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**Bone marrow adipose tissue mass and dipeptidyl peptidase-4 link aging and metabolic health
to biomarkers of bone turnover**

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Running head: Impact of bone marrow adipose tissue on bone health

32 **Abstract**

33 Bone marrow adipose tissue (BMAT) has been linked to negative bone health outcomes and a high
34 level of bone marrow adipocyte accumulation is observed during aging and in individuals with diabetes
35 and obesity. This study explores the relationships between BMAT, age, metabolic health and the
36 impact of their interactions on bone turnover in a cross-sectional cohort of healthy women and men.
37 Levels of bone turnover biomarkers, procollagen type 1 N-terminal propeptide (P1NP) and beta-
38 Crosslaps (CTX) were determined alongside dipeptidyl peptidase-4 (DPP4) concentration and activity
39 as biomarkers of metabolic health. We used magnetic resonance imaging to assess proton density fat
40 fraction (PDFF) to quantify BMAT mass in healthy individuals, and correlated results to sex, age, body
41 mass index (BMI) and glycated hemoglobin A1c (HbA1c), which represents long-term glycemic
42 control. Age was the strongest determinant of increased BMAT mass, explaining more than a third of
43 its overall variation, as well as a robust determinant of bone turnover. A sex-specific correlation pattern
44 was observed between BMAT and bone turnover: Women displayed a trend for a positive correlation
45 of BMAT which depended on age. In men, BMAT mass correlated significantly, but inversely, with both
46 biomarkers, which was also age-dependent. Dipeptidyl peptidase-4 concentration and activity were
47 positively associated with P1NP in both sexes and these relationships were independent of age, BMI
48 or HbA1c. These findings indicate that the impact of BMAT on bone turnover may be age-dependent,
49 whereas metabolic regulator DPP4 is linked to bone turnover independently of metabolic health or
50 aging.

51

52 **Keywords:** Bone marrow adipose tissue, Dipeptidyl peptidase-4, BMAT, aging, bone turnover

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64 **Introduction**

65 Bone health is influenced by a range of systemic pathologies, such as aging and metabolic diseases.

66 It stands to reason that these disorders are interlinked and that diabetes and obesity exacerbate the

67 functional decline of bone health during aging. Bone marrow adipose tissue (BMAT) is increasingly

68 recognized as a potential connecting element between these factors, linking it to clinically relevant

69 conditions, such as osteoporosis and hematological diseases (1). Its expansion occurs progressively

70 with age and it is found to be elevated in individuals with obesity and patients with either type-1 or

71 type-2 diabetes (T1D, T2D) (2). Bone marrow adipocytes (BMAds), however, also exist in healthy

72 bones and some reports challenge the view of an unambiguously inverse relationship between bone

73 marrow adipogenesis and bone health, suggesting that BMAds can constitute a critical component of

74 the healthy bone niche (2, 3). Thus, BMAT's precise role in bone pathophysiology remains unclear and

75 recent findings further illustrate its complex relationship with anatomical location, age and gender (2).

76 For instance, levels of BMAT were found to be increased during aging in proximal-to-distal distribution

77 in both axial and appendicular skeleton, an effect that was more pronounced in women, especially

78 post-menopause (4). These results indicate that BMAT is regulated differently in men and women,

79 likely due to changes in hormonal cues. Moreover, the study highlights that the degree of fatty acid

80 unsaturation in BMAds varies across the skeleton, with higher unsaturated fatty acid levels found in

81 the axial skeleton. While somewhat contradicting the data from animal models, these findings further

82 support the concept of distinct roles for BMAT in different skeletal areas (4).

83 A growing body of research highlights the potential use of biomarkers based on chemical-shift-

84 encoded magnetic resonance imaging (CSE-MRI) in assessing bone health, mainly expressed as

85 proton density fat fraction (PDFF) and its use for quantifying BMAT mass (5, 6). For instance, such

86 analyses have demonstrated that fat depositions in bone and skeletal muscle are positively correlated

87 and are also linked to degenerative disorders, such as intervertebral disc degeneration, altogether

88 linking degenerative processes within the musculoskeletal system to ectopic fat accumulation (7).

89 These findings align with the observation that PDFF measurements could predict vertebral

90 compression fractures (VCFs) (8). Such results suggest that increases of BMAT mass may serve as

91 an early marker of elevated fracture risk, offering a non-invasive tool for assessing bone composition

92 changes linked to VCFs in aging populations.

93 Parallel to the impact of BMAT on bone, metabolic regulators like dipeptidyl peptidase-4 (DPP4) are
94 increasingly recognized as influential in bone health. Our previous studies in pre-clinical animal
95 models have linked expansion of BMAT in aged mice to increased DPP4-release from cells of the
96 bone-resident adipocyte lineage, which resulted in impaired bone healing (9). Detrimental effects of
97 DPP4 have also been reported for hematopoiesis in the bone marrow (10). DPP4, a membrane-shed
98 protease, is responsible for the inactivation of various secreted proteins, including the incretin
99 hormones, glucagon-like peptide 1 (GLP1) and glucose-dependent insulintropic polypeptide (GIP),
100 which are vital for stimulating postprandial insulin secretion. DPP4 inhibitors, known as gliptins, are a
101 class of oral drugs commonly used for the management of T2D (11). Individuals with metabolic
102 diseases, such as diabetes, frequently show inverse relationships between DPP4, its molecular
103 targets, and bone health (12). High DPP4 activity correlates with low bone mineral density (BMD) and
104 increased fracture risk, which may depend on two signaling pathways: the entero-endocrine-osseous
105 axis involving gastrointestinal substrates of DPP4, i.e. GIP and GLP-1, and a pancreatic-endocrine-
106 osseous axis, linking DPP4 to bone and energy metabolism through expression of receptor activator of
107 nuclear factor kappa B ligand (RANKL) and blood glucose regulation (13). Circulating DPP4 is also
108 elevated in patients with osteoporosis, in otherwise metabolically healthy individuals, as well as in
109 newly diagnosed patients with T2D (14, 15). These findings are supported by the observation that
110 gliptins may exert beneficial effects on bone by enhancing the function of osteogenic progenitor cells
111 (9). It should be noted, however, that many drugs that are used to treat T2D are not clearly linked to a
112 reduction in fracture risk. In fact, while some gliptins may produce protective effects on bone health, at
113 least one member of this class, trelagliptin, correlated with increased fracture risk, altogether
114 suggesting that the impact of DPP4 inhibition on bone requires further study (16). On the other hand,
115 the use of GLP-1 receptor agonists in patients with T2D may stabilize or even improve BMD and bone
116 turnover marker profiles, an effect which is also supported by corresponding observations in pre-
117 clinical animal models (17, 18). Conversely, the use of GLP-1 receptor agonists showed no clear risk
118 reduction for fractures per se (17). Interestingly, direct infusions of GLP-1 or GIP suppressed bone
119 resorption, and simultaneous administration of both hormones resulted in a synergistic effect (19). In
120 summary, the interplay between DPP4 activity, BMAT accumulation, and bone pathology in older
121 individuals with or without metabolic disease presents a compelling area of investigation. In order to
122 further investigate these relationships, we here examined the links between markers of bone turnover,
123 MRI-based measurements of BMAT mass and body fat distribution, in combination with analyses of

