bone formation and also regulate glucose homeostasis. In the absence of the NPY receptor at an early stage of osteoblast differentiation, the bone mass increased but insulin secretion was reduced, leading to glucose intolerance [144].

In individuals without cystic fibrosis but with either type 1 or type 2 diabetes mellitus, the risk of fragility fractures is due to low bone turnover and macro and microarchitecture alterations [145]. Along these lines, a cohort of cystic fibrosis patients younger than eighteen years old who had impaired glucose tolerance or had already developed diabetes showed reduced bone mineral density and bone mineral content, indicating that poor glucose control together with other well-recognized factors such as reduced lung function, may impact bone homeostasis [146]. Furthermore, the patients with cystic fibrosis-related diabetes showed reduced bone turnover compared to patients without cystic fibrosis-related diabetes [147]. However, bone mineral density Z-scores were not different between the two groups of cystic fibrosis patients [147].

These observations highlight an intricate crosstalk between bone mass and glucose metabolism, suggesting that cystic fibrosis-related diabetes may be caused by factors other than pancreatic destruction. Additionally, the temporal relationship between cystic fibrosis-related diabetes and bone disease remains uncertain. Further studies will be required to address the relationship between low bone mass and impaired glucose homeostasis.

6. CFTR Modulators and Their Potential Impact in Bone Health

Management of low bone mass in patients with cystic fibrosis relies on empirical non-pharmacological measures to optimize bone health and pharmacological measures such as bisphosphonates [148]. Bisphosphonates have been shown to increase bone mineral density in adults and children with this condition [55,149]. CFTR modulators pose a new therapeutic opportunity to these patients and have been associated with improvements in lung function [150]. On one hand, Interferon γ -mediated responses in cystic fibrosis monocytes were reduced after one week of treatment with ivacaftor [151]. On the other hand, whole-blood transcriptomic analysis revealed that innate and adaptive immune pathways persisted as overexpressed despite lumacaftor/ivacaftor treatment [152].

Even though it is still unclear whether the CFTR modulators ameliorate the disease-associated inflammation, there are some insightful observations regarding CFTR modulators and bone disease. Patients treated with ivacaftor showed an improvement in the microarchitecture of cortical bone [153] and in bone mineral density [154]. Additionally, TNF α and IL-17-induced RANKL production in osteoblast from cystic fibrosis patients was reverted via treatment with a CFTR modulator [114,154]. Oral administration of miglustat, a drug that improves F508del-CFTR function, improved bone mass and microarchitecture in the lumbar spine and femur of F508del-CFTR mice [95]. Pharmacological modulation of CFTR reduced the ratio of RANKL/OPG in osteoblasts with the delF508 mutation in CFTR [155].

7. Challenges in the Management of Bone Health in Cystic Fibrosis

Early detection of cystic fibrosis bone disease allows us to prevent its impact on the patients' quality of life. For that purpose, the American Cystic Fibrosis Foundation and the European Cystic Fibrosis Society recommend the frequent monitorization of bone mineral

density via a bone density scan (DXA) in all individuals with the disease [156]. Bone densitometry using DXA is a useful noninvasive method that allows for the assessment of the fracture risk in these patients [157] but is costly and exposes the patients to X radiation. Another caveat of DXA is the measurement of bone mineral density and the incapacity to detect alterations in the bone microarchitecture. Furthermore, the coverage of bone density screening via DXA is still low, as the median DXA screening rate of adults with cystic fibrosis in the US was only 66% [158]. The missed screening may result in an under diagnosis of cystic fibrosis bone disease, which may explain the decreased incidence of bone disease reported in the 2019 CFFPR report [8]. There is a need to identify better biomarkers and scores to stratify the patients in terms of the risk of bone disease and low impact fractures [159]. Perhaps the monitorization of the inflammatory status may predict the risk of low bone mass as the levels of C-reactive proteins negatively associated with the levels of procollagen type 1 N-terminal propeptide (P1NP) [147]. Thus, the monitorization of the inflammatory status in cystic fibrosis patients may be a tool to predict mineral bone mass.

8. Conclusions

Several factors contribute to alterations in the bone content and architecture in individuals with cystic fibrosis. The available data suggest that abnormal CFTR function causes an intrinsic defect in osteoblastogenesis, hampering new bone formation and the failure to reach an optimal bone mass peak and reduced bone turnover in cystic fibrosis patients. The chronic inflammatory status of these patients together with periods of infective exacerbations drives osteoclast formation and bone loss by further increasing the RANKL/OPG ratio. The impact in bone mass may also be potentiated by dysregulated glucose homeostasis and should be investigated in future studies.

The recently introduced CFTR modulators may be useful in decreasing the basal inflammatory status of people with cystic fibrosis, improving bone mass in these patients. Further studies are required to understand the impact of CFTR modulators in disease-associated inflammation and bone disease and to identify/characterize the relationship between chronic inflammation and reduced bone mass. Additionally, the development of better bone health screening biomarkers will be important for early detection and for acting on cystic fibrosis bone disease to prevent low-impact fractures and the consequent decay in the patients' health and quality of life.

Author Contributions: A.C.G. conceptualized the manuscript and performed the literature search. Ó.F. prepared the figures. Ó.F., M.S.G., M.A.A. and A.C.G. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Annex IV

Serum Amyloid A proteins reduce bone mass during mycobacterial infections

Manuscript under revision in *Frontiers in Immunology*

Serum Amyloid A proteins reduce bone mass during mycobacterial infections

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Abstract

Osteopenia has been associated to several inflammatory conditions, including mycobacterial infections. How mycobacteria cause bone loss remains elusive, but direct bone infection may not be required. Using genetically engineered mice and morphometric, transcriptomic and functional analyses, we found that infection with *Mycobacterium avium* impacts bone turnover by decreasing bone formation and increasing bone resorption, in a IFN γ - and TNF α -dependent manner. IFN γ produced during infection enhanced macrophage TNF α secretion, which in turn increased the production of serum amyloid A (SAA). *Saa3* expression was upregulated in the bone of both *M. avium*- and *M. tuberculosis*-infected mice and SAA proteins were increased in the serum of patients with active tuberculosis. Furthermore, the increased SAA levels seen in active tuberculosis patients correlated with altered serum bone turnover markers. Additionally, human SAA proteins impaired bone matrix deposition and increased osteoclastogenesis in vitro. Overall, we report a novel crosstalk between the cytokine network operating in macrophages and bone homeostasis. These findings contribute to a better understanding of the mechanisms of bone loss during infection and open the way to pharmacological intervention. Additionally, our data disclose SAA proteins as potential biomarkers of bone loss during infection by mycobacteria.

1 Introduction

Bone loss has been reported during several inflammatory syndromes (1), but the underlying mechanisms remain elusive. Mycobacterial infections are good models to elucidate the molecular cross-talk between the immune response and bone homeostasis. During in vivo experimental infection with *Mycobacterium avium*, we noticed that the long bones obtained from infected mice were more brittle and fragile than those obtained from non-infected mice. The presence of mycobacteria in the bone has been traced back to thousands of years ago (2). Mendelian susceptibility to mycobacterial disease is associated with a higher frequency of bone involvement (3). Furthermore, previous mycobacterial infection, even without bone involvement, increases the risk of osteopenia and osteoporosis (4, 5).

A regulated balance between bone degradation and formation maintains an adequate bone mass, conferring the necessary rigidity and strength to the skeleton, to bear weight and avoid low impact fractures. Besides bone forming (osteoblasts and osteocytes) and bone degrading (osteoclasts) cells, bone remodeling is also influenced by immune mediators (1, 6). In this context, we and others have previously shown that mycobacterial infection skews the bone marrow hematopoietic development with important immune and hematological consequences (6, 7, 8, 9, 10). Therefore, the infection of bone cells themselves or the immune modulation of the bone marrow niche during infection may disrupt bone mass (6).

In this study, we investigated the mechanisms underlying reduced bone mass during mycobacterial infection to shed light on how the immune system and bone homeostasis articulate. We combined morphometry, transcriptomic and functional in vitro and in vivo assays to study how infections with the non-tuberculous mycobacteria, *M. avium*, impacted the bone. We established a mechanistic network centered in macrophages and linking IFN γ , TNF α and serum amyloid A (SAA) proteins, which combined to dysregulate bone turnover. The production of SAA by macrophages was confirmed upon direct administration of TNF α to macrophages and during aerosol infection with *M. tuberculosis*. Finally, we showed a systemic increase of SAA levels in patients with active tuberculosis, which

positively correlated with altered bone turnover markers measured in the plasma. Our study advances our understanding on how the immune response to infection impacts bone loss and reveals potential drug targets and clinical biomarkers for this. These findings are particularly important in tuberculosis disease, given the enormous number of cases in the world (11), combined with an aging population more susceptible to bone disease.

2 Materials and Methods

Bacteria

Mycobacterium avium 25291 SmT (obtained from the American Type Culture Collection, Manassas, VA) was grown at 37°C in Middlebrook 7H9 broth (BD Difco, USA), supplemented with 0.05% of Tween 80 (Sigma, St. Louis, MO) and 10% ADC (Albumin-Dextrose-Catalase). *M. avium* were collected during the exponential growth phase, centrifuged, washed twice with saline containing 0.04% Tween 80, re-suspended in the same solution, and briefly sonicated at low power to disrupt bacteria clumps. *M. tuberculosis* HN878 (clinical isolate) was grown to midlog phase in Middlebrook 7H9 broth supplemented with 0.05% Tween80, and 0.5% glycerol. Aliquots were prepared and stored at -80°C, prior to CFU determination or until needed. Just before use, an aliquot was thawed and diluted to the appropriate concentration. Work with *M. avium* and *M. tuberculosis* was performed under ABSL-2 or 3 facilities, respectively.

Mice

C57BL/6J, *Ifng*^{-/-}, MIIG, and *Tnfa*^{-/-} mice were bred and housed under specific pathogen-free conditions at Instituto de Investigação e Inovação em Saúde (i3S) animal facility. Mice were kept inside individually ventilated cages with HEPA filters and fed with sterilized food and water *ad libitum*. Mice were maintained on a 12:12 light cycle at 45-65% humidity and provided *ad libitum* water and standardized synthetic diet (Envigo Teklad Global Rodent Diet, 2014S). Animal experiments were approved by the i3S Animal Ethics Committee and licensed by Portuguese Competent Authority (DGAV), in July 6th, 2016 with reference 0421/000/000/2016. All animals were handled in strict accordance with good animal practice as defined by national authorities (DGAV, Decreto-Lei 113/2013, August 7th) and European Directive 2010/63/EU. The i3S animal facility is AAALAC accredited and follows the Guide for the Care and Use of Laboratory Animals, principle of the Three R's, to replace, reduce, and refine animal use for scientific purposes, as well as FELASA recommendations.

Human samples

The study protocol leading serum isolation was approved by the Portuguese *Comissão de Ética para a Saúde da ARS Norte* (#T792). To ensure confidentiality, each case was anonymized by the assignment of a random identification number. Participants were enrolled at a TB clinic in Porto and provided informed consent. Experiments were conducted according to the principles expressed in the Declaration of Helsinki. 10 healthy controls of unknown IGRA status (6 males and 4 females, 49±7 years old), 20 IGRA+ latent tuberculosis infection (LTBI, 9 males and 11 females, 54±19 years old), and 20 patients with active tuberculosis (9 males and 11 females, 50±17 years old).

114 ***In vivo* infection**

115 8- to 10-week-old mice were infected with 1 million CFU of *M. avium* 25291 SmT intravenously (iv)
116 into one of the lateral tail veins. Control groups were injected with the same volume of saline solution
117 by the same route. At 8-weeks p.i., mice were anesthetized using an isoflurane chamber. Blood was
118 collected by retro-orbital puncture under deep anesthesia, immediately before euthanasia. Long bones
119 were also collected. For *M. tuberculosis* experiments, mice were infected via the aerosol route using
120 an inhalation exposure system, calibrated to deliver ~200 colony forming units to the lung. The initial
121 bacterial load was determined by quantification of colony forming units in the lungs three days after
122 infection. Infected mice were monitored regularly for signs of illness such as wasting, piloerection and
123 hunching. At 27 days post infection, mice were euthanized by CO₂ inhalation and long bones were
124 collected. Long bones from aged-matched non-infected mice collected at the same time and used as
125 controls.

126 **Bone morphometric analysis by microCT**

127 Tibias were harvested, cleaned of muscle, fixed in 4% paraformaldehyde at 4°C, overnight, and
128 scanned at 70Kv, 200 µA with 0.5 mm aluminum filter, and an isotropic resolution of 6 µm, using
129 SkyScan 1276 System (Brucker, Belgium). The obtained projection images were reconstructed with
130 NRecon software, followed by bone alignment along the sagittal axis using DataViewer software.
131 Morphological analysis of both cortical and trabecular bone were performed with CTAnalyser
132 software, following the recommended guidelines (12). Cortical analysis were carried out in 150 slices
133 at the diaphysis (700 slices away from distal growth plate). 3D images of representative samples were
134 generated using CTVol software.

135 **2D Histomorphometry analysis**

136 Tibias/femurs were cleaned from surrounding soft tissue and fixed in 10% (v/v) PFA for 24 h and
137 rinsed in phosphate buffered saline (PBS) solution at 4°C. Samples were then dehydrated in serial
138 ethanol solutions (50–100%) for 3 days each, cleared in xylol for 24 h and further embedded in methyl
139 methacrylate. Undecalcified 7 µm sagittal cuts were stained with modified Masson-Goldner
140 Trichrome, and processed for static histomorphometric analysis as described elsewhere(13), using
141 OsteoMeasure software (OsteoMetrics, Decatur, GA, USA). All histomorphometric analysis were
142 performed by a single blinded operator. Digital images were obtained using NanoZoomer 2.0HT
143 (Hamamatsu) and representative images were acquired.

144 **Dynamic in vivo bone labeling**

145 Double labeling with xylenol orange and calcein was used to study bone turnover during *M. avium*
146 infection(14). Thus, 6 weeks post-infection, a saline solution of xylenol orange (Sigma-Aldrich, USA)
147 was subcutaneously administered at a dose of 80 mg/kg. Two weeks later, a saline solution of calcein
148 green (Sigma-Aldrich, USA) at a dose of 15 mg/kg was administered by the same route as xylenol
149 orange. Three days later (at 8 weeks post-infection), mice were euthanised, and femurs and tibias were
150 collected, fixed in 4% paraformaldehyde (BioOptica, Italy) at 4°C, overnight. Then, the bones were
151 washed 3 times with PBS1x for 10 min, followed by dehydration with PBS1x/ 30% sucrose, at 4°C,
152 overnight. Long bones were embedded in OCT (ThermoFisher, UK), snap-frozen in an ethanol/dry ice
153 bath, and 20-µm sections were cut using the Kawamoto method(15), using a Leica CM 3050S cryostat
154 (Leica Biosystems, Portugal). Sections were kept at -20°C, inside a silica gel chamber, for at least 12
155 hours. Then, slices were warmed to room temperature inside a silica gel chamber and mounted in a

30% glycerin solution. Images were acquired on a Leica SP8 confocal microscope (Leica Microsystems, Germany), using LASX software (Leica Microsystems, Germany). Images were analyzed using the measurement tool of Imaris Software (Oxford Instruments, UK).

Immunofluorescence of femur whole mounts

Long bones were collected from infected mice at 8 weeks post-infection and from littermate non-infected controls, fixed, dehydrated, and embedded in OCT, as described before. Then, bones were trimmed in a cryostat to expose the medullary cavity, and OCT was removed by melting at room temperature. After 3 washes with PBS for 10 minutes, to block the non-specific binding and to permeabilize, whole mounts were incubated for 1 hour at room temperature with PBS containing 20% fetal bovine serum and 0.5% Triton X-100 (Sigma Aldrich, USA). Afterwards, whole mounts were stained for 3 days, at room temperature, with rabbit anti-TRAP antibody (1:50 dilution, Abcam, UK). Whole mounts were washed in PBS for 10 minutes, three-times, and stained with Alexa 647 anti-rabbit antibody (1: 500 dilution, Jackson ImmunoResearch, UK) and DAPI (Sigma-Aldrich) for 3 days, at room temperature and protected from the light. After 3 washes in PBS for 10 minutes, whole mounts were transferred to an imaging chamber (ibidi GmbH, Germany) containing 30% glycerin solution and imaged on a Leica SP8 confocal microscope (Leica Microsystems, Germany). Bone surface expressing TRAP was quantified using Imaris Software (Oxford Instruments, UK).

Histology

Femurs were fixed in 10% neutral buffered formalin solution overnight at 4°C, in EDTA/Glycerol solution for 3 weeks at 4°C, and then included in paraffin blocks. 5-µm thick sections were cut, deparaffinized and processed through downgraded alcohols, and rehydrated.

Immunohistochemistry of F4/80 and Ziehl-Neelsen stain: Sections were deparaffinized and processed as described before. To unmask the antigenic epitope, sections were incubated in citrate buffer pH 6.0 (Thermo Fisher Scientific) in a steamer for 20 min, allowed to cool for 10 min at RT, followed by enzymatic digestion with 0.05% Trypsin (Gibco) in a humidified chamber at 37°C for 30 min. Enzymatic reaction was stopped with incubations in cold water for 5 minutes, twice. After blocking of endogenous peroxidase activity using Ultravision hydrogen peroxide block (Thermo Fisher Scientific), endogenous biotin was blocked (Lab Vision Avidin biotin Blocking Solution, Thermo Fisher Scientific). Unspecific binding sites were blocked with Normal Goat Serum (1:5 dilution, DAKO) in antibody diluent (Thermo Fisher Scientific) at RT for 30 minutes. Rat anti-mouse F4/80 antibody (clone BM8, Biolegend) was added to the sections and incubated in a humidified chamber overnight at 4°C. Then, sections were stained with a biotinylated goat anti-rat IgG (Enzo) in a humidified chamber at room temperature for 30 minutes, followed by incubation with Streptavidin-HRP (Vector Laboratories) in a humidified chamber at RT for 30 minutes. DAB Quanto (Thermo Fisher Scientific) was added to allow the color development for 3 minutes. Slides were stained with Carbol Fuchsin and then heated and rinsed off in tap water. Then, 1% of hydrochloric acid in methanol was added to slides. Slides were counter stained with Hematoxylin, rinsed twice with water, dehydrated (reverse of downgraded alcohols and deparaffinization), and coverslipped in Entellan. Sections were scanned in D-sight Plus f2.0 and analyzed with D-Sight viewer.

198 Flow cytometry

199 Cells were isolated from bone marrow of long bones by flushing in DMEM supplemented with 2%
200 heat-inactivated bovine fetal serum, 5% HEPES, and PenStrep (all from Invitrogen). Cells were
201 counted using the trypan blue (Sigma-Aldrich) exclusion assay.

202 **Myeloid cell analysis:** One million BM cells were stained with anti-Gr-1 (clone RB6-8C5,
203 BioLegend), anti-CD115 (clone AFS98, BioLegend), anti-F4/80 (clone BM8, BioLegend) and anti-
204 CD169 (clone 3D6.112, BioLegend). for 30 min, on ice. Cells were washed twice at 2000 rpm for 2
205 min and fixed with 2% PFA for 10 min at room temperature. Cells were gated as previously
206 described(16). Neutrophils: Gr-1^{hi} CD115⁻; Monocytes: Gr1^{lo} CD115⁺ F4/80⁺; Macrophages: Gr1^{lo}
207 CD115⁻ F4/80⁺ CD169⁺.

208 **Stromal cells analysis:** The isolation of bone marrow stromal cells was performed as described before
209 (17). Briefly, long bones were carefully flushed using HBSS supplemented with 2% heat-inactivated
210 fetal bovine serum, PenStrep, HEPES and 200 U/mL Collagenase IV (Sigma-Aldrich). The flushed
211 bone marrow was then digested at 37°C for 20 minutes. Cells clumps were dissociated by gentle
212 pipetting, followed by a further incubation for 10 min at 37°C. Cells were then washed with
213 HBSS/2%FBS, spun at 1000 rpm for 7 min and stained. LEPR staining was performed using biotin-
214 conjugated anti-LEPR (R&D systems) for 1 hour, on ice. The remaining staining using PE-conjugated
215 anti-CD45 (clone 30-F11, BioLegend) and anti-TER119 (BioLegend) and APC-conjugated anti-CD31
216 (clone 391, BioLegend) and anti-CD144 (BV13, BioLegend) were done for 20 min, on ice, followed
217 by 2 washes at 1000 rpm for 7 minutes. Finally, cells were washed and fixed with 2% PFA for 10 min
218 at room temperature. Cells were analyzed using a BD FACS Canto II flow cytometer and FlowJo.

219 RNA isolation and Quantitative Real Time PCR.

220 For *M. avium*-infected mice and littermate non-infected mice, bones were harvested and snap frozen
221 in liquid nitrogen. Bones were crushed in a liquid nitrogen bath and total RNA was isolated using RNA
222 Mini Kit (Invitrogen), according to manufacturer instructions. For *M. tuberculosis*-infected mice bone
223 marrow was flushed from femurs and tibias and extracted with Trizol. cDNA was then synthesized
224 using NZY First-Strand cDNA synthesis kit (NZYTech, Portugal).

225 Primers sequences: *Acp5* Fw: 5'- GCTGGAAACCATCATCACCT - 3'; *Acp5* Rv: 5'-
226 TGAAGCGCAAACGGTAGT - 3'; *Csfl* Fw: 5'- GGGCCTCCTGTTCTACAAGT - 3'; *Csfl* Rv: 5'-
227 AGGAGAGGGTAGTGGTGGAT - 3'; *Csflr* Fw: 5'-TTGGACTGGCTAGGGACATC-3'; *Csflr* Rv:
228 5'-GGTTCAGACCAAGCGAGAAG-3'; *Gapdh* Fw: 5'- TGTGTCCGTCGTGGATCTGA - 3';
229 *Gapdh* Rv: 5'- CCTGCTTCACCACCTTCTTGA - 3'; *Rank* Fw: 5'- GTGCTGCTGGTTCCACTG -
230 3'; *Rank* Rv: 5'- CCGTCCGAGATGCTCATAAT- 3'; *Saa3* Fw: 5'-ACATGTGGCGAGCCTACTCT-
231 3'; *Saa3* Rv: 5'-GAGTCCTCTGCTCCATGTCC-3'.

232 Targeted Transcriptomic Analysis

233 Whole bone total RNA was isolated from C57BL/6J mice at 8 weeks post-infection and from littermate
234 non-infected mice, as described above. The transcriptomic laboratorial processing was performed by
235 the Genomics Core Facility at i3S (Porto, Portugal). Briefly, RNA concentration was measured using
236 Qubit3.0 fluorometer and total RNA integrity number was determined using the Agilent 2100
237 Bioanalyzer. Libraries were constructed according to Ion AmpliSeq™ Transcriptome Mouse Gene
238 Expression Kit protocol (Thermo Fisher Scientific, USA) and pooled with each sample ligated to a
239 unique barcode. The pooled libraries were processed on Ion Chef™ System and the resulting 540™

chip was sequenced on Ion S5™ XL System. FASTQ and/or BAM files were generated using the Torrent Suit plugin FileExporter v5.12.

The statistical analysis and graphical representations were performed in Rstudio version 1.4.1106. The quality control of the expression profiles of triplicates of each experimental group were investigated through Principal Component Analysis (PCA). Differentially expressed genes in the bone between infected and non-infected bones were identified by the R package DESeq2 package v1.24 with default parameters (Love et al. 2014), considering an adjusted p-value threshold below 0.05 and a $\log_2\text{FoldChange} > 0.5$. Clustering and heatmap representation of these significantly expressed genes, between uninfected and infected tests, were obtained using the R package heatmap version 1.0.12 package. Pre-ranked pairwise gene-set enrichment analyses (GSEA) were conducted in GSEA software for the Gene Ontology (GO) (biological process or BP, cellular component or CC, and molecular function or MF) and KEGG pathway datasets, after converting the mouse genes in the homologous human genes by using information from the Mouse Genome Informatics Web Site (18). The resulting enriched pathways were ordered by the normalized enrichment score (NES) and the false discovery rate (FDR) q-value. Top significantly enriched pathways ($\text{FDR} < 0.05$) were further explored based on information contained in the publicly available database of Ingenuity (<https://targetexplorer.ingenuity.com/index.htm>; Qiagen, Hilden, Germany).

Measurement of SAA, TNF α and CTX-I by ELISA

Blood was collected from the orbital vein to non-heparinized tubes and let to clot and spun at high speed to collect serum. Serum levels of SAA3, TNF α and CTX-I were measured using the mouse SAA-3 ELISA kit (Sigma-Aldrich), LEGEND MAX™ Mouse TNF- α ELISA Kit (BioLegend) and the RatLaps™ EIA CTX-I (Immunodiagnostic Systems, Denmark), following the manufacturer's instruction.

Human peripheral blood was collected by venous puncture and plasma was recovered. SAA and CTX-I were measured using the human SAA ELISA (Sigma-Aldrich) and Serum Crosslaps (CTX-I) ELISA (ImmunoDiagnosticSystems, Denmark), following the manufacturer's instruction. Circulating levels of cytokines/ chemokines and bone biomarkers were determined using the Human Cytokine/Chemokine Panel A 48-Plex Discovery Assay® Array (HD48A) and Human Bone 13-Plex Discovery Assay® Array (HDBN13), respectively (Eve Technologies, Calgary, Canada).

Differentiation of Bone Marrow-derived Macrophages

Bone marrow was harvested from the long bones of C57BL/6 mice by flushing with Hank's Balanced Salt Solution (HBSS, Gibco, UK). Cells were cultured overnight in DMEM containing 10% fetal bovine serum, 1% pyruvate, 1%-glutamine, 1% HEPES, and 10% L929 cell-conditioned medium (LCCM), at 37°C/ 5% CO₂. Non-adherent cells were collected and then plated at a cellular density of 5×10^5 cells/mL. Cells were cultured in DMEM containing 10% fetal bovine serum, 1% pyruvate, 1%-glutamine, 1% HEPES, and 10% L929 cell-conditioned medium (LCCM), at 37°C/5% CO₂. At day 4 of culture, 10% LCCM was added and at day 7, the medium was renewed. At the 10th day, cell culture media was collected to obtain non-infected bone marrow-derived macrophage conditioned media (NI BMDM CM). Then, cells were in vitro infected with 5×10^5 CFU/mL of *M. avium* 25291 SmT, for 4 hours at 37°C/5% CO₂. Afterwards, cells were washed 4 times with warm HBSS and then incubated at 37°C/5% CO₂ for 5 days. For TNF α and IFN γ treatments, a concentration of 10 ng/mL and 16ng/mL, respectively, was added to the cultures for 3 days post-infection. At day 5 post-infection, cell culture medium was removed and filtered to obtain *M. avium*-infected bone marrow-derived macrophages

conditioned medium (*Mav* BMDM CM). Conditioned media were stored at -80°C until used. RNA was harvested from infected and non-infected cultures using RNA Mini Kit (Invitrogen). Gene expression was determined as previously described.

Osteoclast differentiation assays

Hematopoietic progenitors were harvested from the bone marrow by flushing the long bones with α MEM supplemented with 10% fetal bovine serum. After red blood cell lysis, cells were cultured overnight in α MEM supplemented with 10% fetal bovine serum and 10 ng/mL M-CSF, at 37°C/5% CO₂. Non-adherent cells were collected and then cultured at a cell density of 7.5×10^5 cells/mL, with 100ng/mL RANKL (BioLegend), 100 ng/mL M-CSF (BioLegend), and 50% NI or *Mav* BMDM CM. Media was renewed every other day. At the 7th day of culture, cells were fixed with 4% paraformaldehyde (BioOptica, Italy) for 10 min, at room temperature, and permeabilized with 0.1% Triton X-100 in PBS 1x for 15 minutes at room temperature. Nuclei were labelled using DAPI, and cytoplasm was labeled with HCS CellMask (Invitrogen, USA). Imaging was performed using IN CELL Analyzer 2000 microscope (GE Healthcare, USA). Osteoclasts were manually quantified using Image J and Microsoft Excel (Microsoft Corporation, USA). An osteoclast was defined as a multinucleated cell with more than 3 nuclei per cell. Four ranges were defined: 3-4 nuclei per cell, 5-10 nuclei per cell, 11-20 nuclei per cell and more than 20 nuclei per cell. The frequency of each class was calculated. To determine osteoclast resorptive capacity, osteoclast precursors were seeded on top of commercially available bovine cortical bone slices (boneslices.com, Denmark). At day 7, cells were lysed by incubation with water. Bone slices were washed and stained with Toluidine Blue. The total number of pits and trenches per slice was enumerated using an optical microscope.

Osteoblast cultures

Bone marrow stromal cells were flushed from the long bones C57BL6 mice with α MEM supplemented with 10% fetal bovine serum, plated in 75 cm² flasks and cultured for 7 days. Medium was renewed every 2-3 days to remove hematopoietic cells in suspension. Primary osteoprogenitor cells were harvested at pre-confluence using trypsin solution and cell viability was determined by the Trypan Blue Exclusion assay. Then, cells were plated at a 5×10^5 cells/mL, under osteogenic conditions (MesenCult Osteogenic Stimulatory Kit, Stem Cell Technologies, USA). Medium was renewed every 3 days. At day 12 of culture under osteogenic conditions, cells were fixed with 4% paraformaldehyde for 10 min, at room temperature, and stained with alizarin-red staining solution (Sigma-Aldrich, USA) for 30 min, at room temperature with gentle shaking. Then, calcium deposits were eluted with 10% cetylpyridinium chloride in sodium phosphate (pH 7.0) and quantified at 570 nm.

Statistics

Student's two-tailed t test and One-Way ANOVA were performed using GraphPad Prism 9 (GraphPad Software, CA, USA). A p value <0.05 was considered significant.

3 Results

3.1 Mycobacterial infection reduces bone mass and mineral density due to both increased degradation and decreased formation

Our first aim was to comprehensively characterize the alterations induced in the bone during *M. avium* infections. For that, C57BL/6J mice were infected with *M. avium* through the iv route (19). Eight weeks later several parameters in the cortical and trabecular bone were quantified by micro-computerized tomography (microCT, **Fig. S1, A to C**). Even though the marrow volume (M.V.) and periosteal perimeter (Perio. P.) were increased in the cortical bone (**Fig. 1, A and B**), the cortical volume (C.V.), mineral density (C. B.M.D.) and thickness were reduced by infection (**Fig. 1, C to E**). Endocortical perimeter (Endo. P.) and mean polar moment of inertia (MPPI) were not altered (**Fig. 1, F and G**). Additionally, the trabecular bone volume (T.B.V.) was decreased (**Fig. 1H**) whereas the trabecular tissue volume (T.T.V.) was not altered (**Fig. 1I**), which resulted in a reduction of the ratio trabecular bone volume to tissue volume (%B.V./T.V., **Fig. 1J**). Also reduced in infected mice were the trabecular mineral density (T. B.M.D.), and the trabecular thickness and number (#T., **Fig. 1, K to M**), with the concomitant increase in the trabecular separation (T. S.; **Fig. 1N**).

Given these alterations suggestive of bone loss during infection, a dynamic histomorphology analysis was performed (14). Bone surface was fluorescently labeled in vivo by the injection of xylenol orange and calcein green at 6- and 8-weeks post-infection, respectively. Mice were sacrificed 3 days after the latter, and 20- μ m bone sections were analyzed by confocal microscopy. In non-infected mice, the two fluorochrome labels were detected in the bone and were visibly separated by non-labeled (newly formed) bone tissue. However, in the infected bones, even though both calcein and xylenol orange were detected, some regions were not labeled with xylenol orange, or the two layers of dyes overlapped (**Fig. 1O**). The distance between the xylenol and calcein layers in cortical and trabecular bone, indicative of the degree of bone formation, was measured and found to be significantly decreased in infected as compared to non-infected mice. Concomitantly, the mineral appositional rate (M.A.R.) was significantly decreased in infected mice (**Fig. 1P**).