124 DPP4 concentrations and activities in men and women across different ages and levels of overweight
125 and obesity.

126

127 **Methods**

128 **Study cohort and assessment of clinical and anthropometric data**

129 The study cohort consisted of 76 healthy volunteers (40 women, 36 men) without bone pathologies
130 (e.g. osteoporosis) or overt T2D in their medical history. Specifically, exclusion criteria were as follows:
131 acute illness or infection within the last four weeks; conditions, as assessed by a study physician, that
132 call into question the success of the study or indicate a risk to the participant; existing Type-1 diabetes;
133 patients with Type-2 diabetes and pharmacological diabetes therapy other than Metformin and DPP4
134 inhibitors; HbA1c >10.0%; Diabetes duration \geq 2 years. All enrolled participants completed the study,
135 i.e., there was no attrition. Groups assignments in this study were generated retroactively, as
136 determined by sex, age, and/or BMI. Therefore, no randomization or blinding during recruitment and
137 data collection was performed. Plasma analyses were performed in a blinded manner. Upon inclusion,
138 individuals' information on age, gender, and body mass index (BMI) was recorded. Glycosylated
139 hemoglobin A (HbA1c) was determined in the majority of individuals to monitor metabolic health
140 status. While some cases of pre-diabetes, based on HbA1c levels, were included in the study (9
141 women, 6 men), no individuals surpassed the diabetes-defining threshold of 48 mmol/mol (Table 1).
142 The study and its protocol were reviewed and approved by the Ethics Committee of the University of
143 Tübingen and written informed consent was obtained from all subjects prior to participation.

144

145 **MR imaging protocol and image analysis**

146 MR measurements were performed in the early morning after overnight fasting on a 3T whole-body
147 imager (Magnetom Vida, Siemens Healthineers, Erlangen, Germany). For the first part of the
148 examination, volunteers were placed head first in supine position and the spine-array coil in
149 combination with two body-array coils placed on the trunk were applied as receiver coils. For
150 assessment of adipose tissue distribution in the trunk, a 3D 2-point volumetric interpolated breath-hold
151 examination (VIBE) Dixon sequence was applied for quantification of visceral (VAT) and subcutaneous
152 adipose tissue (SAT) of the abdominal (aSAT) and thoracic (tSAT) region. Segmentation was
153 performed as previously described (20). Bone marrow PDFF was quantified in vertebral bodies
154 applying a 3D 6-point VIBE CSE sequence covering the lumbar spine (6). Additionally, intrahepatic

155 lipids (IHL) were determined by localized proton MR spectroscopy in the posterior part of segment 7
156 applying a single voxel STEAM technique (21).

157

158 **Plasma analyses**

159 Beta-CrossLaps (CTX) and procollagen type 1 N-terminal propeptide (P1NP) were assessed by Labor
160 Berlin – Charité Vivantes GmbH (Berlin, Germany) using standardized procedures for clinical markers.
161 DPP4 activity was measured by enzymatic conversion of the substrate Gly-Pro p-nitroanilide (Gly-Pro-
162 pNA) into p-nitroaniline (pNA) as published before (22). Briefly, 40 µl of plasma was diluted 1:5 in
163 assay buffer (50 mM glycine, 1mM EDTA, pH 8.7). A standard dilution series was prepared by serial
164 dilution of 1 mM pNA (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany; RRID: SCR_008988). 90 µl
165 of diluted samples, standards and blanks were measured in duplicates at 405 nm as kinetic over 30
166 min, starting directly after addition of 10 µl 5mM Gly-Pro-pNA (Sigma-Aldrich, Merck KGaA,
167 Darmstadt, Germany; RRID: SCR_008988). The DPP4 activity was calculated by the slope of the
168 standard series in nmol/min/ml. For DPP4 concentrations, we used the Human DPPIV/CD26
169 Quantikine ELISA Kit (Bio-Techne GmbH, Wiesbaden, Germany) according to the manufacturers'
170 instructions.

171

172 **Statistical analysis**

173 For initial group-based analyses, women (W) and men (M) were considered separately. We next
174 separated our individuals by the mean age of 46 years into individuals belonging to a younger and an
175 older subgroup. The cohort was additionally stratified by BMI into individuals with healthy body weight,
176 i.e. with a BMI <25 kg/m², and individuals with overweight/obesity, including all individuals with a BMI
177 ≥25 kg/m². Accordingly, the resulting eight groups consisted of (1) women up to 46 years of age with a
178 BMI <25 kg/m², (2) women up to 46 years of age with a BMI ≥25 kg/m², (3) women older than 46 years
179 of age with a BMI <25 kg/m², (4) women older than 46 years of age with a BMI ≥25 kg/m², (5) men up
180 to 46 years of age with a BMI <25 kg/m², (6) men up to 46 years of age with a BMI ≥25 kg/m², (7) men
181 older than 46 years of age with a BMI <25 kg/m², and (8) men older than 46 years of age with a BMI
182 ≥25 kg/m² (Table 1; Figure 1A). The software GraphPad Prism (versions 10.4.0 and 10.5.0; RRID:
183 SCR_002798) was used to conduct grouped analyses by two- and three-Way analyses of variance (2-
184 Way ANOVA, factors: age, BMI; 3-Way ANOVA, factors: age, BMI, sex) with full model fitting of
185 individual factor interactions and the Tukey test for multiple comparisons correction. To further

186 investigate the relationship between age, BMI and HbA1c with the biomarkers of interest, Spearman
187 correlations were performed using software R (version 4.4.1; RRID: SCR_001905). Standard pairwise
188 Spearman correlations without controlling for co-variables were computed using the cor() function
189 among selected variables. Statistical significance was evaluated using permutation-based tests
190 implemented via cor.test(). Semi-partial Spearman correlations were calculated using the ppcor
191 package's pcor.test() function, controlling for age, BMI, or HbA1c. All individuals lacking data for
192 individual parameters were excluded for correlation analyses.

193

194 **Results**

195 **Cohort characteristics and stratification into subgroups: impact of age and BMI on BMAT**

196 A total of 76 individuals (40 women, 36 men) were included in the study. Age-stratification by the
197 cohort's mean age of 46 years yielded 33 individuals (16 women, 17 men) up to 46 years of age, and
198 43 individuals (24 women, 19 men) that were older than 46 years. BMI-stratification resulted in 37
199 individuals with healthy BMI, i.e. BMI <25 kg/m² (21 women, 16 men), and 39 individuals with a BMI
200 ≥25 kg/m², indicating overweight/obesity (19 women, 20 men; all data are summarized in Table 1;
201 Figure 1A). HbA1c, as a standard diagnostic marker of long-term glycemic control, was mostly similar
202 between groups, but we observed significantly higher levels in women of both older groups with either
203 healthy or overweight-indicating BMI, compared to women in the younger group with a healthy BMI.
204 Despite its common use as diagnostic marker in diabetes care, we found no BMI-dependent
205 differences for HbA1c in any of the groups. This may be due to the fact that none of the participants in
206 our cohort met the T2D criterion for HbA1c, which is defined by a value above 48 mmol/mol (Table 1).
207 Using three-way analysis of variance (3-Way ANOVA), we compared the eight subgroups to examine
208 which of the three factors, age, BMI and sex, contributed to variation in BMAT mass in our study
209 participants. While in this statistical model BMI did not associate with BMAT mass, age as
210 independent variable was the most significant determinant of BMAT mass, explaining 36% of its
211 variation. Sex also contributed statistically significantly to variation, but only explained 3.1% of the
212 variation in BMAT mass. There were significant interactions between BMI and sex, as well as all three
213 factors together regarding BMAT mass (Figure 1B). These findings indicate that distinct, sex-specific,
214 biological processes could be regulating marrow adipogenesis.

215

216 **Analysis of biomarkers of bone turnover and metabolic health**

217 To examine the link between bone health, BMAT and age-related metabolic dysfunction, we next
218 assessed markers of bone turnover in women and men separately. Procollagen type 1 N-terminal
219 propeptide (P1NP) and beta-CrossLaps (CTX), are clinical markers of bone formation and resorption,
220 respectively, and were analyzed alongside concentration and activity of circulating DPP4. Among the
221 various biomarkers of bone turnover, CTX and P1NP are widely used with a broad availability of
222 reference datasets (23). Overall, no major sex-specific differences were found: when comparing all
223 women to all men in our cohort, we observed no significant differences for all four markers (P1NP
224 $P=0.3136$; CTX $P=0.7741$; DPP4 concentration $P=0.9238$; DPP4 activity $P=0.7537$; by Mann Whitney
225 test; data not shown). No significant differences were observed when comparing measurements of the
226 four markers in women and men in the stratified subgroups (as listed in Table 2), with the exception of
227 older women with BMI <25 kg/m² displaying a significantly higher value compared to men ($P=0.0162$).
228 For P1NP, women in the older group with a normal BMI had significantly higher levels of this marker in
229 comparison to women in the other three groups, while no significant differences between strata were
230 found in men (Figure 2A). 2-Way ANOVA, however, showed that age was a significant factor in
231 predicting P1NP, explaining 18% and 14% of its variance, in women and men, respectively. BMI was
232 only a significant variable in women, explaining 23% of variance (Figure 2A). For bone resorption
233 marker CTX, we observed similar results, with age and BMI explaining a significant proportion of its
234 variance in women (12% and 16%, respectively), while only age was a significant predictor of CTX in
235 men (19% explained variance; Figure 2B). We next assessed whether age or overweight predicted
236 the concentration or activity of circulating DPP4. However, no major statistically significant differences
237 were observed between the subgroups. Analysis of variance showed that age explained a significant
238 proportion of variance of DPP4 concentration and activity in women (10% and 23% explained
239 variance, respectively), but no statistically significant differences were observed in men (Figures 2C
240 and 2D).

241

242 **Correlational analysis of the link between BMAT, bone turnover and metabolic health**

243 To further evaluate the relationship of DPP4 and bone health in the context of aging and metabolic
244 disease, we used Spearman correlations to examine the relationship of age, BMI, and HbA1c with the
245 vertebral PDFF as a marker of BMAT mass. To evaluate bone health, bone turnover markers P1NP
246 and CTX were included, as well as DPP4 concentration and activity to further evaluate the impact of
247 metabolic health status. DPP4 concentration and activity correlated significantly with each other in

248 women and men in all analyses, indicating that both biomarkers are useful as robust representations
249 of circulating DPP4. To better estimate the impact of each variable on these correlations, we
250 subsequently adjusted all correlations for either of the individual variables age, BMI or HbA1c (Figure
251 3).

252 In women, we observed a positive correlation of age with BMAT mass, which is consistent with the
253 literature on aging and BMAc accrual (Figure 3A). Moreover, we observed a trend for a positive
254 correlation between both bone turnover markers and BMAT mass ($P=0.067$ and $P=0.099$ for P1NP
255 and CTX, respectively). Regarding the relationship to DPP4, we observed significant positive
256 correlations between BMAT mass and DPP4 concentration and activity. Of note, DPP4 was also
257 positively correlated with bone turnover markers, suggesting that these three biomarker categories, i.e.
258 of BMAT, of bone turnover, and of metabolic health, are linked with each other. BMI correlated
259 inversely with bone turnover and showed no association with DPP4 or BMAT mass (Figure 3A).
260 HbA1c was significantly associated with age and BMAT mass, and showed a less strong, but
261 significant association with both DPP4 markers (Figure 3A). When adjusting these correlations for age,
262 the positive correlation between BMAT mass and DPP4 markers became less evident, suggesting a
263 high degree of age-dependency for this relationship (Figure 3B). The significant positive correlations
264 between P1NP and both DPP4 markers were retained upon adjustment for age, and CTX was now
265 also significantly correlated to DPP4 concentration (Figure 3B). Age is therefore not a strong
266 determinant of the correlation between bone turnover markers and DPP4 markers. Adjustment for BMI
267 retained or increased most positive correlations and significance levels. For instance, the correlation
268 between BMAT mass and both bone turnover markers became significant, suggesting that BMI is a
269 mild confounder of the impact of age on the correlations (Figure 3C). Correction for HbA1c had a small
270 effect on the correlations, especially between DPP4 and BMAT mass or bone turnover markers
271 (Figure 3D).

272 A different pattern emerged when performing the same set of correlations in men. Age was also
273 significantly and positively correlated to BMAT mass (Figure 3E). Contrasting the observations in
274 women, in men a significant inverse relationship was observed between age and bone turnover
275 markers, P1NP and CTX, and this inverse relationship was also evident between both turnover
276 markers and BMAT mass (Figure 3E). While DPP4 markers did not correlate with age or BMAT mass,
277 a positive association, similar to women, was found between DPP4 markers and bone turnover
278 biomarker P1NP (Figure 3E). Further paralleling the observation in women, age-adjustment mitigated

279 most correlations, with the exception of the positive correlations between bone turnover markers and
280 DPP4, which were partially retained (Figure 3F). Adjustments for BMI or HbA1c only had limited
281 impact (Figures 3G and 3H). In summary, the positive correlation between DPP4 concentration and
282 activity on one side and bone turnover marker P1NP on the other side remained robust in men,
283 regardless of adjustment, thus displaying a similar pattern as in women. A similar effect, albeit
284 somewhat less pronounced, was evident for CTX.

285

286 **Correlation analyses of BMAT, bone turnover and markers of adiposity**

287 BMADs represent a type of fat cell that is distinct from other adipocytes, displaying different responses
288 to some typical physiological stimuli (2, 24). We therefore asked whether the relationship between
289 bone turnover and other adipose tissue depots would be comparable to those observed for BMAT. For
290 this purpose, we examined the correlations between BMAT mass and other adipose tissue depots
291 throughout the body, i.e. aSAT, tSAT, VAT, and IHL, to bone turnover markers, P1NP and CTX. The
292 adipose tissue compartments displayed highly significant correlations among each other, to IHL and to
293 BMI in men and women (Figure 4). Conversely, while significant for tSAT, VAT and IHL, there were
294 only moderate positive correlations with BMAT mass in women (Figure 4A) and none in men (Figure
295 4B). Mirroring the findings for BMI in the previous group-based analysis, bone turnover markers were
296 negatively correlated with most adipose tissue depots, but showed some sex-specific differences:
297 while P1NP reached statistical significance for aSAT, tSAT and VAT in women, only CTX displayed
298 significant correlations for all four depots and BMI in men (Figure 4A and 4B). Upon correction for
299 either age or HbA1c as independent variables, similar correlation patterns between adipose tissue
300 compartments among each other, and the inverse correlations to bone turnover markers were largely
301 retained, while significances of correlations to BMAT mass were lost, altogether indicating that only
302 limited connection exists between vertebral marrow adipogenesis and fat accumulation in other depots
303 (Figures 4C-4F).