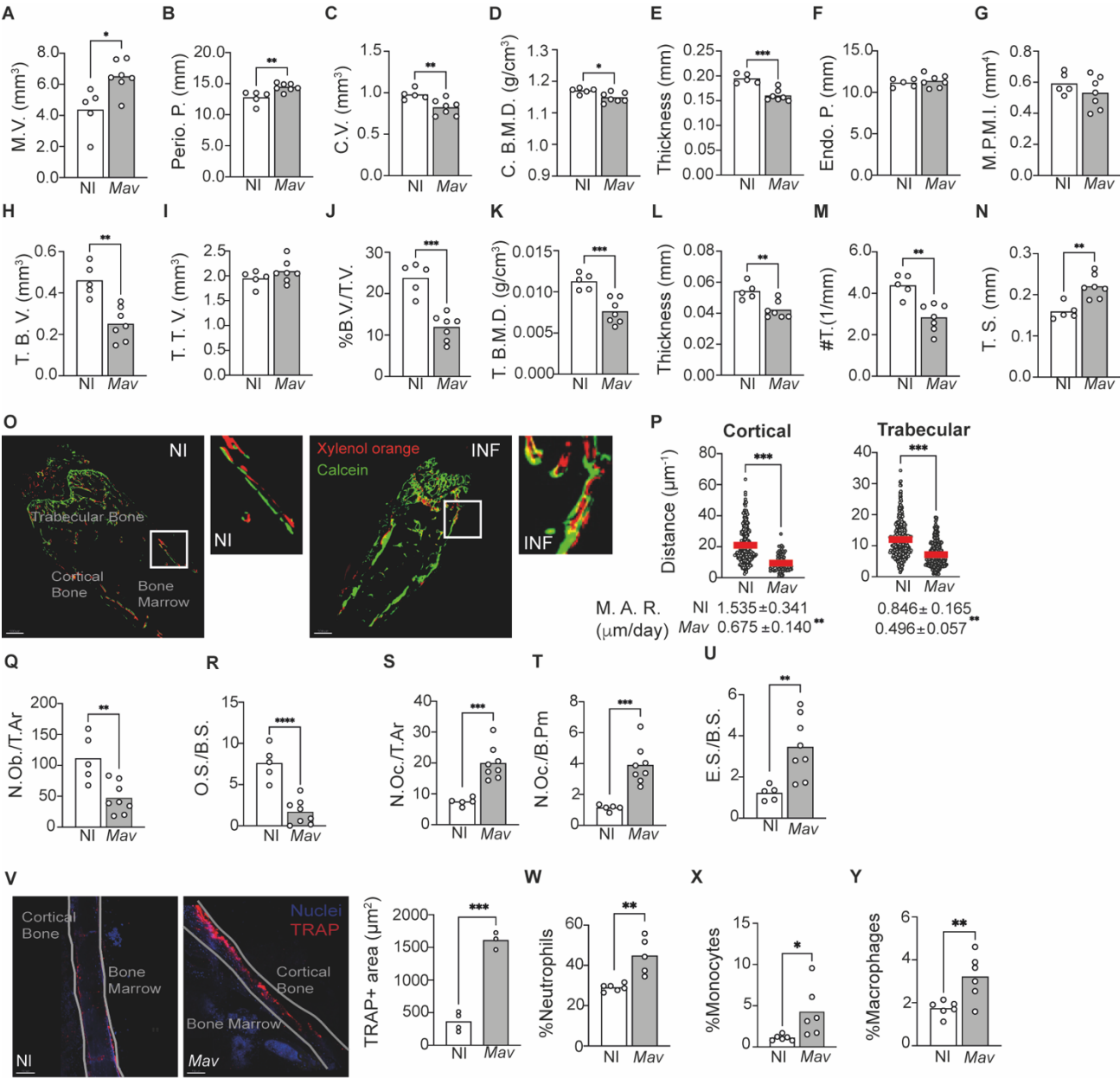
In what regards the cellularity of the bone, we found that infection reduced the number of osteoblasts (N.Ob./T.Ar.) and osteoid volume per bone surface (O.S./B.S.) (**Fig. 1, Q and R**), as well as the number of osteoblasts lining the bone surface (N.Ob./B.Pm.; **Fig. S1D**). The alterations in osteoblast lineage could also be detected at the LEPR⁺ mesenchymal stem cells (MSC) level, whose frequency was increased in the bone marrow of infected mice (**Fig. S1E**). LEPR⁺ MSC were described as the major source of osteoblasts and adipocytes in the adult bone marrow (20). The increase in the frequency of LEPR⁺ MSC may indicate a blockade in their differentiation towards osteoblasts.

Finally, histomorphology analysis of the bone revealed an increased number of osteoclasts per total area (N.Oc./T.Ar.) and per total bone perimeter (N.Oc./B.Pm., **Fig. 1, S and T**), which led to an increased eroded bone surface per bone surface (E.S./B.S.; **Fig. 1U**). Together these findings indicate that bone loss is happening due to exacerbated osteoclast activity. To test this hypothesis, we measured the area of TRAP⁺ bone, which is indicative of osteoclast activity as TRAP is an enzyme secreted by osteoclasts during bone resorption. The area of TRAP⁺ bone surface was increased in infected mice (**Fig. 1V**). Furthermore, significantly higher levels of C-terminal telopeptides of Type I collagen (CTX-I) were detected in the serum of infected mice (**Fig. S1F**), an indicator of increased bone resorption.

Osteoclasts have a myeloid origin. For this purpose, myeloid populations were identified as previously described (16). We found an increased frequency of neutrophils, monocytes and macrophages are present in the bone marrow of infected mice (**Fig. 1, W to Y**). These data are in agreement with

366 previous reports that mycobacterial infections skew the differentiation of hematopoietic stem cells
367 towards the myeloid lineage (8, 9, 10).

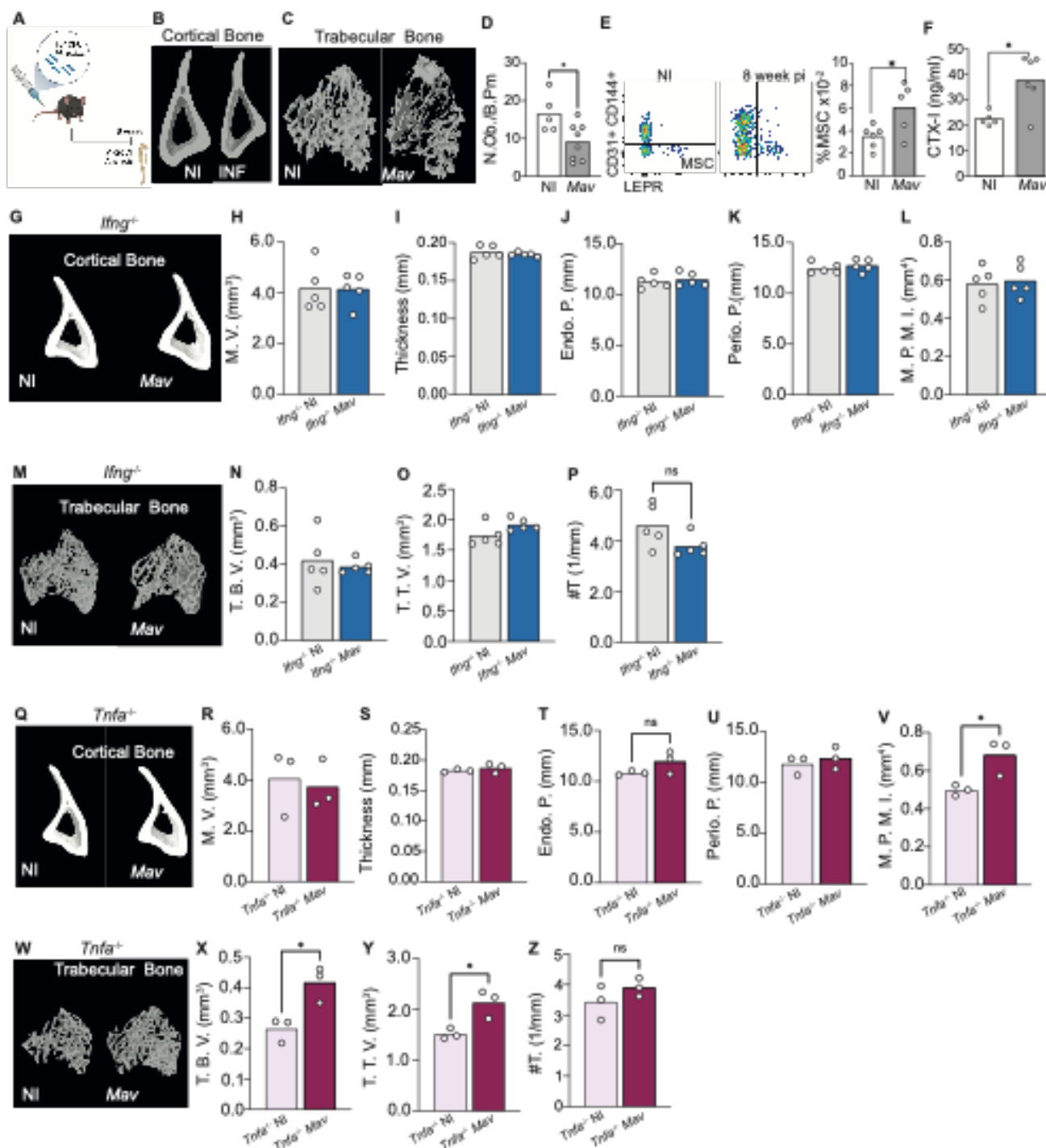
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369

370 **Figure 1. *Mycobacterium avium* infection alters bone turnover, reducing bone mass.** A-G) Measurement of the
371 marrow volume (M.V., A), periosteal perimeter (Perio. P., B), cortical volume (C.V., C), cortical bone mineral density
372 (C. B. M. D., D), cortical thickness (E), endocortical perimeter (Endo. P., C), and polar mean moment of inertia (M.
373 P. M. I., G) in *M. avium*-infected mice (Mav bars) compared to non-infected littermate controls (NI bars). H-N)
374 Measurement of the trabecular bone volume (T.B.V., H), trabecular tissue volume (T.T.V., I), ratio of bone volume
375 to tissue volume (%B.V./T.V., J), trabecular bone mineral density (T. B. M. D., F), trabecular thickness (L),
376 trabecular number (#T, M) and trabecular separation (T.S.,N) in *M. avium*-infected mice (Mav bars) and non-infected
377 controls (NI bars).N=5-7 mice per experimental group; data representative of two independent experiments. Bars
378 indicate average of the group, and each circle depicts each individual mouse analyzed. O) 20-μm-thick sections of
379 femoral bone labeled with xylenol orange (red) and calcein (green). Scale bar: 100μm. P) Distance between xylenol

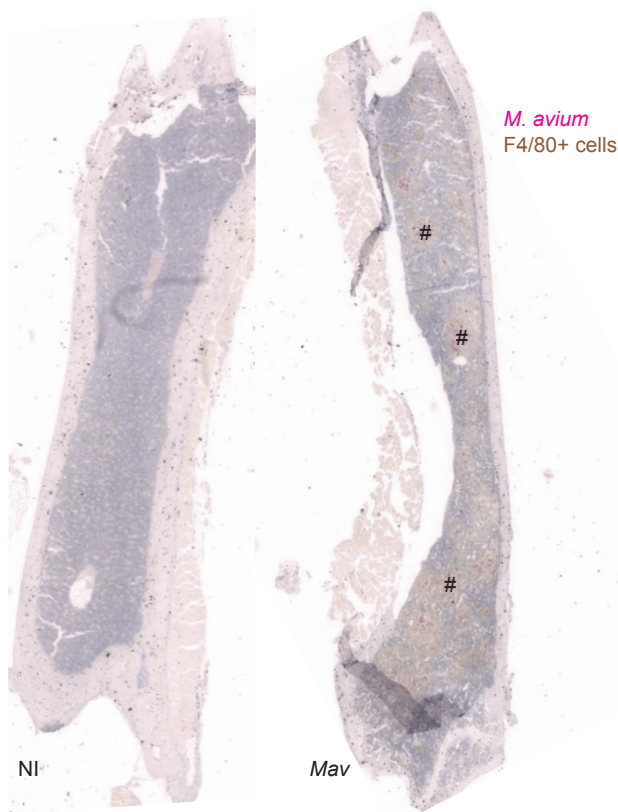
orange and calcein layers on the bone surface of non-infected (NI) and infected (*Mav*) mice. Bellow the graphs, the mineral apposition rate (M.A.R) is indicated. N=5-6 mice per experimental group. Red lines indicate average, and circles depict each measurement. **Q-U**) Histomorphometry analysis of the long bones of *M. avium*-infected mice (*Mav* bars) compared with non-infected controls (NI bars). Osteoblast number per total area (N.Ob/T.Ar, **Q**), Osteoid surface per bone surface (OS/BS, **R**), Number of osteoclast per total area (N.Oc/T.Ar, **S**), Number of osteoclasts per bone perimeter (N.Oc./B.Pm., **T**), Eroded surface per bone surface (ES/BS, **U**). N=5-8 mice per experimental group. Representative of two independent experiments. Bars indicate the average; circles depict each individual mouse. **v**) Quantification of the bone area expressing TRAP. TRAP-expressing bone surface was identified in the bones of infected (*Mav*) and non-infected (NI) mice and the area of those regions was then measured. TRAP staining in red; nuclei in blue; white line shows the limits of the bone; NI, non-infected; *Mav*, infected for 8 weeks; Scale bar: 150µm. Bars represent the average of the experimental group; each dot corresponds to one individual animal. **W-Y**) Frequency of neutrophils (Gr-1^{hi} CD115⁻; **W**), monocytes (Gr1^{lo} CD115⁺ F4/80⁺; **X**), and macrophages (Gr1^{lo} CD115⁻ F4/80⁺ CD169⁺; **Y**) in the bone marrow. N=6 mice per experimental group. Representative of three independent experiments. Bars indicate the average; circles depict each individual mouse. *, p<0.05, ** p<0.01, ***, p<0.001 by unpaired Student's *t* test.



Supplementary Figure 1. *Mycobacterium avium* infection increases bone degradation and reduces bone formation. **A)** Representative scheme of in vivo *M. avium* infection. **B-C)** Representative image of the cortical (**B**) and trabecular (**C**) tibial bone of *M. avium*-infected (*Mav* bars) and non-infected mice (NI bars) scanned by micro-CT. **D)** Enumeration of the osteoblast number per bone perimeter (N.Ob./B.Pm.) in infected mice (*Mav* bars) vs non-infected controls (NI bars). **E)** Enumeration of the frequency of LEPR⁺ MSC in the bone marrow of infected and non-infected mice by flow cytometry. **F)** Quantification of CTX-I in serum. N=5-8 mice per experimental group; data representative of two independent experiments. Bars indicate average and each dot represents each mouse. **G)** Representative image of the cortical tibial bone of *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Ifng*^{-/-} mice scanned by micro-CT. **H-L)** Measurement of the marrow volume (M. V., **A**), cortical thickness (**I**), endocortical perimeter (Endo. P., **J**), periosteal perimeter (Perio. P., **K**), and mean polar moment of inertia (M. P. M. I., **L**) in *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Ifng*^{-/-} mice. **M)** Representative image of the trabecular tibial bone of *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Ifng*^{-/-} mice scanned by micro-CT. **N-P)** Measurement of the trabecular volume (T. B. V., **N**), trabecular thickness (T. T. V., **O**), and trabecular number (#T., **P**) in *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Ifng*^{-/-} mice. **Q)** Representative image of the cortical tibial bone of *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Tnfr*^{-/-} mice scanned by micro-CT. **R-V)** Measurement of the marrow volume (M. V., **R**), cortical thickness (**S**), endocortical perimeter (Endo. P., **T**), periosteal perimeter (Perio. P., **U**), and mean polar moment of inertia (M. P. M. I., **V**) in *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Tnfr*^{-/-} mice. **W)** Representative image of the trabecular tibial bone of *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Tnfr*^{-/-} mice scanned by micro-CT. **X-Z)** Measurement of the trabecular volume (T. B. V., **X**), trabecular thickness (T. T. V., **Y**), and trabecular number (#T., **Z**) in *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Tnfr*^{-/-} mice.

406 *avium*-infected *Infgamma*^{-/-} mice (*Mav* bars) compared to non-infected littermate controls (NI bars). **M)**
407 Representative image of the trabecular tibial bone of *M. avium*-infected (*Mav* bars) and non-infected (NI bars) *Ifng*^{-/-}
408 mice scanned by micro-CT. **N-P)** Measurement of the trabecular bone volume (T. B. V., **N**), trabecular tissue volume
409 (T. T. V., **N**), trabecular number (#T, **P**) in *M. avium*-infected *Ifng*^{-/-} mice (*Mav* bars) and non-infected controls (NI
410 bars). **Q)** Representative image of the cortical tibial bone of infected and non-infected *Tnfa*^{-/-} mice scanned by micro-
411 CT. **R-V)** Measurement of the marrow volume (M. V., **R**), cortical thickness (**S**), endocortical perimeter (Endo. P.,
412 **T**), periosteal perimeter (Perio. P., **U**), and mean polar moment of inertia (M. P. M. I., **V**) in *M. avium*-infected *Tnfa*^{-/-}
413 mice (*Mav* bars) compared to non-infected littermate controls (NI bars). **W)** Representative image of the trabecular
414 tibial bone of infected and non-infected *Tnfa*^{-/-} mice scanned by micro-CT. **X-Z)** Measurement of the trabecular bone
415 volume (T. B.V., **X**), trabecular tissue volume (T.T.V., **Y**), trabecular number (#T, **Z**) in *M. avium*-infected *Tnfa*^{-/-}
416 mice (*Mav* bars) and non-infected controls (NI bars). N=3-5 mice per experimental group; data representative of two
417 independent experiments. Bars indicate average; each dot depicts each individual mouse analyzed *, p<0.05, **
418 p<0.01, ***, p<0.001 by Student's *t* test.

420 Disseminated *M. avium* infection leads to the colonization of macrophages within the bone marrow
421 parenchyma (19) instead of a direct colonization of bone cells (**Fig. S2**), suggesting that the altered
422 bone turnover and reduced bone mass that we now show during infection might be a consequence of
423 the ongoing immune response instead of a direct colonization of bone cells.



425 **Supplementary Figure 2. In infected bones, *M. avium* resides mostly inside macrophages distributed**
426 **throughout the bone marrow parenchyma.** 5-μm thick sections of non-infected (NI, left panel) and infected (*Mav*,
427 right panel) femurs stained with anti-F4/80 and Ziehl-Neelsen. (#) mark F4/80+ (brown) cells infected with *M. avium*
428 (red rods).