304

305 **Discussion**

306 In the present study, we investigated the relationship between aging and biomarkers of bone turnover
307 and the modulating effects of bone marrow adipogenesis, as marker of bone disorder, and circulating
308 DPP4, which is frequently considered a marker of metabolic health and an established target of
309 diabetes therapies. Age correlated positively with vertebral BMAT mass in women and men, while

310 markers of metabolic health, BMI and HbA1c, did not, suggesting that they may only have more limited
311 impact on marrow adipogenesis. It is noteworthy, however, that other studies report significant
312 associations between various markers of obesity, such as BMI or visceral fat, and BMAT, suggesting
313 that the small group size of our subgroups may somewhat limit data interpretation (25, 26). However,
314 one study exclusively assessed postmenopausal women, limiting full comparability to our dataset (25).
315 Another study showed significant positive associations between spinal PDFF and certain adiposity
316 traits, i.e. visceral fat mass in particular, while the positive association with BMI became fully evident
317 only after correction for BMD (26). Interestingly, other BMAT depots, in the femoral head, diaphysis
318 and total hip, showed significant inverse correlations between PDFF and BMI, altogether presenting a
319 highly complex relationship between obesity, regional fat distribution and BMAT accumulation
320 throughout the body, whereas age was consistently positively associated with PDFF measurements in
321 all bone regions. Age also emerged as the main parameter that determined the correlations between
322 BMAT mass and bone turnover markers. These associations displayed a sex-dimorphism and were
323 positively correlated in women and inversely correlated in men. Conversely, significant positive
324 correlations between DPP4 and bone turnover markers, mainly P1NP, were observed in women and
325 men, and these associations were independent of age, BMI or HbA1c, suggesting a direct connection
326 between bone health and DPP4.

327 Our findings on the age-related increase of marrow adipogenesis are consistent with previous studies,
328 recapitulating the established consensus of increased bone marrow adipocyte accumulation in
329 individuals with increased age, despite differences having been reported in men and women and in
330 different anatomical locations (2, 4). Conversely, the link of obesity parameters, such as BMI and other
331 fat depots, to marrow adipocyte accumulation is comparably weak in women and essentially absent in
332 men in our dataset, further supporting the notion that BMADs represent a distinct type of fat cells with
333 unique metabolic properties and distinct regulatory mechanisms (24). While the impact of age on
334 BMAT mass is well-documented in men, menopause has been identified as an independent factor that
335 increases marrow adipogenesis. In women, we can, therefore, not fully separate the impacts of these
336 two processes, aging and menopause, on marrow adipogenesis, and, therefore, also not on other
337 biomarkers assessed in our study. The literature consensus for women is that the two markers of bone
338 turnover assessed in our study, P1NP and CTX, increase markedly in women at the onset of
339 menopause. Similar dynamics are also described for other markers, such as osteocalcin and bone
340 alkaline phosphatase (23). A decline of these markers is mainly observed in early life, plateauing for

341 some markers in adult women prior to menopause. We observed mild age-dependent increases for
342 P1NP and CTX in the age-stratified groups, i.e. individuals aged either above or below 46 years, which
343 likely split the female sub-cohort into women before and after menopause, and this was true for both
344 BMI-separated subgroups, i.e. health BMI weight vs. overweight-indicating BMI. Thus, stratification of
345 our cohort using age as categorical variable was able to explain a significant proportion of bone
346 turnover variance in women. Age as continuous variable, as used in our Spearman correlations, also
347 robustly correlated with bone turnover markers, which was statistically significant upon BMI-
348 adjustment, altogether suggesting that age might be partially responsible for increased levels of bone
349 turnover markers in women, but that menopause status is a major main driver of this effect. This
350 assumption is supported by a study that linked aging to elevated levels of bone turnover biomarker
351 bone alkaline phosphatase in postmenopausal women, i.e. after the age of 50 years. The increase
352 could be explained by the absence of estrogen, as estrogen treatments resulted in reduced levels in
353 post-menopausal individuals (27).

354 In men, the correlation of age with bone turnover biomarkers seems to be inverse for most individual
355 markers, although some are reported to be unchanged and there is some contradictory literature
356 suggesting that mild increases in elderly men may occur, depending on estrogen availability (23, 28).
357 For instance, P1NP was found to be decreased, but only until approximately 50 years of age, with little
358 further change after this (29). This observation is in principle consistent with our own data, as only age
359 explained a significant proportion of variation for P1NP and CTX in the age- and BMI-stratified
360 subgroups in men, while BMI had no significant effects. While no data on CTX were reported in the
361 article, markers like osteocalcin and urinary N-telopeptides of type I collagen (NTX) displayed similar
362 patterns. Other markers, like bone alkaline phosphatase, showed no age-related changes in men (29).
363 These observations are consistent with other datasets (27).

364 Unlike women, DPP4 was not affected by age in men, and no link to BMAT mass was evident in men
365 in our dataset, regardless of whether stratified group analyses or correlation analyses were performed.
366 These data taken together show a sex dimorphism for the age-dependency of bone turnover
367 biomarkers, although it stands to reason that some of this is determined by menopause in women,
368 rather than a generalized aging process. In opposition to this, the impact of obesity and the associated
369 measurements in our study, i.e. the adipose tissue volumes and liver fat content, were more consistent
370 between sexes, showing a significant negative correlation between bone turnover biomarkers, P1NP
371 in women and CTX in men, and obesity, with little impact of age- or HbA1c-adjustments.

372 In our study, we observed significant positive correlations between the two DPP4 markers and the
373 bone turnover markers. This was particularly pronounced for bone formation marker P1NP in women,
374 but also evident in men, showing a significant correlation with DPP4 concentration and a trend with
375 DPP4 activity ($P=0.057$). Similar correlations were also observed for CTX, although not always
376 statistically significant. These effects were retained after adjustments for age, BMI or HbA1c,
377 altogether suggesting that DPP4 may affect bone turnover markers and that its effect is independent of
378 metabolic health and age-related pathological factors. The impact of DPP4-associated molecular
379 signaling pathways, such as incretin hormones, on bone turnover is well documented, but mainly
380 exists in the context of diabetes. Our pre-clinical studies in mice showed that DPP4, secreted from
381 adipogenic cells of the bone marrow, could contribute to impaired bone healing whereas
382 administration of sitagliptin, which inhibits DPP4, resulted in activation of osteogenic progenitor cells
383 and improved bone healing (9). This is consistent with a meta-analysis showing that diabetes drugs
384 targeting GLP1 signaling promote osteogenic differentiation while inhibiting adipogenesis of
385 mesenchymal stromal cells (30). Corresponding results have also been reported in clinical settings,
386 although these data for the most part focus on patients with diabetes, where ongoing treatments may
387 confound the relationship between DPP4, bone health/turnover and BMAT. In patients with newly
388 diagnosed T2D, DPP4 activity correlated with severity of osteoporosis and fracture risk, suggesting
389 that DPP4 may impact on bone health in the context of metabolic disease (15). While DPP4 has a
390 wide range of substrates for proteolytic cleavage, the available literature primarily addresses incretin
391 hormones, as GLP1 and GIP are also key targets for diabetes treatment. Incretin hormone receptors
392 are widely expressed in the skeletal system and promote bone formation, suggesting that an elevated
393 DPP4 activity might negatively impact bone health (31). While mouse models with deletion of the
394 individual incretin receptors display signs of compensation, deletion of the GIP receptor in mice
395 resulted in lower bone size and mass and altered bone metabolism, while deletion of the GLP1
396 receptor impaired bone mechanical quality (18, 32). Mice with simultaneous deletion of both receptors
397 also showed impaired bone health (33). Lastly, DPP4 may target certain immune cells and impact on
398 bone metabolism through immunomodulatory mechanisms (31). Altogether these data support the
399 conclusion that changes in DPP4 may directly impact on bone health by altering incretin hormone
400 activity. Further studies are needed to evaluate the impact of this endocrine axis on marrow
401 adipogenesis. Moreover, the understanding of the impact of more recently approved diabetes drugs,
402 like liraglutide and semaglutide, on bone remains scarce and is limited by several factors, including

403 lack of bone turnover marker analyses as primary endpoints and short observation periods. Moreover,
404 the analysis of the impact of such drugs has frequently been conducted in pre-clinical models using
405 much higher concentrations than those used in clinical practice [reviewed in: (17)]. Both incretin
406 hormones and their signaling cascades have also been implicated in bone health and maintenance
407 (18, 34). It is therefore conceivable that correlations between bone turnover and DPP4 levels, as
408 observed in our study, may be linked to the impact of DPP4 on incretins and their ability to regulate
409 bone formation and resorption. For instance, direct infusion of both hormones in non-diabetic men
410 showed a significant acute impact on bone resorption (19).

411 Our study has limitations. The cohort size is relatively limited, which could impact on broad
412 applicability, for instance to various ethnically diverse populations. The small group size also resulted
413 in our single BMI threshold for the stratified group analyses by age, sex, and BMI. Since none of the
414 participants in our study had manifest diabetes, the findings on the relationships between BMAT,
415 DPP4 and bone turnover may not extend to patients with T2D. Moreover, our study does not consider
416 individual participants' medication, lifestyle interventions and bone mineral density as a marker of bone
417 health, which should be assessed more precisely in future studies. The cross-sectional design of our
418 study also limits interpretation of longitudinal effects of age and diabetes on bone turnover and follow-
419 up studies are needed to further establish the role of such changes over time. DPP4's connection with
420 bone turnover markers is presently correlative, and our study does not explore mechanisms or provide
421 insight into how DPP4 could influence bone or marrow health. Additional mechanistic studies are
422 necessary to clarify the physiological role of DPP4 in this context. The study applied complex
423 statistical adjustments to isolate the effects of variables like age, BMI, and HbA1c, which could
424 introduce interpretation challenges. Although these adjustments provide insights, they also increase
425 the likelihood of statistical noise, making it harder to draw definitive conclusions about the relationships
426 observed. Related to this, a limitation of this study is the relatively small number of participants in each
427 of the eight subgroups, potentially limiting the statistical power of subgroup analyses and increasing
428 the likelihood of failing to detect true differences (Type II errors). The study's findings suggest that age,
429 rather than BMI, plays a prominent role in determining marrow adipogenesis and bone health, in
430 women and men. Sex-specific differences indicate that aging processes affecting bone health
431 biomarkers, such as P1NP and CTX, are complex and vary significantly between sexes. Of note,
432 DPP4 correlated with bone turnover markers in both sexes and this relationship appears unrelated to
433 age or metabolic health. Overall, the study emphasizes the need to consider sex and age

434 independently when examining the link between bone health and marrow adipogenesis. Future
435 research may explore the molecular mechanisms driving these sex-specific differences and the
436 potential utility of biomarkers like DPP4 in predicting bone health outcomes across the lifespan.

437

438 **Data availability**

439 Data will be made available upon reasonable request

440

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453

454 **Disclosures**

455 No conflicts of interest, financial or otherwise, are declared by the authors.

456

457 **Author contributions**

458 S.H., S.G., J.M., and T.J.S. conceived and designed research. S.H., S.G., and T.H., performed
459 experiments. S.H., S.G., O.K., T.H., J.M. and T.J.S. analyzed data. S.H., S.G., N.S., A.L.B., J.M. and
460 T.J.S. interpreted results of experiments. S.H., S.G., and T.J.S. prepared figures. J.M. and T.J.S.
461 drafted manuscript. S.H., S.G., O.K., T.H., N.S., A.L.B., J.M. and T.J.S. approved final version of the
462 manuscript

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569 **Figure legends**

570 **Figure 1. Bone marrow adipogenesis is influenced by age and sex. (A)** Distribution of body mass
571 index (BMI) and age in study cohort (men: gray circles; women: black circles; summarized in Table 1).
572 Broken lines indicate stratification into individuals with a healthy BMI (BMI <25 kg/m²) or a BMI
573 indicating overweight/obesity (BMI ≥25 kg/m²), and individuals grouped into the younger (age ≤46
574 years) and older (age >46 years) subgroups. **(B)** Quantification of MR-based measurements of bone
575 marrow proton density fat fraction (PDFF) as marker of BMAT mass in the groups separated by BMI
576 (healthy: BMI <25 kg/m²) and (overweight/obese: BMI ≥25 kg/m²), sex (men: gray open / filled circles;
577 women: black open / filled circles) and age (age ≤46 years [46y]: open circles; age >46y: filled circles).
578 Data are depicted as grouped scatter plots with lines indicating medians for each group; **P*<0.05;
579 ***P*<0.01; ****P*<0.001; *****P*<0.0001 indicate significant differences by multiple comparisons testing
580 after 3-Way ANOVA. Source of variation analysis summarized in box below plot for significances for
581 the independent variables' (body weight, sex, age) contribution to variation and for the pairwise and
582 triplewise interactions (i.e. BMI x Sex x Age) between factors (ns, not significant; **P*<0.05; ***P*<0.01;
583 ****P*<0.001; *****P*<0.0001).

584

585 **Figure 2. Analysis of biomarkers of bone turnover, P1NP and CTX, and DPP4-concentration**
586 **and activity in stratified subgroups. (A-D)** Analysis of the two bone turnover biomarkers, P1NP (A)
587 and CTX (B), as well as DPP4 concentration (C) and activity (D) in plasma samples. Stratified groups
588 are separated into women (F, black open / filled circles, left panels) and men (M, gray open / filled
589 circles, right panels), and subdivided by BMI (healthy: BMI <25 kg/m² and overweight/obese [O/wt]:
590 BMI ≥25 kg/m²) and age (age ≤46y: open circles; age >46y: filled circles). Data are depicted as
591 grouped scatter plots with lines indicating medians in each group, **P*<0.05; ***P*<0.01; ****P*<0.001
592 indicate significant differences by multiple comparisons testing after 2-Way ANOVA. Source of
593 variation analysis summarized in box below plot for significances (Sig) for the two independent
594 variables' (BMI, age) contribution to variation (%Var) and for interaction between both factors (BMI x
595 Age; ns, not significant; **P*<0.05; ***P*<0.01; ****P*<0.001).