3.2 IFN γ and TNF α production during *M. avium* infection lead to bone resorption

To gain insight into the molecular mechanisms behind reduced bone mass during mycobacterial infection, we performed targeted RNAseq of whole bone and compared the gene expression between non-infected and infected mice. We found that 1822 genes were statistical and differentially expressed (adjusted p -values of ≤ 0.05) in the bone of infected mice (**Fig. 2A**). Within the top upregulated differentially expressed genes (DEG) were several immune mediators, such as *Cxcl9*, *Cxcl10* and *Nos2* (**Fig. 2A**). Furthermore, pathway-enrichment analysis revealed that the most upregulated (and statistically significant) pathways during *M. avium* infection were “response to IFN γ ” and “IFN γ mediated signaling pathway” (**Fig. 2B**). Next, we investigated whether this cytokine was also involved in bone loss during infection. For that, *Ifng*^{-/-} mice were infected with *M. avium*. We showed previously that the proliferation of the *M. avium* strain used in this study is not significantly different in *Ifng*^{-/-} as compared to *Ifng*^{+/+} mice (19). MicroCT analysis revealed that most of the alterations in cortical and trabecular bone found in infected of *Ifng*^{+/+} (**Fig. 1**) were absent in mice lacking *Ifng* (**Fig. 2, C to F**; and **Fig. S1, G to P**). Thus, IFN γ is a central piece mediating bone loss during *M. avium* infection.

The analysis also highlighted several pathways related to myeloid and monocytic function, which we hypothesized could be related to osteoclast differentiation (1, 6, 21, 22, 23). In fact, *M. avium* infection induced the up-regulation of *Csfl*, *Csflr*, *Rank*, and *Acp5* in the bones of *Ifng*^{+/+} but not of *Ifng*^{-/-} mice (**Fig. 2, G to J**). Overall, our data indicate that osteopenia during *M. avium* infection depends on IFN γ production, which mediates the upregulation of genes related to osteoclast formation and activity. These observations agree with previous studies reporting that IFN γ is an indirect promoter of osteoclastogenesis and bone resorption (24).

Besides IFN γ -related pathways, our RNA-Seq data highlighted an upregulation of pathways associated with TNF, NIK and NF κ B signaling in the bone of infected mice (**Fig. 2B**). Thus, we decided to investigate the contribution of TNF α to the bone alterations induced by *M. avium* infection. In mice lacking the *Tnfa* gene, *M. avium* infection did not generally result in reduction of cortical and trabecular bone mass and mineral density (**Fig. 2, K to N**; **Fig. S1, Q to Z**). Of note, cortical bone volume, trabecular bone volume and trabecular total volume were significantly increased in *Tnfa*^{-/-} infected mice (**Fig. 2K**; **Fig. S2, X and Y**). Together, the data suggest that osteopenia during *M. avium* infection requires both IFN γ and TNF α .

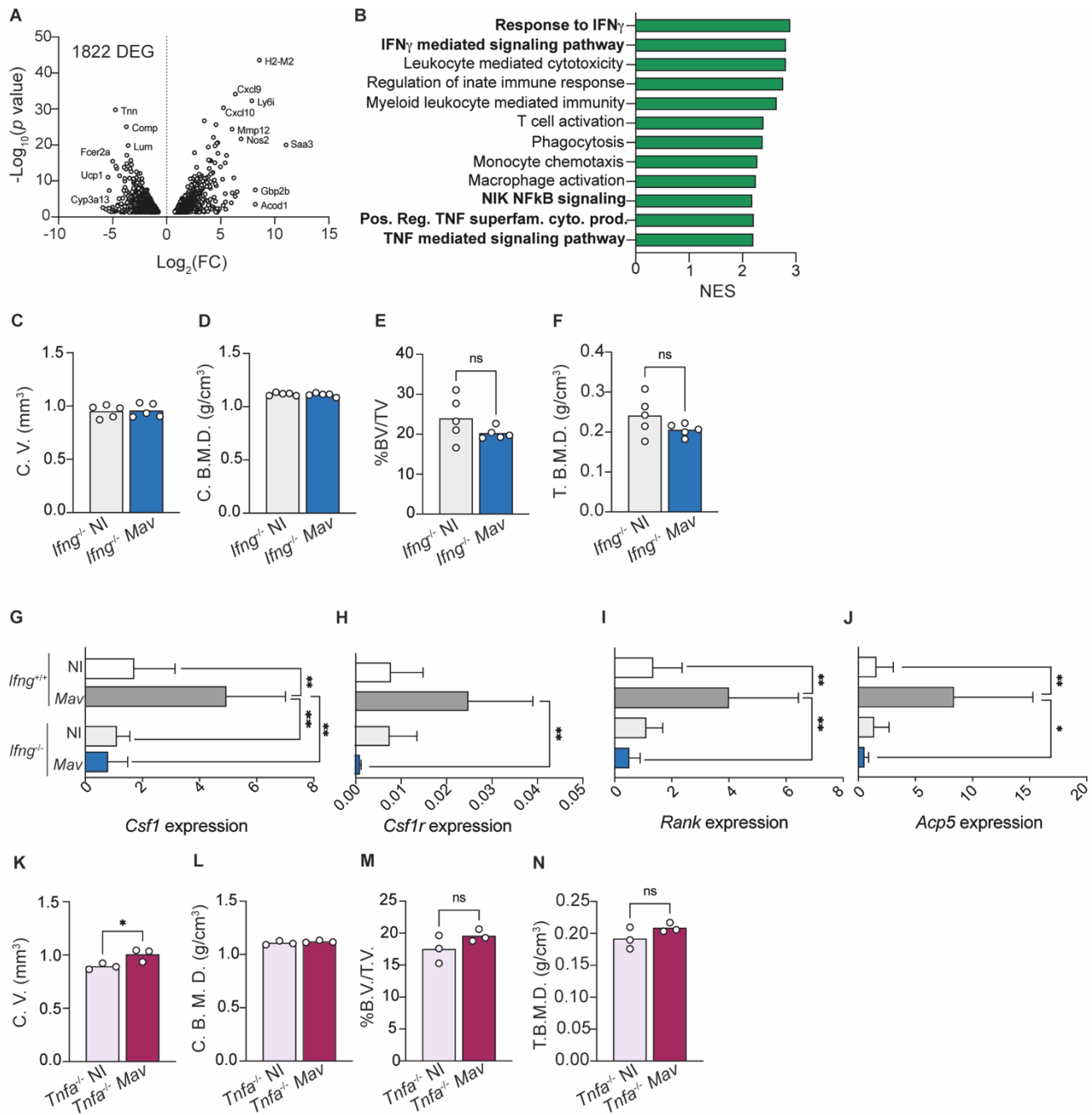


Figure 2. Bone mass reduction during chronic *M. avium* infection is dependent on $\text{IFN}\gamma$ and $\text{TNF}\alpha$ production.

A) Volcano plot of the differentially (and statistically significant) expressed genes in the bone of *M. avium*-infected mice compared to non-infected littermate control mice. **B**) Differentially upregulated pathways (from the GO Biological Process dataset) in the bone of infected mice compared with non-infected controls. **C-D**) Measurement of the cortical volumes (C. V., **C**), and cortical bone mineral density (C. B. M. D., **D**) in *M. avium*-infected (*Mav* bars) compared to non-infected mice (NI bars). **E-F**) Measurement of the ratio of bone volume to tissue volume (%BV/TV, **E**), and trabecular mineral bone density (T. B. M. D., **F**). N=5 mice per experimental group; data representative of two independent experiments. Bars indicate average; circles depict each individual mouse analyzed. n.s., $p > 0.05$ by unpaired Student's *t* test. **G-J**) Expression of *Csfl* (**G**), *Csflr* (**H**), *Rank* (**I**), *Acp5* (**J**), in the bones of infected (*Mav* bars) and non-infected (NI bars) *Ifng*^{-/-} and *Ifng*^{+/-} mice. Bars represent the average $\pm$ standard deviation of the experimental group. *, $p < 0.05$, ** $p < 0.01$ by two-way ANOVA. **K-L**) Measurement of the cortical volumes (C. V., **K**), and cortical bone mineral density (C. B. M. D., **L**). **M-N**) Measurement of the ratio of bone volume to tissue volume (%BV/TV, **M**), and trabecular mineral bone density (T. B. M. D., **N**). N=3 mice per experimental group; data

representative of two independent experiments. Bars indicate average; circles depict each individual mouse analyzed. n.s., $p > 0.05$ by unpaired Student's t test.

3.3 Soluble factors produced by *M. avium*-infected macrophages increase osteoclastogenesis

In vivo data indicated that IFN γ and TNF α were involved in osteoclastogenesis and bone resorption during infection. We thus decided to use in vitro systems to investigate whether these cytokines had a direct effect on osteoclastogenesis.

Firstly, osteoclasts were differentiated in vitro with RANKL and MCSF, the two osteoclastogenesis instructing cytokines. The baseline of differentiation was determined for the condition RANKL and MCSF alone. IFN γ addition to these in vitro osteoclast cultures did not alter osteoclastogenesis relatively to baseline conditions (**Fig. 3, A and B**). Secondly, 3D cultures of osteoclasts on bone slices were performed to determine the bone resorption capacity of the formed osteoclasts. Osteoclasts formed in the presence of IFN γ similarly resorbed bone compared to the cultures without IFN γ addition (**Fig. 3C**). These data suggest that the role of IFN γ in bone loss in vivo is most likely indirect.

Because mycobacteria reside mostly inside macrophages, we hypothesized that osteoclastogenesis could be modulated by soluble factors produced by infected macrophages in the bone marrow parenchyma. So, we next repeated our in vitro experiments supplementing the osteoclast cultures with conditioned media obtained from non-infected or infected BMDM. In the presence of conditioned media from infected macrophages, the frequency of osteoclasts with more than 20 nuclei increased (**Fig. 3, D and E**). Moreover, in 3D cultures of osteoclasts on top of bone slices, more pits and trenches were formed in the presence of conditioned media from *M. avium*-infected macrophages (**Fig. 3F**). Together, our data indicate that the local production of cytokines and other soluble factors by macrophages during infection leads to an increased osteoclast production.

Taking our above findings showing that both IFN γ and TNF α are required as in vivo mediators of bone loss, that IFN γ likely acts through indirect mechanisms and that soluble factors produced by infected macrophages induce osteoclast production, we hypothesize that IFN γ may act on macrophages during infection to potentiate TNF α production, which in turn directly or indirectly promotes osteoclast activity. Thus, we firstly measured TNF α production by *M. avium* infected macrophages in the absence or presence of IFN γ . As expected (25), the production of TNF α was elevated in the presence of IFN γ (**Fig. 3G**). TNF α treatment of osteoclast cultures reduced the number of nuclei per osteoclast (**Fig. 3, H and I**), whilst inducing osteoclasts to perform more resorption events, namely trenches (**Fig. 3G**), as compared to baseline conditions. Overall, our results indicate that TNF α directly promotes osteoclast activity by increasing the proportion of trenches formed on the bone surface. Contrary to the standard mode of bone resorption (the pit mode), the trenches result from a faster and longer resorption of bone as the osteoclasts move, creating deeper and parallel erosions on the bone surface (26, 27), suggesting a more aggressive resorption mode. Additionally, our data also indicate that other soluble factors in produced by infected macrophages, in addition to TNF α , may impact osteoclast formation.

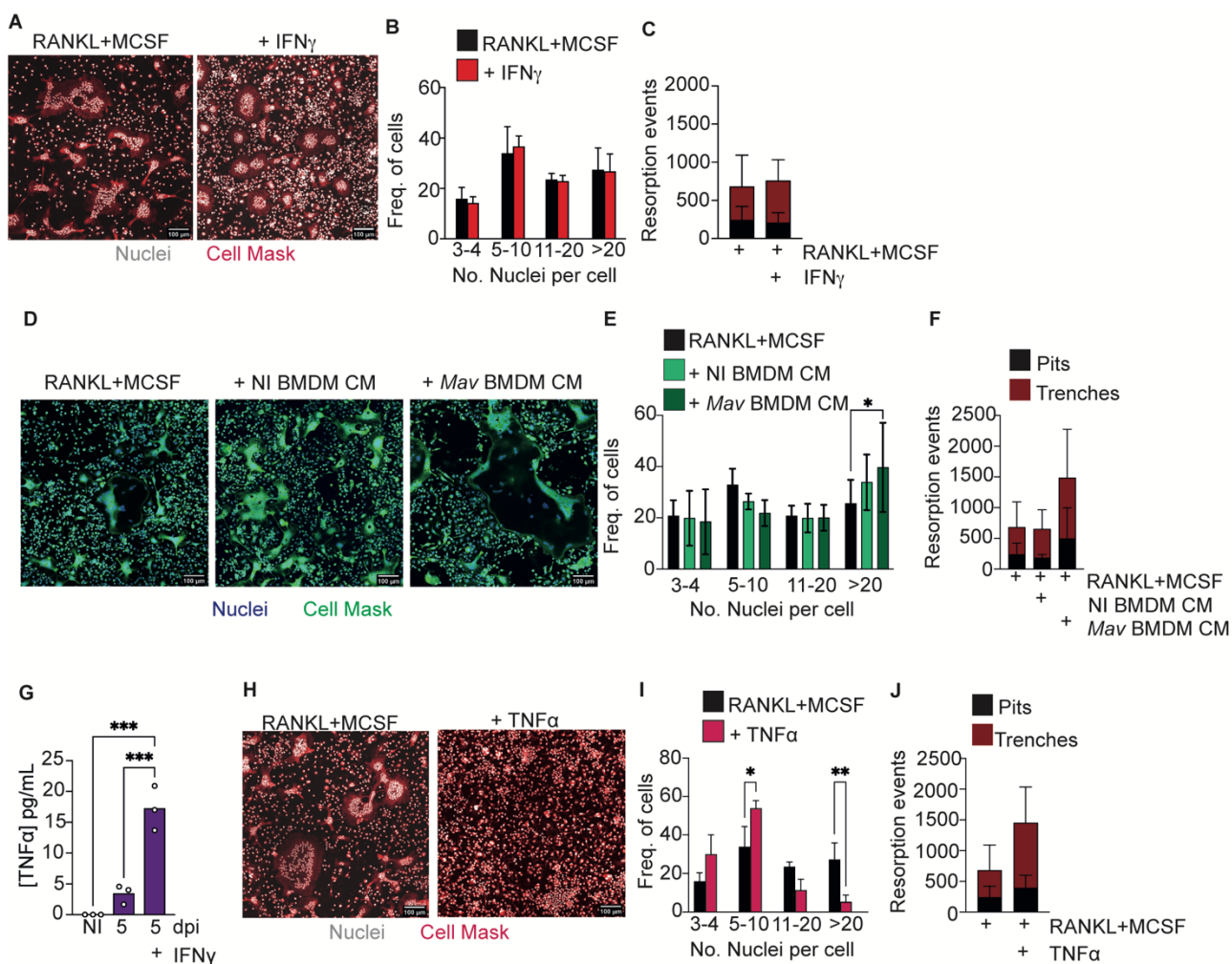


Figure 3. Soluble factors produced by *M. avium*-infected macrophages increase osteoclastogenesis. **A)** Osteoclast differentiation in the presence of saturating amounts of M-CSF and RANKL (left panel) and with the addition of 1 ng/mL of IFN γ . **B)** Frequency of multinucleated cells grouped by number of nuclei per cell. Data are pooled from three independent experiments. Bars represent the average $\pm$ standard deviation of the three independent experiments. **C)** Enumeration of pits (black bars) and trenches (red bars). Data are representative of two independent experiments. Bars represent the average $\pm$ standard deviation of 4 replicates in each experimental group. **D)** Osteoclast differentiation in the presence of saturating amounts of M-CSF and RANKL (left panel) and with the addition of conditioned media (CM) from non-infected (NI BMDM, middle panel) and *M. avium*-infected bone marrow-derived macrophages (*Mav* BMDM) (right panel). **E)** Frequency of multinucleated cells grouped by number of nuclei per cell. Data are pooled from six independent experiments. Bars represent the average $\pm$ standard deviation of the six independent experiments. *, $p < 0.05$, by Two-way ANOVA. **F)** Enumeration of pits (black bars) and trenches (red bars). Data are representative of two independent experiments. Bars represent the average $\pm$ standard deviation of 4 replicates in each experimental group. **G)** Measurement of TNF α in conditioned media from bone marrow derived and *M. avium* infected-macrophages. **H)** Osteoclast differentiation in the presence of saturating amounts of M-CSF and RANKL (left panel) and with the addition of TNF α (right panel). **I)** Frequency of multinucleated cells grouped by number of nuclei per cell. Data are pooled from four independent experiments. **J)** Enumeration of pits (black bars) and trenches (red bars). Data are representative of two independent experiments. Bars represent the average $\pm$ standard deviation of the experimental group. *, $p < 0.05$, ** $p < 0.01$ by Two-way ANOVA.