596

597 **Figure 3. Interactions between bone marrow adipogenesis, bone turnover and DPP4 occur and**
598 **are mainly affected by age. (A-D)** Spearman correlation between age, BMI, HbA1c (Hba) and
599 multiple biomarkers representing bone marrow adipogenesis (PDFF), bone turnover (P1NP, CTX),
600 and DPP4 concentration (DPP4) and DPP4 activity (D4A), as summarized in Table 2, in women
601 without (A), and after adjustment for the factors age (B), BMI (C), or HbA1c (D). (E-H) Corresponding
602 correlation analyses in men without (E) adjustment, and after adjustment for the factors age (F), BMI
603 (G), or HbA1c (H). Purple color indicates positive correlations with a correlation coefficient >0,
604 turquoise color indicates negative correlations with a correlation coefficient <0. Statistically significant
605 correlations are indicated as **P*<0.05; ***P*<0.01; ****P*<0.001; strong trends are indicated as numbers
606 (*P*<0.1; ns, not significant).

607

608 **Figure 4. Sex-specific correlations of adiposity and bone turnover markers CTX and P1NP. (A-**
609 **F)** Spearman correlation between BMAT mass (PDFF), bone turnover markers (P1NP, CTX) and
610 obesity markers (BMI, aSAT, tSAT, VAT) and IHL in women (panels A, C, E) and men (panels B, D, F)
611 without adjustment (panels A, B) and after adjustment either for age (panels C, D) or for HbA1c
612 (panels E, F). Purple color indicates positive correlations with a correlation coefficient >0, turquoise
613 color indicates negative correlations with a correlation coefficient <0. Statistically significant

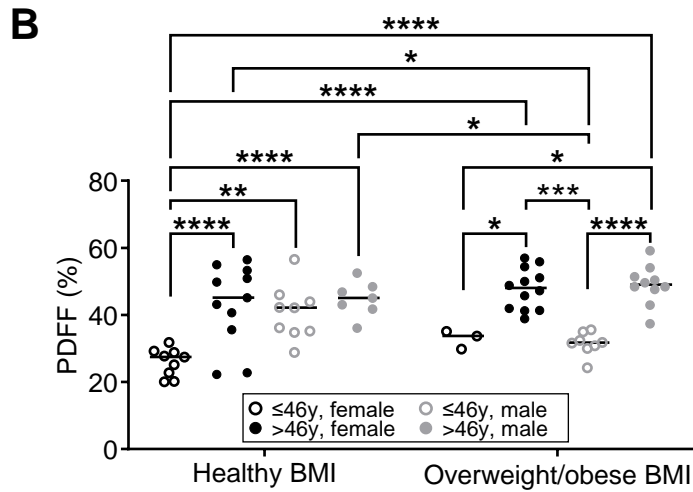
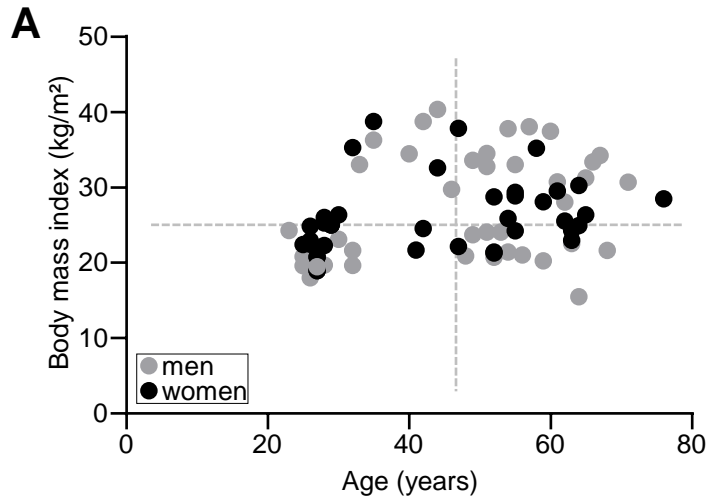
614 correlations are indicated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; strong trends are indicated as numbers
615 ($P < 0.1$; ns, not significant).

616

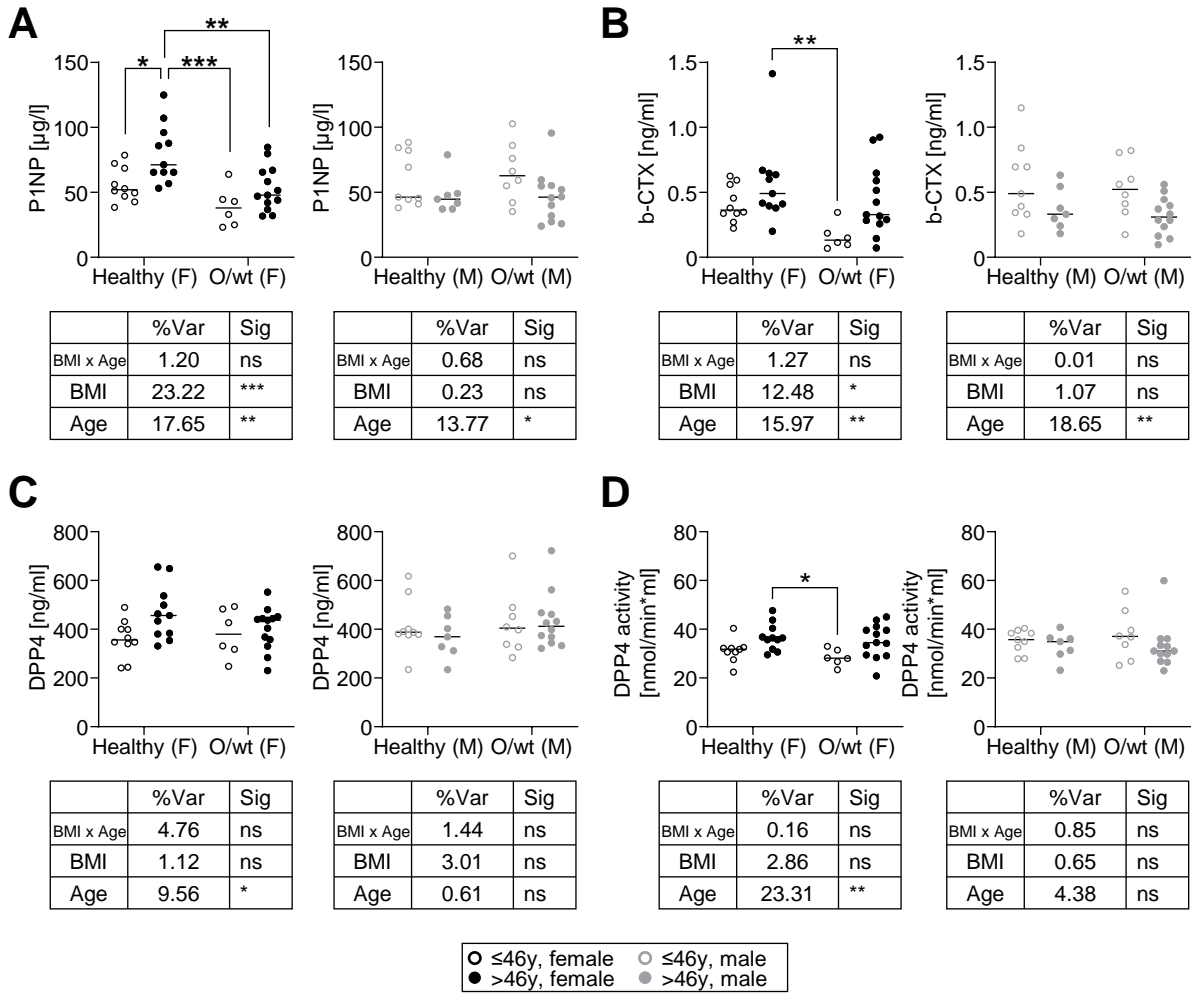
617 **Table 1: Overview of subgroups after stratification by age and BMI.** Women (F) and men (M)
618 were separated into subgroups of individuals either aged 46 years or below or older than 46 years of
619 age, and secondly by BMI, indicating either normal body weight (BMI $< 25 \text{ kg/m}^2$) or overweight /
620 obesity (BMI $> 25 \text{ kg/m}^2$). Data are depicted as mean \pm standard error of mean (SEM) whenever
621 applicable. Letters and symbol indicate statistical significance by 1-Way ANOVA ($P < 0.05$) as follows: a
622 - compared to group: Age ≤ 46 / BMI < 25 (within same sex); b - compared to group: Age ≤ 46 / BMI > 25
623 (within same sex); c - compared to group: Age > 46 / BMI < 25 (within same sex); § - women compared
624 to men within age- and BMI-matched group. Values in parentheses depict instances when dataset is
625 incomplete, i.e. deviating from regular n with actual number of individuals in group indicated in
626 'participants' row.