3.4 SAA3 levels increase both locally and systemically during mycobacterial infection

In the search for factors other than TNF α involved in increased osteogenesis, we noted that the most differentially upregulated gene in the bones of *M. avium*-infected mice was *Saa3* (**Fig. 4A**). This gene codes for the Serum Amyloid A (SAA)3 protein, which has been recently implicated in bone homeostasis (28, 29, 30, 31), namely by enhancing osteoclastogenesis (30). Quantitative real-time PCR analysis confirmed that the expression of *Saa3* is upregulated in the bone during *M. avium* infection (**Fig. 4B**). Moreover, the expression of *Saa3* in the bone was dependent on the production of both IFN γ and TNF α , as mice deficient in either of these cytokines do not upregulate *Saa3* expression in the bone during *M. avium* infection (**Fig. 4, C and D**). Importantly, in mice infected with a low dose of *M. tuberculosis* by the aerosol route, the expression of *Saa3* in the bone marrow was also upregulated as compared with non-infected mice (**Fig. 4E**).

We also found that *Saa3* was upregulated in BMDM 5 days after *M. avium* infection and was further augmented by the addition of TNF α to the macrophages' cultures (**Fig. 4F**). Corroborating the gene expression data, SAA3 was present in the conditioned media of infected macrophages, and the levels were further increased when infected macrophages were treated with TNF α (**Fig. 4G**), as expected (32, 33, 34, 35).

To determine whether the alterations in *Saa3* expression in the bone correlated with the systemic levels of the cytokine, SAA3 levels were measured in the serum of *M. avium*-infected and non-infected mice. Indeed, high concentrations of SAA3 were found in the serum of infected mice at 8 weeks post-infection (**Fig. 4H**).

Thus far, our results suggest that during *M. avium* infection, the production of IFN γ leads to the activation of macrophages with the concomitant production of TNF α and SAA3. In the absence of TNF α , SAA3 was not expressed in the bone and the bone mass was not altered. Additionally, SAA3 was produced by TNF α -stimulated *M. avium*-infected macrophages.

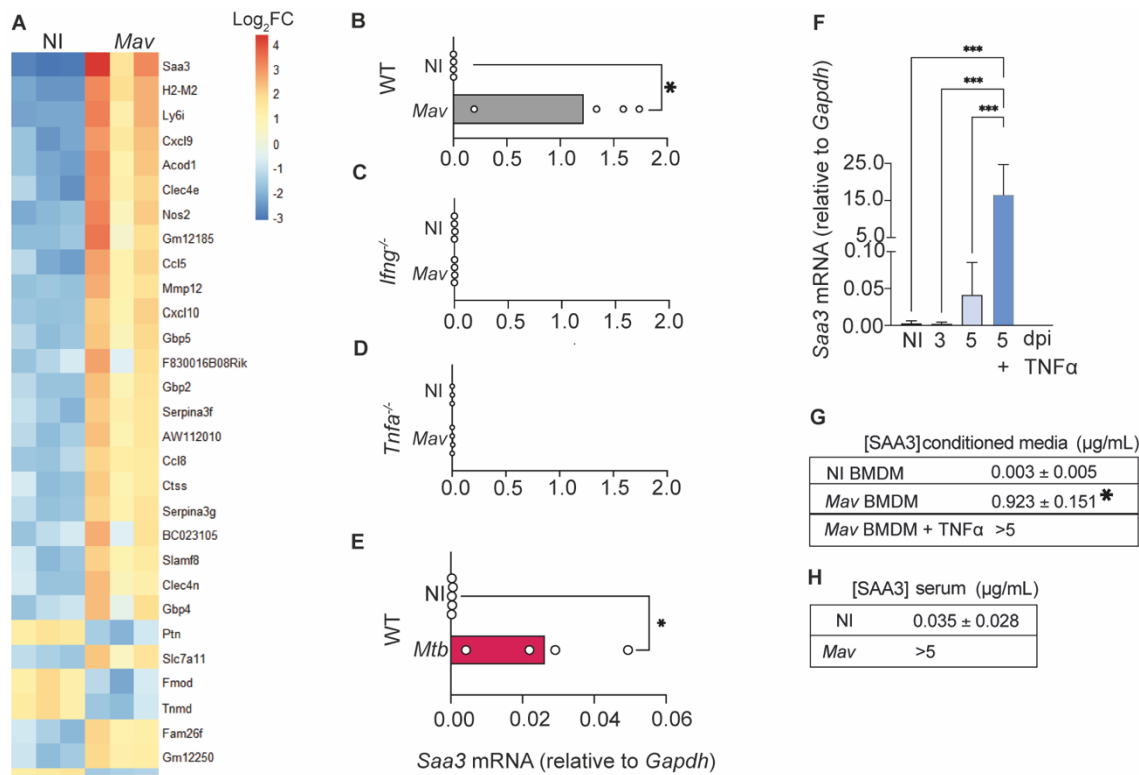


Figure 4. *M. avium*-infected macrophages produce SAA3. **A)** Heatmap representing the 30 most differentially expressed genes in the femurs of *M. avium*-infected mice (*Mav*) compared with non-infected controls (NI). **B-D)** *Saa3* expression relative to *Gapdh* in the femurs of non-infected (white bars) and *M. avium*-infected (gray bars) wild-type (WT, **B**), *Ifng*^{-/-} (**C**), and *Tnfa*^{-/-} (**D**) mice. Bars indicate mean and circles depict individual mice analyzed. **E)** *Saa3* expression in the bone marrow of mice infected with *M. tuberculosis* by the aerosol route. Bars indicate the average and circles depict each individual mouse. *, p < 0.05, by Student's *t* test. **F)** *Saa3* expression relative to *Gapdh* in bone marrow derived macrophages. Bars represent the average ± standard deviation of the 4 replicates in each experimental condition. **G)** Quantification of SAA3 in conditioned media from bone marrow derived macrophages, expressed as average ± standard deviation of the experimental condition. Results are pooled from 3 independent experiments. *, p < 0.05, ***, p < 0.001 by two-way ANOVA. **H)** Measurement of SAA3 in the serum of *M. avium*-infected mice (*Mav*) compared to non-infected controls (NI) expressed as average ± standard deviation of the experimental group. N = 5 mice per group.

3.5 SAA proteins directly impact bone turnover and are a potential biomarker of bone loss in patients with active Tuberculosis

We next decided to test whether SAA proteins directly impact bone turnover. SAA proteins are well-conserved throughout the mammalian radiation (36) and have several isoforms (37). Although the human *Saa3* is a non-translated pseudogene (38), the human SAA1 and SAA2 display a remarkable homology to mouse SAA3 (30). To test if human SAA would also influence osteoclast differentiation, we used our previously described in vitro system incubating osteoclasts in the presence of human recombinant SAA1 and SAA2 proteins (human SAA). Osteoclastogenesis was increased (**Fig 5, A and B**) but the number of resorption events was decreased (**Fig. 5C**), indicating that besides SAA other soluble factors are important for the increased bone resorption. According to our previous data, TNFα is a likely candidate. Indeed, in osteoclasts cultured in the presence of TNFα and human SAA, we not only observed an increase of osteoclastogenesis, but also of osteoclast activity, measured as the

proportion of trenches formed (**Fig. 5, D and F**). Overall, these results indicate that SAA and TNF α , which are produced by infected macrophages during mycobacteria infection, co-operate as pro-osteoclastogenic factors, which during mycobacteria infection are produced by infected macrophages.

Besides increasing osteoclastogenesis, SAA3 was also reported to inhibit osteoblastogenesis (28, 30, 31). Thus, we interrogated our transcriptomic data (**Fig. 2A**) for possible differences in bone morphogenesis, bone development and growth, and ossification pathways and found that they were among the most differentially and statistically downregulated in the femurs of *M. avium*-infected mice compared with the femurs of littermate control mice (**Fig. 5G**). This finding led to the hypothesis that osteoblastogenesis may also be impaired during mycobacterial infections. To test whether human SAA might also impact on osteoblastogenesis, MSC from the bone marrow of non-infected mice were isolated and osteoblast formation in vitro assays were performed in the presence and absence of human SAA. Osteoblast formation and bone matrix mineralization was detected by alizarin red staining. The addition of human SAA significantly reduced the calcium deposits in the cultures (**Fig. 5H**).

The incidence of non-tuberculous mycobacterial infections, as *M. avium*, has been rising (39), namely in cystic fibrosis patients (40) and even in seemingly healthy children and adults (41). However, *M. tuberculosis* remains one of the deadliest human pathogens (11). Given the data suggesting a role for SAA in *M. avium* and *M. tuberculosis* mouse infection, as well as for human SAA in modulating osteoclast/osteoblast homeostasis, we went on to investigate whether the levels of SAA might change during active tuberculosis. For that, circulating levels of TNF α and SAA were measured in the plasma of a cohort of patients with active tuberculosis compared to those detected in healthy controls of unknown IGRA status or for IGRA+ (latent tuberculosis infection, LTBI) individuals. While TNF α serum levels were similar between the three groups (**Fig. 5I**), patients with active tuberculosis showed higher systemic SAA levels as compared to both healthy and LTBI controls (**Fig. 5J**). To correlate the alterations seen in SAA proteins to bone loss, we next measured the circulating levels of CTX-I and osteopontin (OPN) as biomarkers of bone degradation, and of osteocalcin (OC) as a biomarker of bone formation (42), in the plasma of the study cohort. We found an increased amount of CTX-I in active tuberculosis patients which did not reach statistical significance (**Fig. 5K**). The circulating levels of OPN was higher in the LTBI and active TB groups compared with healthy controls (**Fig. 5L**). Bone formation was also impaired in LTBI and TB active groups as OC levels were reduced in these individuals compared to healthy controls (**Fig. 5M**). Of note, there was a good correlation between the systemic levels of SAA, CTX-I, OPN and TNF α in patients with active TB (**Fig. 5N**). These findings highlight the potential for the use of the systemic levels of SAA proteins as a biomarker of increased risk of bone loss during chronic mycobacterial infections.

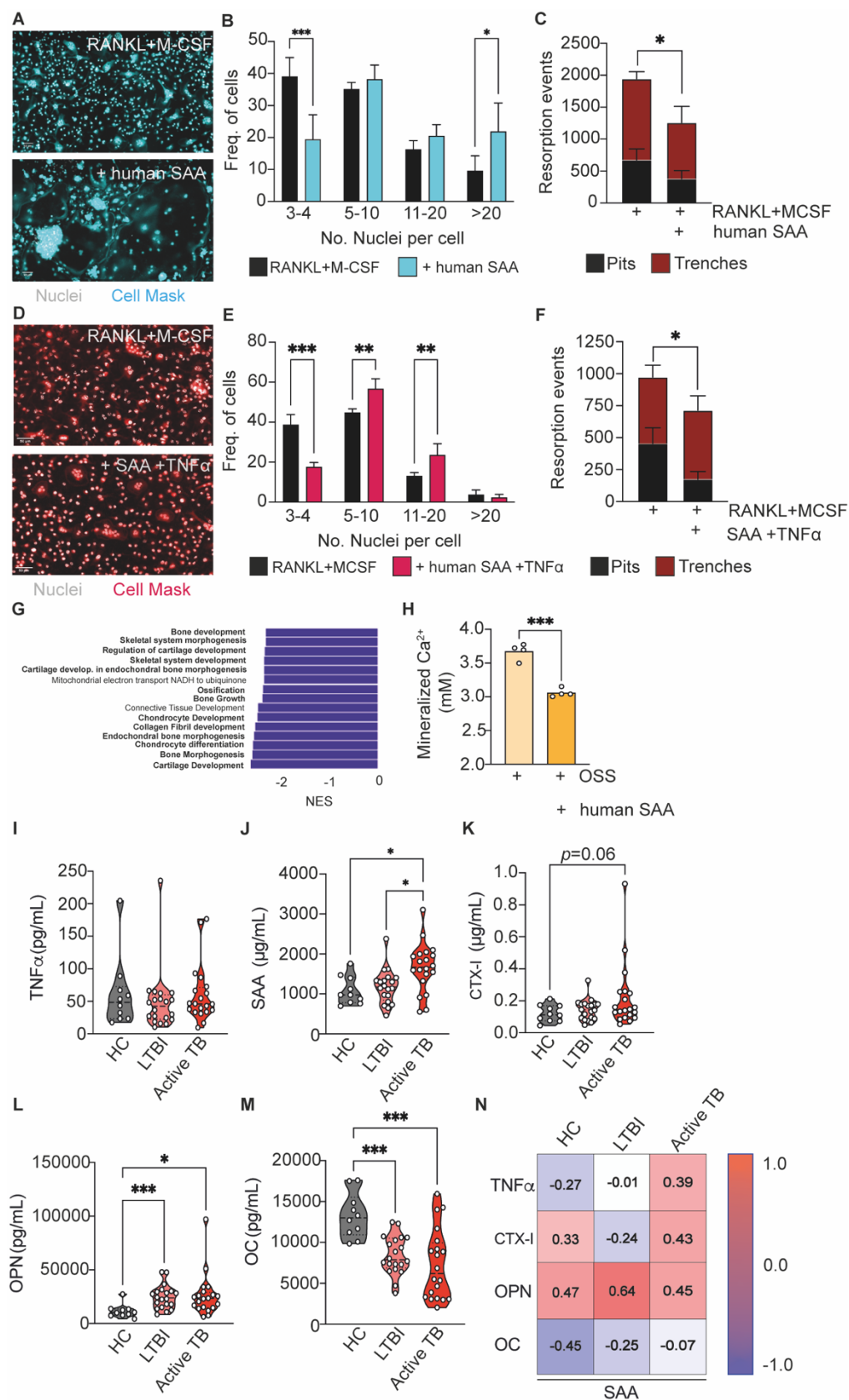


Figure 5. Human SAA increase osteoclastogenesis and decrease osteoblastogenesis and correlate with altered markers of bone turnover. **A)** Osteoclast differentiation in the presence of saturating amounts of M-CSF and RANKL (top panel) and with the addition of human SAA (bottom panel). Scale bar: 50 μm **B)** Frequency of

multinucleated cells grouped by number of nuclei per cell. Data are pooled from three independent experiments. Bars represent the average $\pm$ standard deviation of the three independent experiments. **C)** Enumeration of pits (black bars) and trenches (red bars). Data are representative of two independent experiments. Bars represent the average $\pm$ standard deviation of 4 replicates in each experimental group. **D)** Osteoclast differentiation in the presence of saturating amounts of M-CSF and RANKL (left panel) and with the addition of human SAA and TNF α (right panel). Scale bar: 50 μ m **E)** Frequency of multinucleated cells grouped by number of nuclei per cell. Data are pooled from two independent experiments. Bars represent the average $\pm$ standard deviation of the two independent experiments. **F)** Enumeration of pits (black bars) and trenches (red bars). Data are representative of two independent experiments. Bars represent the average $\pm$ standard deviation of 4 replicates in each experimental condition. *, $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$ by Two-way ANOVA. **G)** Top15 of most differently enriched pathways (from the GO Biological Process dataset) in the bones of infected mice compared to non-infected littermate controls. **H)** Quantification of deposited calcium of murine bone marrow MSC cultured with osteoblast stimulatory supplement (OSS) plus 5 μ g/mL human SAA. Bars indicate average; each circle depicts each of 4 replicates per experimental condition. Data are representative of two independent experiments. ***, $p < 0.001$ by Student's t test. **I-M)** Measurement of TNF α (**I**), SAA (**J**), CTX-I (**K**), Osteopontin (OPN, **L**), Osteocalcin (OC, **M**) in the serum of healthy controls (HC), IGRA+ with latent tuberculosis infection (LTBI) individuals, and patients with active Tuberculosis (active TB). Violin plots depict the density, and each circle represents each patient. *, $p < 0.05$, ***, $p < 0.001$ by two-way ANOVA. **N)** Pearson correlations between circulating levels of SAA and TNF α , or each of the different bone turnover markers.

4 Discussion

Although it is well established that mycobacteria can infect the bone (2) and that previous mycobacterial infection increases the risk of osteoporosis (4, 5), very little evidence of the pathophysiology of these alterations has been gathered. Our study shows that during chronic disseminated *M. avium* infection, IFN γ and TNF α induce bone loss, due to both increased resorption and decreased new bone formation. Our work advances the growing body of evidence supporting a reciprocal interaction between bone homeostasis and hematopoietic development. On one hand, alterations in LEPR⁺ MSC correlated with decreased bone formation. LEPR⁺ MSC were described as the major source of osteoblasts and adipocytes in the adult bone marrow (20) while they nurse hematopoietic maintenance and differentiation (17, 43), reinforcing the close relationship between hematopoiesis and the bone (3). In line with our observations, a previous study demonstrated that sepsis altered both hematopoietic stem cells and hematopoiesis supporting-MSC leading to a reduction in lymphocyte and osteoblast production (44). Previous reports have implied the bone marrow as a niche for mycobacteria (45, 46), even during intranasal infection (47). Besides macrophages, hematopoietic (48) and mesenchymal (49) stem cells are colonized by mycobacteria in the bone marrow, which may explain the hematopoietic (7) and bone dysfunctions.

Our study reveals that both IFN γ and TNF α are critically involved in the pathophysiology of bone loss during mycobacterial infection, which is consistent with their recognized effects on the hematopoiesis and the above-mentioned interconnections between hematopoiesis and bone homeostasis. Interestingly, it was demonstrated that patients with autosomal dominant IFN γ R1 or STAT1 deficiency showed enhanced osteoclastogenesis (3), which could be contradictory with our findings. However, this observation may be explained by the variable penetrance of disease (50) and by the fact that T cells from these patients still produce TNF α upon stimulation (51), which could mediate the increased osteoclastogenesis. The study of possible bone loss in these patients warrants further research and may shed light into alternative or complementary mechanisms to those reported here.

The study of gene expression in the bones of mice revealed that one of the genes which is most upregulated by mycobacterial infection is *Saa3*. This is in agreement with previous observations that

Saa3 expression is upregulated in bone marrow-derived macrophages and in the lungs after infection with *M. tuberculosis* (46, 48). SAA proteins are described as acute phase proteins that are expressed mostly by hepatic macrophages in the liver (32) but also by monocytes and macrophages in other tissues, chondrocytes, adipocytes, and osteoblasts in response to inflammatory stimuli (52, 53, 54, 55, 56, 57, 58, 59, 60, 61). More recently, SAA were shown to have both catabolic and anti-anabolic roles in bone turnover (28, 29, 30, 31). Here, we showed that SAA proteins are produced by macrophages after infection with mycobacteria and that the levels of these proteins are significantly increased in circulation, both in experimental models of infection with mycobacteria and in humans with LTBI or active TB. We experimentally confirmed that SAA, together with inflammatory cytokines, directly contributes to increased bone degradation and decreased bone formation (**Figure 5**). Additionally, we found evidence that SAA proteins strongly associate with bone alterations in both *M. avium* and *M. tuberculosis* infections, being also altered in the serum of TB patients.

Biochemical markers of bone turnover such as CTX-I, OPN and OC provide a non-invasive tool for the clinical assessment and for guiding and monitoring of bone loss treatment (42). Nevertheless, some studies have reported that despite no alteration in bone turnover markers levels, alterations in the microstructure and mineral density may be already observed by high resolution peripheral computed tomography (HR-pQCT) and dual X-ray absorptiometry (DXA), respectively (62, 63, 64), in the absence of alterations in bone turnover markers. As DXA and HR-pQCT are not routinely performed on tuberculosis patients, relying on CTX-I, OPN and OC in the serum may underestimate the impact of infection on the bone health of these individuals. Our results are the first evidence correlating the circulating levels of SAA proteins and bone turnover markers in humans with active tuberculosis. A prospective study to correlate the circulating SAA levels and the bone mineral density in a cohort of tuberculosis patients is warranted, to assess the utility of SAA as a new bone loss marker.

Furthermore, as we show that SAA3 production and bone loss are dependent on inflammatory cytokines, the clinical relevance of SAA proteins as a biomarker of bone loss could be extended to other pathologies such as chronic cardiovascular diseases (65, 66), inflammatory rheumatic diseases as well as COVID-19 (67), in which the systemic levels of SAA are elevated. In the long run, these results will also contribute to a deeper understanding of the mechanisms underlying bone loss during inflammatory diseases and consequently to the proposal of new therapeutic strategies to prevent or cure these disorders.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

ACG, ML and MSG supervised the study. ACG conceptualized, designed and performed the experiments. ACG, MS and MSG wrote the manuscript. DMS conceptualized and performed some experiments. TCO, OF, DS, ACM and TS performed experiments. AF, MJT and MS provided human samples. RP and LP performed the transcriptomic analysis.

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10 Data availability

The RNAseq data is available in GEO under the accession number GSE215856 and can be accessed using the following token: edwpcwserhgxf.

Annex V

Ethical Committee approval documentation

23 de Fevereiro de 2022

A Diretora do Centro de Epidemiologia Hospitalar

(Prof.ª Doutora Ana Azevedo)



DIRECÇÃO CLÍNICA

23/2/2022

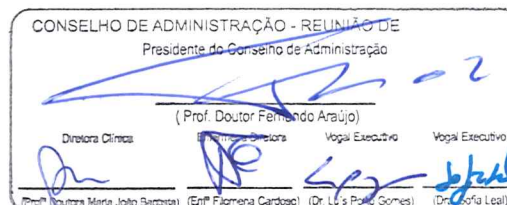
n.º 407 / 21

PEDIDO DE AUTORIZAÇÃO

Realização de Investigação

Exmo. Senhor Presidente do Conselho de Administração
do Centro Hospitalar de São João

Nome do Investigador Principal:
Ana Rita Azevedo Cordeiro Gomes



Título da Investigação:

IDENTIFICAÇÃO DE NOVOS BIOMARCADORES PARA
DETEÇÃO PRECOCE DE REDUÇÃO DA MASSA ÓSSEA
DURANTE INFEÇÕES PULMONARES CRÓNICAS (BONE
SAAv3r)

Pretendo realizar no(s) Serviço(s) de:
Pneumologia e Reumatologia

a investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador/Promotor, autorização para a sua efetivação.

Para o efeito, anexo toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de São João/Faculdade de Medicina da Universidade do Porto respeitante à investigação, à qual enderecei pedido de apreciação e parecer.

Com os melhores cumprimentos.

O Investigador/Promotor

Porto, 12 de novembro de 2021.

assinatura



Encarregado
de Proteção de Dados
Data Protection Officer

Entrada

5/01/2022

• Centro Hospitalar São João •
Centro de Epidemiologia Hospitalar

14/2/2022

Parecer da Comissão de Ética do

Centro Hospitalar Universitário de São João / Faculdade de Medicina da Universidade do Porto

Título do Projeto: Identificação de novos biomarcadores para deteção precoce de redução da massa óssea durante infeções pulmonares crónicas (BONE SAAv3r)

Nome da Investigadora Principal: Doutora Ana Rita Azevedo Cordeiro Gomes (investigadora do i3S)

Onde decorre o Estudo: Nos Serviços de Pneumologia e Reumatologia do CHUSJ. Apresentou declaração do Prof. Doutor Venceslau Hespanhol e da Dra. Lúcia Costa. A profissional de ligação será a Dra. Maria Adelina Azevedo Amorim Carneiro (Pneumologia).

Objetivos do Estudo:

Identificar as proteínas SAA1 e SAA2 como potenciais biomarcadores de distúrbios no metabolismo ósseo em pacientes com fibrose quística (FQ).

Conceção e Pertinência do estudo:

Os pacientes com FQ têm um risco aumentado de perda de massa óssea, o que aumenta o risco de fraturas com consequente declínio na função pulmonar, perda da qualidade de vida, mortalidade e sobrecarga económica para os indivíduos e para o SNS.

A deteção das proteínas SAA em soro de pacientes com FQ poderá constituir um biomarcador de alterações no metabolismo ósseo.~

*No questionário para submissão de investigação, está referido que o estudo é sem intervenção, mas da análise do protocolo de estudo, trata-se de um estudo **com intervenção**, pelo que deve ser alterado.*

Benefício/risco:

Os doentes terão que ser sujeitos a uma colheita de sangue adicional, feita após a consulta de rotina no Serviço de Pneumologia, não implicando deslocações adicionais.

Confidencialidade dos dados:

As amostras serão identificadas exclusivamente por um código que inclui a sigla FQ e um número correspondente à ordem de recrutamento do doente no estudo.

Apresentou um pedido de reutilização de registos clínicos para Investigação e Desenvolvimento ao RAI.

Falta entregar o documento de avaliação de impacto sobre a prestação de dados (EPD).

Respeito pela liberdade e autonomia do sujeito de ensaio:

Dispõe de uma adequada informação ao participante e modelo de CI do CHUSJ.

Curriculum da investigadora: Adequado à investigação.

Data previsível da conclusão do estudo: dezembro de 2022

Conclusão: Proponho um parecer favorável à realização do estudo, após resposta às questões em itálico.

Porto, 17 de dezembro de 2021

O Relator da CE, Dr. John Preto



Todas as questões foram esclarecidas.

04/01/2022

John Preto



Questionário para submissão de Investigação

Exmo. Sr. Presidente da Comissão de Ética do Centro Hospitalar de São João/
Faculdade de Medicina da Universidade do Porto,

Pretendendo realizar a investigação infracitada, solicito a V. Exa., na qualidade de Investigador, a sua apreciação e a elaboração do respetivo parecer. Para o efeito, anexo toda a documentação requerida.

IDENTIFICAÇÃO DO ESTUDO

Título da investigação: Identificação de novos biomarcadores para deteção precoce de redução na massa óssea durante infeções pulmonares crónicas (BONE SAAv3r)

Nome do investigador: Ana Rita Azevedo Cordeiro Gomes

Endereço eletrónico: ana.c.gomes@i3s.up.pt

Contacto telefónico: 964812310

Caracterização da investigação:

- ☐ Estudo retrospectivo ☐ Estudo observacional ☒ Estudo prospetivo
☐ Inquérito ☐ Outro. Qual? _____

Tipo de investigação:

- ☐ Com intervenção ☒ Sem intervenção

Formação do investigador em boas práticas clínicas (GCP): ☐ Sim ☒ Não

Promotor (se aplicável): _____

Nome do orientador de dissertação/tese (se aplicável): Dra. Maria Adelina Azevedo Amorim Cordeiro

Endereço eletrónico: maria.cordeiro@chs.j.min-saude.pt

Local/locais onde se realiza a investigação: Serviços Pneumologia e Reumatologia CHUSJ e i3S - Universidade do Porto

Data prevista para início: 01 / 01 / 2022

Data prevista para o término: 31 / 12 / 2022

PROTOCOLO DO ESTUDO

Síntese dos objetivos:

O objetivo do projeto BONE SAAv3r é:

Identificar as proteínas SAA1 e SAA2 como potenciais biomarcadores de distúrbios no metabolismo ósseo em pacientes com Fibrose Cística.

Fundamentação ética (ganhos em conhecimento/ inovação; ponderação benefícios/ riscos):

Os pacientes com Fibrose Quística tem um risco aumentado de perda de massa óssea, o que aumenta o risco de fraturas com consequente declínio na função pulmonar, perda da qualidade de vida, mortalidade e sobrecarga económica para os indivíduos e para o Sistema Nacional de Saúde. Embora de causalidade multifactorial, a redução da massa óssea poderá ser agravada pela infeção pulmonar crónica. A infeção sistémica em murganhos por Mycobacterium avium cursa com diminuição da massa e mineralização óssea e aumento sistémico das proteínas SAA, que por sua vez, promovem a degradação óssea e inibem a formação óssea. Assim, a deteção das proteínas SAA em soro de pacientes com Fibrose Quística poderá constituir um biomarcador de alterações no metabolismo ósseo. Trata-se de um estudo não intervencional, com colheita de dados que fazem parte do seguimento rotineiro destes doentes, acrescido apenas da colheita de um tubo de sangue adicional no serviço de Pneumologia durante a consulta de rotina dos pacientes, não envolvendo, portanto, deslocações adicionais. A participação dos doentes será completamente voluntária e sujeita ao seu consentimento informado.

CONFIDENCIALIDADE

De que forma é garantida a anonimização dos dados recolhidos de toda a informação?

As amostras serão identificadas exclusivamente por um código que inclui a sigla FQ e um número correspondente à ordem de recrutamento do doente no estudo.

O investigador necessita ter acesso a dados do processo clínico?

☒ Sim ☐ Não

Está previsto o registo de imagem ou som dos participantes?

☐ Sim ☒ Não

Se sim, está prevista a destruição deste registo após o sua utilização?

☐ Sim ☐ Não

CONSENTIMENTO

O estudo implica recrutamento de:

Doentes: ☒ Sim ☐ Não

Voluntários saudáveis: ☐ Sim ☒ Não

Menores de 18 anos: ☐ Sim ☒ Não

Outras pessoas sem capacidade do exercício de autonomia: ☐ Sim ☒ Não

A investigação prevê a obtenção de Consentimento Informado: ☒ Sim ☐ Não

Se não, referir qual o fundamento para a isenção:

Existe informação escrita aos participantes: ☒ Sim ☐ Não

PROPRIEDADE DOS DADOS

A investigação e os seus resultados são propriedade intelectual de:

☐ Investigador ☐ Promotor ☒ Ambos ☐ Serviço onde é realizado

☐ Não aplicável Outro: _____

BENEFÍCIOS, RISCOS E CONTRAPARTIDAS PARA OS PARTICIPANTES

Benefícios previsíveis:

A identificação das proteínas SAA como biomarcador do risco de desenvolvimento de fraturas constituirá um marco na abordagem e deteção da redução da massa óssea em pacientes com bronquiectasias complicadas por infeção crónica. Assim, poderá levar a uma melhoria da qualidade de vida e redução da morbilidade e mortalidade nestes pacientes.

Riscos/incómodos previsíveis:

Os doentes terão que ser sujeitos a uma colheita de sangue adicional. Esta colheita será feita após a sua consulta de rotina no Serviço de Pneumologia do CHUSJ, não implicando, portanto, quaisquer deslocações adicionais para o paciente.

São dadas contrapartidas aos participantes:

· pela participação ☐ Sim ☒ Não ☐ Não aplicável

· pelas deslocações ☐ Sim ☐ Não ☒ Não aplicável

· pelas faltas ao emprego ☐ Sim ☐ Não ☒ Não aplicável

· por outras perdas e danos ☐ Sim ☐ Não ☒ Não aplicável

CUSTOS / PLANO FINANCEIRO

Os custos da investigação são suportados por:

☒ Investigador ☐ Promotor ☐ Serviço onde é realizado

☐ Não aplicável Outro: _____

Existe protocolo financeiro? ☐ Sim ☒ Não

LISTA DE DOCUMENTOS ANEXOS

- ☒ Pedido de autorização ao Presidente do Conselho de Administração do Centro Hospitalar de São João (se aplicável)
- ☐ Pedido de autorização à Diretora da Faculdade de Medicina da Universidade do Porto (se aplicável)
- ☒ Protocolo do estudo
- ☒ Declaração do Diretor de Serviço onde decorre o estudo
(sendo um estudo na área de enfermagem deve anexar também a concordância da chefia de enfermagem)
- ☒ Profissional de ligação
- ☒ Informação dos orientadores
- ☒ Informação ao participante
- ☒ Modelo de consentimento
- ☐ Instrumentos a utilizar (inquéritos, questionários, escalas, p.ex.): _____
- ☒ Curriculum Vitae abreviado (máx. 3 páginas)
- ☐ Protocolo financeiro
- ☐ Outros:

COMPROMISSO DE HONRA E DECLARAÇÃO DE INTERESSES

Declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (1960 e respetivas emendas), e da Organização Mundial da Saúde, Convenção de Oviedo e das "Boas Práticas Clínicas" (GCP/ICH) no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo, nos últimos três meses. Comprometo-me a entregar à CES o relatório final da investigação, assim que concluído.

Porto, 12 de novembro de 2021

Nome legível: Ana Rita Azevedo Cordeiro Gomes



assinatura

Parecer da Comissão de Ética do Centro Hospitalar de São João/FMUP

Emitido na reunião plenária da CE de 17 / 12 / 2021

Aguardar esclarecimentos.

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

Centro Hospitalar São João.

CONSIDERADOS QUE FORAM COMO SATISFATÓRIOS OS
ESCLARECIMENTOS PRESTADOS PELO(A)
INVESTIGADOR(A), A CES APROVA POR UNANIMIDADE O
PARECER DO RELATOR, PELO QUE NADA TEM A OPOR À
REALIZAÇÃO DESTA PROJETO DE INVESTIGAÇÃO.

04 / 01 / 2022

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

RESPONSÁVEL PELO ACESSO À INFORMAÇÃO



Pedido de Reutilização de Registos Clínicos para Investigação e Desenvolvimento (I&D)

Exmo. Senhor
Responsável pelo Acesso à Informação
(Artigo 9.º da Lei n.º 26/2016, de 22 de agosto)
Dr. Rui de Vasconcellos Guimarães



Número do Pedido

22000123
(A preencher pelo Gabinete de Apoio ao RAI)

AUTORIZADO

RAI Responsável pelo Acesso à Informação no Centro Hospitalar de São João
Art. 9º, Lei 26/2016, de 22/8)

06/01/2022

1. Identificação do(s) Investigador(es) Preenchimento Obrigatório

1.1. Investigador Principal No caso de haver mais do que um investigador mencionar nas observações

Nome Ana Rita Azevedo Cordeiro Gomes

Contacto telefónico 9 6 4 8 1 2 3 1 0

Endereço eletrónico ana.c.gomes @i3s.up.pt

1.2. Investigador(es) Associado(s)

Número Total: 1

Nome Maria Adelina Azevedo Amorim Cordeiro

Contacto telefónico 9 6 5 5 9 1 5 2 4

Endereço eletrónico maria.cordeiro @chsj.min-saude.pt

Nome Georgina Terroso

Contacto telefónico 9 6 3 9 8 9 5 9 3

Endereço eletrónico georgina.terroso @chsj.min-saude.pt

Nome Maria Salomé Gomes

Contacto telefónico

Endereço eletrónico sgomes @ibmc.up.pt

1.3. Afiliação Institucional do Investigador Principal

1.3.1. Grupo Profissional

☐ Médico(a) ☐ Enfermeiro(a) ☐ Docente ☒ Estudante
☒ Outro. Qual? Investigadora - i3S

1.3.2. Documento de identificação pessoal ou profissional

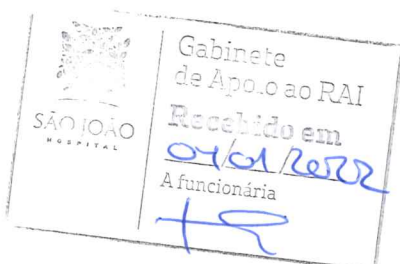
☒ Cartão de Cidadão ☐ Bilhete de Identidade ☐ Cédula Profissional
☐ Cartão de Docente ☐ Cartão de Estudante ☐ Outro. Qual?