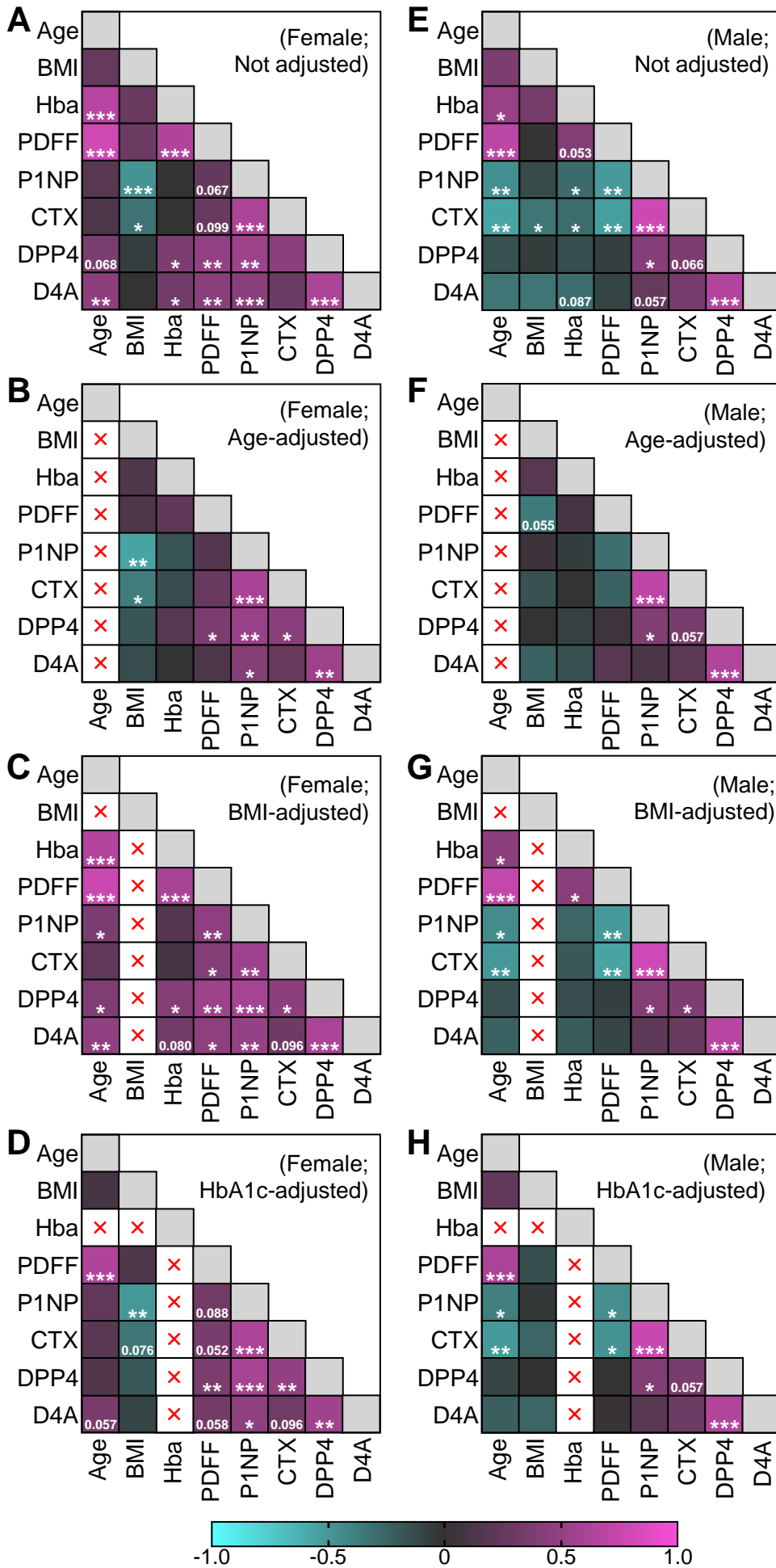
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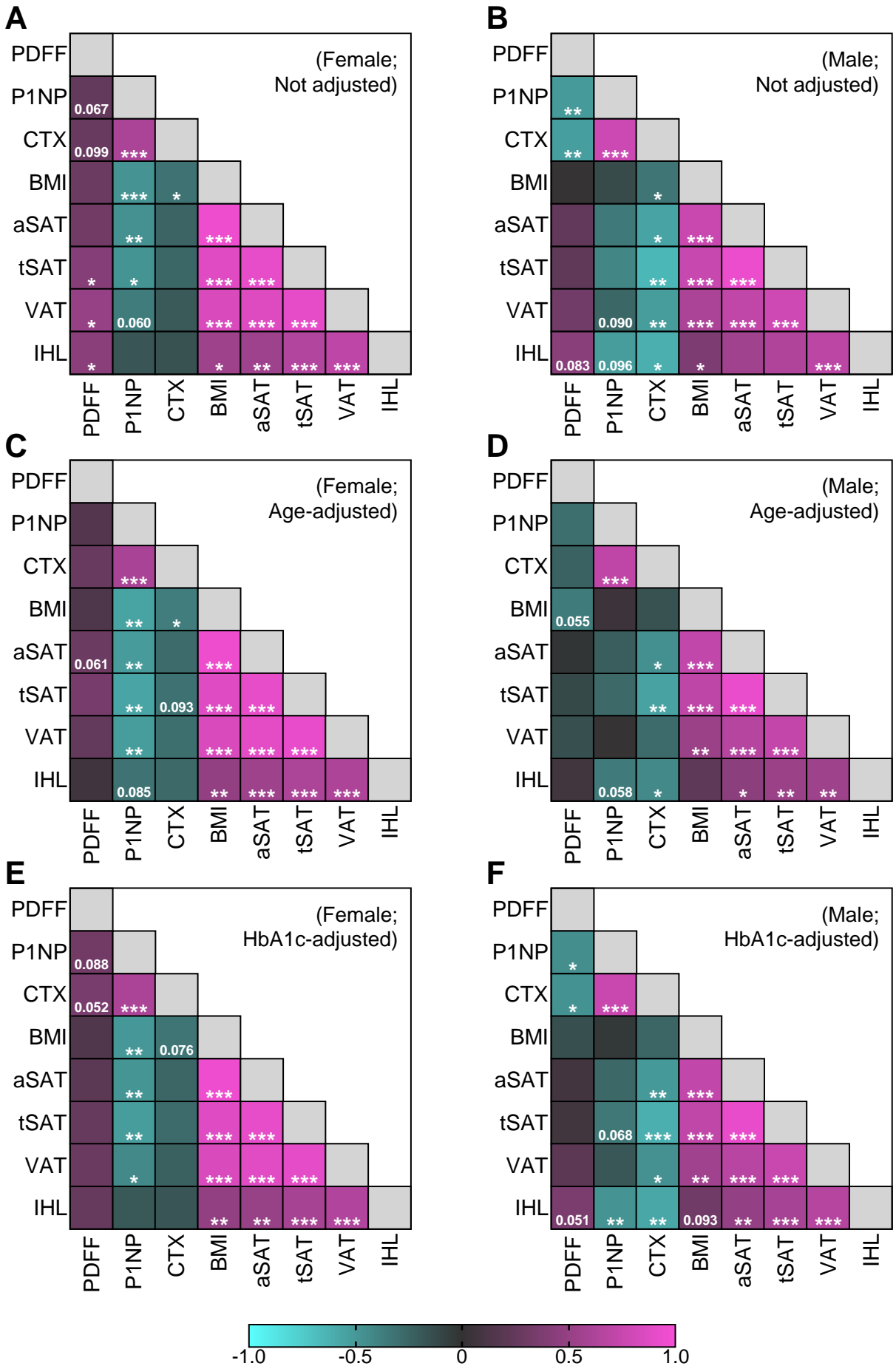
628 **Table 2: Summary of subgroup MRI-quantifications and biomarker measurements.** Analyses of
629 biomarker levels CTX, P1NP, DPP4 concentration and activity as well as quantifications from the MRI-
630 based analyses in the stratified subgroups. Study participants were divided into individuals separated
631 by BMI (healthy: BMI $< 25 \text{ kg/m}^2$) and overweight / obese (BMI $> 25 \text{ kg/m}^2$) or age, i.e. individuals up to
632 46 years of age or individuals older than 46 years. Data are depicted as mean \pm standard error of
633 mean (SEM) whenever applicable. Values in parentheses depict instances when dataset was
634 incomplete, i.e. deviating from regular n with actual number of individuals in group indicated in row
635 'Sex'.