Número de Documento 1 3 1 8 2 3 6 8

2. Enquadramento e Identificação do Trabalho de Investigação e Desenvolvimento Preenchimento Obrigatório

2.1. Enquadramento da investigação

☒ Projeto ou trabalho académico de investigação e desenvolvimento:
☐ Não conferidor de grau
☒ Conferidor de grau: ☐ Licenciatura ☒ Mestrado ☐ Doutoramento



2.2. Entidade(s) que tutela(m) a investigação

☒ Centro Hospitalar de São João
Serviço: Pneumologia e Reumatologia

☒ Universidade do Porto
Faculdade / Instituto: i3S - Instituto de Investigação e Inovação em Saúde - Universidade do Porto

☐ Outra Instituição. Qual? _____

Há alguma parceria entre instituições?

☒ Não ☐ Sim. Qual(is)? _____

2.3. Orientador Se Aplicável

Contacto telefónico 9 6 5 5 9 1 5 2 4

Endereço eletrónico maria.cordeiro @ chsj.min-saude.pt

2.4. Título provisório

Identificação de novos biomarcadores para deteção precoce de redução da massa óssea durante
infecções pulmonares crónicas (BONE SAAv3r)

*Deverá posteriormente indicar o título definitivo para emissão do Certificado de Reutilização pelo RAI –
DAta REuse Certificate for Research – DARE através dos contactos disponíveis no fim deste formulário.*

2.5. Acesso requerido

☐ Ficheiro

Descrição do património informacional a que pretende ter acesso, identificando a informação a obter, i.e. nome, morada, diagnóstico, idade, códigos dos distritos, entre outros.

☐ Consulta de processos clínicos em ambiente papel:

☐ Bloco

☐ Consulta Externa

☐ Hospital de Dia

☐ Internamento

☐ MCDT

☐ Urgência

*Deverá anexar ficheiro(s) contendo a identificação do pretendido, i.e. números de processos,
episódios, números de utente, entre outros.*

Anexar ficheiro no ato de envio

☒ Consulta de registos clínicos eletrónicos

Especificar os Sistemas de Informação:

S Clínico

Data previsível de fim de utilização das credenciais de acesso 2 | 0 | 2 | 2 - 1 | 2 - 3 | 1

☐ Outro Acesso. Qual? _____

2.3. Pareceres e Autorizações

☒ Autorização da Hierarquia

☐ Protocolo Científico Aprovado¹

☒ Parecer da Comissão de Ética para a Saúde (CES)¹

☐ Parecer do Centro de Epidemiologia Hospitalar¹

Deverá anexar ficheiro(s) contendo cópia dos documentos referentes às opções selecionadas.

Anexar ficheiro no ato de envio

¹ Obrigatório quando aplicável.

3. Observações Preenchimento Facultativo

Margarida Redondo: 964010652; margarida.redondo@chsj.min-saude.pt

Rita Boaventura: 964878798; rita.p.boaventura@chsj.min-saude.pt

Salomé Garcia: salome.garcia@chsj.min-saude.pt

4. Aceitação dos Termos e Condições da Reutilização

Cumulativamente com as obrigações decorrentes da lei já citada (n.º 2 e 3 do art.º 21 e o n.º 1 e 2 do art.º 12, ambos da Lei n.º 26/2016, de 22 de Agosto) ao submeter o presente pedido concordo e fico ainda vinculado, juridicamente, aos seguintes termos e condições:

- Comprometo-me a manter confidencial toda a informação à qual vou ter acesso;
- Após explicação do RAI do CHUSJ, embora a Lei 26/2016, de 22 de agosto, imponha como requisito a anonimização sem possibilidades de reversão, tal desiderato, é não só uma impossibilidade matemática já comprovada, como ainda resulta num prejuízo para a investigação, face à quantidade e qualidade da informação a retirar à fonte, razão pela qual, concordando com o RAI, assumimos como compromisso a pseudo anonimização, o que impõe uma avaliação e gestão do risco, num quadro ético-jurídico que aceitamos e nos comprometemos a colaborar;
- Não vou elaborar registos, suscetíveis de identificar ou tornar identificável a identidade das pessoas a quem os mesmos dizem respeito;
- Comprometo-me a consultar os processos clínicos nos termos e locais que me forem indicados para o efeito;
- Além do presente pedido para reutilizar registos clínicos, dirigido ao RAI, comprometo-me a obter os necessários pareceres quer do Encarregado da Proteção de Dados, quer da Comissão de Ética do Hospital, quer ainda do Centro de Epidemiologia Hospitalar;
- Comprometo-me a citar as fontes, sempre que publicitar o trabalho de investigação, independentemente de requerer a Certidão de Reutilização (**DAtaREuseCertificate for Research – DARE**).
- Tomei conhecimento, que a violação de qualquer dos compromissos aqui assumidos, resultará no apuramento de responsabilidades disciplinares, civis e penais e ainda, à impossibilidade futura de aceder a informação de saúde para fins de investigação.

5. Decisão do investigador sobre requerer a DAta REuse Certificate for Research – DARE Preenchimento Obrigatório

☒ Pretendo desde já requerer a Certidão de Reutilização (DARE) cujo sentido, valor e significado consultei em <http://portal-chsj.min-saude.pt/pages/710>.

☐ Não pretendo requerer a Certidão de Reutilização (DARE) cujo sentido, valor e significado consultei em <http://portal-chsj.min-saude.pt/pages/710>.

Na circunstância do requerente não indicar nenhuma opção, presumimos que pretende requerer o DARE.

6. Assinatura

Nota 1: Se o presente pedido for submetido eletronicamente ou faz assinatura digital qualificada; ou posteriormente vem ao Centro Hospitalar de São João exibir o seu documento de identificação pessoal; ou no âmbito do seu espaço de liberdade e como manifestação expressa do seu consentimento envia cópia do referido documento, neste caso, concluído o processo ser-lhe-á devolvida ou eliminada a cópia do documento de identificação pessoal, conforme as indicações que dê.

Nota 2: Se o presente pedido for entregue presencialmente, assina e exibe o documento de identificação a quem recebe o pedido.

Data 2021 - 11 - 12



Investigador Principal

Em caso de dúvida no preenchimento contacte através dos endereços eletrónicos
rai.reutilizacao.id@chsj.min-saude.pt ou ruiguimaraes@chsj.min-saude.pt
ou pelos números de telemóvel 962 204 194 ou 918 880 299

SUBMITER

Título do Projeto	Identificação de novos biomarcadores para deteção precoce da redução da massa óssea durante infeções pulmonares crónicas		
Responsável pelo tratamento	Ana Rita Azevedo Cordeiro Gomes		
Instituição	Centro Hospitalar Universitário de São João (CHUSJ) Instituto de Investigação e Inovação em Saúde (i3S)		
Investigador	Interno	Externo	
Contacto telefónico	964812310	Endereço Electrónico	ana.c.gomes@i3s.up.pt
Profissional de Ligação	Maria Adelina Azevedo Amorim Cordeiro (Assistente Hospitalar Graduado Pneumologia)		
Amostra	50		
Análise de Risco	Tolerável	Baixo	Elevado Muito Elevado

Parecer do EPD:

Data: 10/02/2022

Finalidade: Os principais objetivos deste projeto são: 1) Identificar as proteínas SAA1 e SAA2 como potenciais biomarcadores de distúrbios no metabolismo ósseo em pacientes com FQ. 2) Determinar se os pacientes com FQ tem uma capacidade de reabsorção óssea aumentada através da realização de estudos in vitro de diferenciação e atividade osteoclástica a partir de células mononucleares do sangue periférico.

Licitude: fundamento previsto no artigo 6(1)(a) e 9(2)(a) do RGPD.

Categorias de dados pessoais: variáveis identificadas com detalhe na AIPD, datada de 07/01/2022, ponto 13 e protocolo de investigação, tendo presente o princípio da minimização dos dados.

Conservação: os dados serão alvo de pseudonimização, armazenados em local seguro, em área restrita ao Investigador Principal e Equipa de Investigação, com acesso a ficheiros protegido por palavra-passe, efetuando-se a conservação até a conclusão da investigação, durante o prazo máximo de dez (10) anos. Os dados recolhidos serão destruídos após a finalização do estudo.

Comunicação de Dados: não há partilha de dados pessoais.

Face ao exposto, e observadas as recomendações, entende-se que a presente AIPD apresenta os elementos necessários para assegurar que o tratamento é realizado em conformidade com o RGPD.

Recomendações:

- Sempre que o consentimento do titular for o fundamento de legitimidade adequado para o tratamento de dados, dever-se-á assegurar que o mesmo é válido nas condições legalmente exigíveis: *uma manifestação de vontade livre, específica, informada e inequívoca*. Neste contexto, o consentimento deverá ser objeto de reflexão por parte do participante fora do ambiente hospitalar, devendo para tal, existir a possibilidade de o mesmo remeter o consentimento por correio em envelope RSF.
1. Reforçar as medidas de segurança previstas para a conservação dos documentos em formato de papel que impeçam o acesso à informação a pessoas não autorizadas, bem como o seu manuseamento indevido;
 2. Garantir medidas de segurança adicionais para o envio da informação por correio eletrónico, nomeadamente através de medidas de encriptação / codificação dos dados a tratar;
 3. Garantir medidas de segurança adicionais no transporte dos dados com recurso a dispositivos electrónicos de armazenamento (Laptop), nomeadamente através de medidas de cifragem e autenticação;
 4. Em caso de necessidade de extensão de prazo e/ou de qualquer alteração dos pressupostos atinentes ao presente parecer o Investigador Principal deverá solicitar a reapreciação do projeto de investigação junto do EPD.

Revisão AIPD:

☐ Data da próxima revisão: ____ / ____ / ____

☒ Não carece de revisão.

Anexos:

1. Processo CES n.º 407/2021
2. Parecer CES (04/01/2022)
3. AIPD (07/01/2022)
4. Consentimento Informado e Informação ao Participante
5. Protocolo de Investigação

Assinado por: **PAULO ALEXANDRE MOTA DA SILVA**
Num. de Identificação: 12061004
Data: 2022.02.10 11:07:24+00'00'



CARTÃO DE CIDADÃO
* * * *

IDENTIFICAÇÃO DE NOVOS BIOMARCADORES PARA DETECÇÃO PRECOCE DE REDUÇÃO DA MASSA ÓSSEA DURANTE INFEÇÕES PULMONARES CRÓNICAS (BONE SAAv3r)

INVESTIGADORES E AFILIAÇÃO:

Ana Cordeiro Gomes, i3S, Universidade do Porto

Adelina Amorim, CHUSJ, FMUP, Universidade do Porto

Margarida Redondo, CHUSJ

Rita Boaventura, CHUSJ

Georgina Terroso, CHUSJ

Salomé Garcia, CHUSJ

Maria Salomé Gomes, ICBAS e i3S, Universidade do Porto

RESUMO

A prevalência de osteoporose nos doentes de fibrose quística é muito elevada. A osteoporose nestes pacientes é de causa multifatorial, nomeadamente, insuficiência pancreática, diabetes, puberdade tardia, imobilização, malnutrição, bem como a infeção pulmonar crónica. Assim, é de interesse público a identificação precoce dos indivíduos em risco de fratura óssea e consequente prevenção quer farmacológica quer comportamental das fraturas. Para tal, torna-se imperativo a identificação de um biomarcador do risco de perda óssea. Os nossos estudos anteriores em murganho identificaram as proteínas Serum Amyloid A (SAA) como marcadores da perda de massa óssea durante infeções crónicas. No presente estudo, pretende-se correlacionar os níveis séricos das proteínas SAA 1 e 2 em pacientes com fibrose quística com a densidade óssea e com outros parâmetros conhecidos do metabolismo ósseo, de modo a propor as proteínas SAA 1 e 2 como potenciais biomarcadores do risco de fratura óssea. Para tal, propõem-se a avaliação prospetiva de doentes com fibrose quística, com colheita anonimizada de dados demográficos, antropométricos, clínicos, funcionais, radiológicos (como indicadores da extensão da doença pulmonar), e microbiológicos da expetoração, bem como a quantificação de proteínas SAA circulantes e densidade óssea. Também serão recolhidas células mononucleares do sangue periférico para diferenciar em osteoclastos (células responsáveis pela reabsorção óssea) e determinar a sua capacidade reabsortiva. Serão incluídos doentes adultos da consulta de fibrose quística do Serviço de Pneumologia do CHUSJ durante um período de 12 meses. Esperam os autores que, com o estudo desenhado, seja possível otimizar a deteção precoce do risco de fraturas, diminuindo a morbilidade e mortalidade associada a estas.

INTRODUÇÃO

A FQ é uma doença autossômica recessiva causada por mutações patogénicas no gene *CFTR* que codifica um canal iónico epitelial de transporte de cloro e bicarbonato (Shteinberg et al, 2021, Lancet). Embora exista variabilidade na apresentação e gravidade da doença, a FQ classicamente caracteriza-se pela ocorrência de infeção pulmonar crónica (Shteinberg et al, 2021, Lancet), isto é, existe persistência ao longo do tempo de culturas de expetoração positivas. Os agentes mais comuns de infeção crónica em pacientes com FQ são *Pseudomonas aeruginosa* e *Staphylococcus aureus*. Porém, as micobactérias não tuberculosas têm sido cada vez mais identificadas na expetoração de adultos e crianças com FQ (Floto et al, 2016, *Thorax*). As melhorias no tratamento dos pacientes com FQ têm aumentado a sua longevidade. Porém, o aumento da longevidade destes pacientes associa-se à identificação de outras co-morbilidades, nomeadamente, o aparecimento de osteoporose. A redução da massa óssea cursa com um aumento do risco de fraturas, pior prognóstico e diminuição da qualidade de vida (Putman et al, 2019, *J. Cystic Fibrosis*; Contreras-Bolívar et al, 2019, *Scientific Reports*). A massa óssea é regulada pelos osteoblastos que depositam matriz mineral e pelos osteoclastos que degradam osso (Oliveira et al, 2020, *Microorganisms*).

Na investigação prévia dos mecanismos responsáveis pelo desenvolvimento de anemia durante a infeção sistémica por *M. avium* em murganhos, observamos que os fémurs e tíbias dos animais infetados eram mais suscetíveis a fraturas. Estudos por micro-tomografia computadorizada revelaram uma diminuição no osso cortical e trabecular dos animais infetados, acompanhada pela redução da densidade mineral óssea (Cordeiro Gomes et al, resultados não publicados). Embora a infeção óssea por micobactérias seja possível, mas rara nos países desenvolvidos, no nosso modelo de infeção, as micobactérias não infetavam diretamente o osso. As micobactérias foram observadas no interior de macrófagos distribuídos pelo parênquima medular contidos em lesões semelhantes a granulomas (Cordeiro Gomes et al, 2019, *J. Immunology*). Estudos *in vitro* demonstraram que os macrófagos infetados com *M. avium* produzem fatores solúveis que induzem a diferenciação de progenitores hematopoiéticos em osteoclastos (células de origem mieloide, responsáveis pela degradação da matriz óssea) (Cordeiro Gomes et al, resultados não publicados). Simultaneamente, estudos *in vivo* de *turnover* ósseo revelaram um aumento da reabsorção óssea durante a infeção sistémica com *M. avium* que era acompanhado de uma diminuição da formação óssea (Cordeiro Gomes et al, resultados não publicados). Consequentemente, estudos de histomorfometria indicaram que o número de osteoclastos e superfície óssea erodida estavam aumentados ao fim de 2 meses de

infecção sistémica com *M. avium*, enquanto o número de osteoblastos e de osteoide estavam diminuídos. Para identificar os mecanismos moleculares responsáveis pela redução da massa óssea durante a infeção, foi analisado o transcriptoma de osso total de animais infetados em comparação com controlos não infetados, sendo que o gene mais sobreexpresso no osso de animais infetados foi o *Saa3*, que codifica para a proteína Serum Amyloid A3 (SAA3) em murganhos (Cordeiro Gomes et al, resultados não publicados).

A SAA3 foi recentemente envolvida no metabolismo ósseo, mediando a interação dos osteoclastos com os osteoblastos. Especificamente, osteoclastos ativados produzem SAA3 que por ligação ao recetor FPR2, expresso à superfície pelas células estaminais do mesênquima, precursores dos osteoblastos, inibem a sua diferenciação (Choudhary et al, 2016, J. Bio. Chem.). O papel da proteína SAA3 no metabolismo ósseo pode não se restringir à inibição da formação de novo tecido ósseo mas também à indução da própria degradação óssea por mecanismos parácrinos (Thaler et al, 2015, FASEB J.). Portanto, a quantificação desta proteína poderá constituir um biomarcador de defeitos no metabolismo ósseo induzidos pela infeção crónica.

Nos humanos, a família das proteínas SAA compreende 4 isoformas principais: SAA1, SAA2 e SAA3 são proteínas de fase aguda e a SAA4 é constitutivamente expressa. A SAA3 é produzida principalmente a nível extrahepático, sendo um pseudogene (Kluve-Beckerman et al, 1991, DNA Cell Biol.; Sellar et al, 1994, Genomics). Em humanos, SAA1 e SAA2 são produzidas em resposta a estímulos inflamatórios por tecidos extra-hepáticos (Uhlir et al, 1999, Eur. J. Biochem.). A nível filogenético e funcional, a SAA3 de murganhos tem maior semelhança com as proteínas SAA1 e 2 humanas, sugerindo que a proteínas SAA1 e 2 humanas tem a mesma função biológica que a proteína SAA3 murina (Thaler et al, 2015, FASEB Journal). Determinações dos níveis de SAA no soro de doentes com várias patologias pulmonares como DPOC e sarcoidose foram realizadas e sugerem que estas proteínas de fase aguda podem ser potenciais biomarcadores de doença pulmonar (Vietri et al, 2019, Respiratory Investigation). Em doentes com FQ e com infeções pulmonares crónicas, nomeadamente com *Pseudomonas aeruginosa*, foi observado um aumento dos níveis séricos de SAA (Marhaug et al, 1983, Acta Paediatr Scand; Smith et al, 1992, J Clinical Laboratory Analysis; Ratjen et al, 2012, Chest). Por conseguinte, a determinação dos níveis serológicos das proteínas SAA em humanos com FQ, poderá ser uma forma potencial de detetar e intervir precocemente na perda da massa óssea, prevenindo assim o desenvolvimento de fraturas, deterioração da qualidade de vida e aumento da mortalidade bem como o aumento dos custos económicos associados a estas condições.

Caso se verifiquem níveis plasmáticos aumentados de SAA, torna-se pertinente investigar se existe um aumento da reabsorção óssea por aumento da formação das células responsáveis por este processo, os osteoclastos. Os osteoclastos são células multinucleares com

origem mieloide e facilmente deriváveis de células mononucleares do sangue periférico por cultura in vitro com concentrações saturantes da citocina RANKL e do fator de crescimento M-CSF. Assim, na colheita de sangue periférico dos pacientes com FQ é possível obter soro e separar as células mononucleares que serão posteriormente usadas em culturas in vitro para determinar quer a capacidade de formação de osteoclastos quer a atividade de reabsorção óssea.

APRESENTAÇÃO DO PROBLEMA

A perda de massa óssea nos doentes com FQ aumenta o risco de fraturas com potencial agravamento da doença respiratória e consequente perda da qualidade de vida, mortalidade e sobrecarga económica para os indivíduos e para o Sistema Nacional de Saúde. O risco de osteopenia e osteoporose em doentes com FQ é amplamente reconhecido e considerado multifatorial, sendo considerados como fatores de risco: o estado de desnutrição, insuficiência de vitamina D e/ou K, corticoterapia e infeção pulmonar, entre outros (Sermet-Gaudelus et al, 2011, J. Cystic Fibrosis). Na FQ, a baixa densidade óssea resulta do desequilíbrio na remodelação óssea com diminuição da deposição de matriz óssea pelos osteoblastos e aumento da reabsorção óssea pelos osteoclastos, especialmente na presença de infeções pulmonares (Sermet-Gaudelus et al, 2011, J. Cystic Fibrosis). Este mecanismo é semelhante ao observado no nosso modelo de infeção crónica sistémica com *Mycobacterium avium* em murganhos: um aumento da degradação óssea e simultânea diminuição da formação óssea, conduzindo a uma diminuição da massa e mineralização óssea com consequente aumento sistémico de SAA (Cordeiro Gomes et al, resultados não publicados). Embora o principal patógeno identificado nos doentes com FQ seja *Pseudomonas aeruginosa* (Doring et al, 2012, J. Cystic Fibrosis), a identificação de micobactérias não-tuberculosas na expetoração de adultos e crianças com FQ tem aumentado (Floto et al, 2016, *Thorax*). É, portanto, pertinente estudar em doentes com FQ se a produção de SAA poderá constituir um potencial biomarcador para identificar precocemente os indivíduos com infeção pulmonar crónica em risco de desenvolverem osteopenia/osteoporose. A identificação precoce destes doentes é fulcral para permitir uma intervenção precoce a nível de adoção de medidas e/ou terapêuticas preventivas.

A deteção das proteínas SAA1 e SAA2 em soro de pacientes com infeções pulmonares crónicas poderá constituir um biomarcador de alterações no metabolismo ósseo e consequente risco de fratura óssea. No seguimento destas observações torna-se pertinente perguntar se os níveis aumentados de SAA3 correlacionam-se com um aumento da capacidade de formação de osteoclastos e consequente reabsorção óssea. Assim, o isolamento de células mononucleares do sangue periférico simultaneamente ao isolamento de plasma destes pacientes permite responder a estas duas questões sem aumentar a intervenção nos pacientes. O estudo destas proteínas como biomarcadores do metabolismo ósseo e a identificação de uma maior atividade osteoclástica nestes pacientes não foram realizados anteriormente.

Objetivos

Os objetivos do projeto BONE SAAv3r são:

- 1) Identificar as proteínas SAA1 e SAA2 como potenciais biomarcadores de distúrbios no metabolismo ósseo em pacientes com FQ.
- 2) Determinar se os pacientes com FQ tem uma capacidade de reabsorção óssea aumentada através da realização de estudos in vitro de diferenciação e atividade osteoclástica a partir de células mononucleares do sangue periférico.

DESENHO DE ESTUDO E MÉTODOS

Tipo de estudo – Estudo prospetivo intervencional.

Doentes a incluir:

Doentes com FQ com mais de 18 anos, após obtenção de consentimento informado.

Doentes a excluir:

Doentes em exacerbação.

Doentes com comorbilidades como diabetes relacionada com FQ, doenças autoimunes.

Doentes a realizar terapêutica com corticosteroides orais.

Doentes a realizar terapêutica com bisfosfonatos.

Parâmetros a avaliar:

- Densitometria óssea bifotónica (DXA), de acordo com as *guidelines* internacionais de seguimento destes pacientes.
- Doseamento sérico de vitamina D, de cálcio, fósforo, paratormona, fósforo inorgânico, osteocalcina, fosfatase alcalina, beta *crosslaps*, de acordo com o Protocolo de Saúde Óssea no doente adulto com Fibrose Quística do Centro de Referência de Fibrose Quística do Centro Hospitalar de São João.
- Quantificação plasmática por ELISA das proteínas SAA1 e SAA2 – a encargo da equipa de investigação no i3S.
- Determinação da capacidade de formação de osteoclastos e atividade destes usando estudos in vitro com células mononucleares do sangue periférico – a encargo da equipa de investigação no i3S.

Recolha e processamento dos dados:

O volume de sangue a colher será de 9 mL. As amostras serão colhidas na consulta do doente no Serviço de Pneumologia do CHUSJ, após aprovação da Comissão de Ética e após consentimento informado do paciente. Estas amostras serão identificadas exclusivamente por um código que inclui as letras FQ e um número que corresponde ao doente, sendo esse número atribuído pela ordem de entrada no estudo.

Será criada uma base de dados anonimizada, que será partilhada dentro da equipa. Serão realizadas reuniões periódicas para analisar os dados obtidos. Na base de dados, para cada doente, para além dos parâmetros analíticos descritos na secção anterior serão incluídos dados clínicos, incluindo:

fatores de risco e comorbilidades, existência de fraturas ósseas prévias, e também dados antropométricos (peso, altura, IMC).

Duração do projeto:

Serão incluídos todos os doentes elegíveis, pelos critérios descritos acima, que sejam seguidos na consulta de fibrose quística do Serviço de Pneumologia do CHUSJ, no decurso de 12 meses.

Resultados esperados:

1 – Correlacionar os níveis séricos das proteínas SAA com a densidade óssea dos pacientes e com outros indicadores do metabolismo ósseo.

2- Correlacionar os níveis séricos das proteínas SAA com a atividade de osteoclastos.

3- Correlacionar os resultados referidos acima com o padrão de infecção e tipo de agentes infecciosos isolados nos exames bacteriológicos de expectoração regularmente colhidos.

Mediante os resultados obtidos, poderão ser propostas novas determinações.

Deverão resultar duas publicações deste trabalho.

CONSIDERAÇÕES ÉTICAS

Trata-se de um estudo não intervencional, com colheita de dados que fazem parte do seguimento de rotina dos doentes com FQ, acrescido apenas da colheita de um tubo de sangue adicional, no momento da colheita das análises anuais, recomendado pelas *guidelines* de seguimento de doentes com FQ. O tubo adicional será analisado no i3S para separação e recolha de plasma e células mononucleares do sangue. A colheita desta amostra ocorrerá no serviço de Pneumologia durante a consulta de rotina dos pacientes, não envolvendo, portanto, deslocações adicionais.

A participação dos doentes será completamente voluntária e sujeita ao seu consentimento informado.

IMPLICAÇÃO PARA A SAÚDE DAS POPULAÇÕES

Os dados do estudo são inovadores e poderão constituir um marco na abordagem e detecção da redução da massa óssea em pacientes com FQ.

ORÇAMENTO

Sem custos adicionais para o CHUSJ.

Dados relativos aos valores analíticos que fazem parte do seguimento habitual e adequado do doente com fibrose quística.

Transporte e análise das amostras para parâmetros específicos do estudo assegurados pelo i3S.



SÃO JOÃO

PARA INVESTIGAÇÃO CLÍNICA

Considerando a "Declaração de Helsínquia" da Associação Médica Mundial (Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996; Edimburgo 2000; Seoul 2008; Fortaleza 2013)

Designação do Estudo (em português)

Confirmando que expliquei ao participante/representante legal, de forma adequada e compreensível, a investigação referida, os benefícios, os riscos e possíveis complicações associadas à sua realização.

Informação escrita em anexo: ☐ Não ☐ Sim (Nº de páginas _____)

O Investigador responsável

Nome: _____ legível


assinatura

Identificação do participante

Nome: _____

BI/CC nº: _____

Participante/ Representante legal

- Compreendi a explicação que me foi facultada acerca do estudo que se tenciona realizar: os objetivos, os métodos, os benefícios previstos, os riscos potenciais e o eventual desconforto.
- Solicitei todas as informações de que necessitei, sabendo que o esclarecimento é fundamental para uma boa decisão.
- Fui informado da possibilidade de livremente recusar ou abandonar a todo o tempo a participação no estudo, sem que isso possa ter como efeito qualquer prejuízo na assistência que é prestada.
- Declaro não ter sido incluído em nenhum outro projeto de investigação nos últimos três meses.

Concordo com a participação neste estudo, de acordo com os esclarecimentos que me foram prestados, como consta neste documento, do qual me foi entregue uma cópia.

Data: ____ / ____ / ____

assinatura

Nome (Pais/Representante legal): _____

BI/CC nº: _____ Grau de parentesco: _____

Data: ____ / ____ / ____

assinatura

IDENTIFICAÇÃO DE NOVOS BIOMARCADORES PARA DETECÇÃO PRECOCE DE REDUÇÃO DA MASSA ÓSSEA DURANTE INFECÇÕES PULMONARES CRÔNICAS (BONE SAAv3r)

Estamos a convidá-lo a participar de um projeto de investigação, mas antes de decidir se quer ou não participar, queremos que entenda os objetivos e implicações do estudo. Não precisa de decidir imediatamente, devendo colocar qualquer dúvida que tenha. Se aceitar participar, poderá interromper o estudo a qualquer momento, sendo que o seu atendimento médico não será afetado. A participação no estudo é totalmente voluntária, sendo a decisão de participar ou não no estudo totalmente sua. Pode refletir sobre o pedido de participação, inclusive pode ouvir a opinião de familiares e/ou amigos, dando a sua resposta na próxima consulta de rotina, se assim pretender. Em qualquer momento, e caso seja essa a sua vontade, pode pedir para ser retirado do estudo, sem que tal comprometa o seu relacionamento com a equipa médica nem o respeito pelos direitos à assistência que lhe é devida.

Porque pretendemos fazer este estudo?

Os pacientes com Fibrose Quística tem um risco aumentado de desenvolver osteoporose. O desenvolvimento de osteoporose pode estar relacionado com a existência de infeções pulmonares. O projeto BONE SAAv3r pretende identificar substâncias que existem no sangue nos pacientes com Fibrose Quística que possam ser usados como indicadores do risco de reduzida massa óssea. A validação destas substâncias como indicadores do risco de osteoporose permitirá no futuro o tratamento precoce destes doentes com consequente melhoria da sua qualidade de vida. Esta pesquisa irá recrutar cerca de 50 pessoas deste Centro Hospitalar. Este estudo foi aprovado pela Comissão de Ética do Centro Hospitalar S. João.

Como é que o estudo irá decorrer?

Numa primeira visita, iremos explicar-lhe o estudo e terá a oportunidade de fazer as perguntas que quiser. Se decidir participar, será solicitado que assine um formulário de consentimento, e receberá esta folha de informações e uma cópia do formulário do consentimento assinado.

Se você estiver disposto a participar será realizada uma colheita de sangue. Nessa amostra de sangue serão quantificadas as substâncias relacionados com a saúde óssea e serão isoladas células para determinar a capacidade destas de degradarem o osso. Os seus dados pessoais e identificativos não serão usados posteriormente. Não será necessário efetuar nenhuma deslocação adicional ao hospital para a participação no estudo.

Quais são as possíveis desvantagens ou riscos de participar?

A participação no estudo não tem quaisquer custos nem são esperados quaisquer riscos. Participar do estudo não terá implicações para si ou seu tratamento. Haverá algum desconforto habitual associado à colheita de sangue.

Quais são os possíveis benefícios de participar?

O resultado deste estudo poderá ajudar a prestar melhores cuidados aos doentes com Fibrose Quística no futuro. No entanto, não prevemos um benefício clínico direto para si.

O que acontecerá com as amostras recolhidas como parte do estudo?

As suas amostras de sangue serão armazenadas pelo Hospital Universitário S. João e pelo i3S, que são instalações seguras para armazenar amostras de pacientes anónimos. As amostras armazenadas incluirão plasma sanguíneo e células do sangue e isso pode ser usado em pesquisas futuras. Depois de analisadas, as amostras restantes serão armazenadas anonimamente por um período de até 10 anos.

O que acontecerá com as informações recolhidas?

As informações clínicas serão registadas num banco de dados num computador, no Hospital S. João. A sua equipa local terá acesso a informações como o seu nome, mas apenas outras informações como a idade, sexo, resultados de exames de sangue e densitometria óssea, dados sobre o tratamento e os resultados de sangue, serão partilhados com o i3S. Os dados mantidos no registo serão armazenados usando um código identificável apenas para o seu médico. Apenas o seu médico e a equipe responsável pelo registo terão acesso aos seus dados pessoais. Quando os dados do estudo forem fornecidos aos investigadores para serem analisados, as suas informações pessoais serão removidas, para que você não possa ser identificado. Nenhum resultado que possa identificá-lo pessoalmente será divulgado ao público ou publicado. Todas as informações recolhidas durante o curso da pesquisa serão mantidas estritamente confidenciais.

Caso tenha dúvidas sobre a forma como os seus dados pessoais são tratados, para saber mais sobre os seus direitos no âmbito da proteção de dados, pode entrar em contacto com o Encarregado de Proteção de Dados do CHUSJ através do endereço de email: epd@chsj.min-saude.pt.

Caso considere que os seus dados não estão a ser objeto de tratamento legítimo, pode, a todo o momento, apresentar uma reclamação junto da autoridade competente, a Comissão Nacional de Proteção de Dados (www.cnpd.pt).

Se tiver uma reclamação sobre a sua participação no estudo, deve apresentá-la ao investigador principal (Ana Cordeiro Gomes).

O que acontecerá com os dados após a conclusão do estudo?

Os seus dados serão mantidos durante 10 anos após o término do estudo. Depois disso, os seus dados serão permanentemente separados da identificação. Isso significa que os dados ainda serão armazenados, mas não estarão mais vinculados a suas informações pessoais e nem os pesquisadores conseguirão identificar os dados referentes a si.

Equipa:

O estudo é liderado pela Doutora Ana Cordeiro Gomes, investigadora no i3S – Universidade do Porto e estudante de Medicina na Faculdade de Medicina da Universidade do Porto, que poderá ser contactada pelo seguinte email: ana.c.gomes@i3s.up.pt.

Fazem também parte do estudo a Dra. Adelina Amorim, do Serviço de Pneumologia do CHUSJ e da Faculdade de Medicina da Universidade do Porto, a Dra. Margarida Redondo e a Dra. Rita Boaventura do Serviço de Pneumologia do CHUSJ, a Dra. Georgina Terroso e Dra. Salomé Garcia do Serviço de Reumatologia do CHUSJ, e a Professora Doutora Maria Salomé Gomes, docente no Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.