Source of Variation	% variation	Significance
BMI	0.5194	ns (0.3875)
Sex	3.151	* (0.0360)
Age	35.68	**** (<0.0001)
BMI x Sex	3.430	* (0.0290)
BMI x Age	1.483	ns (0.1466)
Sex x Age	1.363	ns (0.1636)
BMI x Sex x Age	3.042	* (0.0393)







Heider et al., Table 1

Parameter/Group	Group: Age ≤ 46 / BMI < 25		Group: Age ≤ 46 / BMI > 25		Group: Age > 46 / BMI < 25		Group: Age > 46 / BMI > 25	
	F	M	F	M	F	M	F	M
Sex								
Participants	10	9	6	8	11	7	13	12
Age [years]	27.4 ± 3.1	29.8 ± 6.7	40.0 ± 5.1 ^a	31.9 ± 5.4	56.1 ± 6.6 ^{a,b}	56.6 ± 6.8 ^{a,b}	59.2 ± 7.0 ^{a,b}	59.0 ± 7.5 ^{a,b}
BMI [kg/m²]	20.8 ± 1.9	22.3 ± 1.8	35.4 ± 3.9 ^{a,§}	29.3 ± 5.5 ^a	21.4 ± 2.4 ^b	23.0 ± 1.5 ^b	33.5 ± 3.0 ^{a,c,§}	29.5 ± 3.6 ^{a,c}
HbA1c [mmol/mol]	33.0 ± 1.9 (9)	35.4 ± 4.1 (8)	37.8 ± 3.1 (5)	33.7 ± 2.7 (7)	37.7 ± 4.2 ^a (11)	35.4 ± 3.6 (7)	38.9 ± 4.4 ^a (10)	37.8 ± 3.3 (10)

a: vs. Group: Age ≤ 46 / BMI < 25 (within same sex)

b: vs. Group: Age ≤ 46 / BMI > 25 (within same sex)

c: vs. Group: Age > 46 / BMI < 25 (within same sex)

§: M vs. F (within same group)

Heider et al., Table 2

Parameter	Group: Age ≤ 46 / BMI < 25		Group: Age ≤ 46 / BMI > 25		Group: Age > 46 / BMI < 25		Group: Age > 46 / BMI > 25	
	M (n=9)	F (n=10)	M (n=8)	F (n=6)	M (n=7)	F (n=11)	M (n=12)	F (n=13)
Sex	M (n=9)	F (n=10)	M (n=8)	F (n=6)	M (n=7)	F (n=11)	M (n=12)	F (n=13)
CTX [ng/ml]	0.57 ± 0.30	0.41 ± 0.14	0.53 ± 0.22	0.16 ± 0.10	0.37 ± 0.16	0.57 ± 0.32	0.32 ± 0.15	0.44 ± 0.27
P1NP [µg/l]	59.9 ± 20.8	55.8 ± 13.2	65.3 ± 22.5	38.8 ± 15.2	48.0 ± 14.3	80.0 ± 22.5 (p0.0162)	46.5 ± 19.8	53.0 ± 17.1
DPP4 [ng/ml]	413.4 ± 111.0	360.5 ± 76.9	425.2 ± 130.5	383.3 ± 99.3	369.5 ± 86.1	467.5 ± 110.1	434.4 ± 113.4	401.8 ± 86.1
DPP4 [nmol/min/ml]	35.1 ± 4.7	31.1 ± 4.7 (9)	37.7 ± 10.1	28.4 ± 3.4	33.2 ± 5.7	36.8 ± 5.5	33.0 ± 9.3	35.1 ± 6.9
PDFF [%]	40.7 ± 8.1	26.0 ± 4.1 (9)	31.4 ± 3.5	32.9 ± 2.7 (3)	44.8 ± 5.2	43.2 ± 12.0	49.0 ± 5.8 (10)	47.8 ± 6.1 (12)
Abdomen SAT [L]	4.1 ± 1.5	4.3 ± 2.0	7.2 ± 4.9	16.6 ± 2.3	4.4 ± 0.7	4.5 ± 1.7	8.3 ± 5.0	15.3 ± 5.3
Thorax SAT [L]	1.7 ± 0.6	1.7 ± 1.0	2.6 ± 1.5	5.8 ± 1.3	1.9 ± 0.4	2.1 ± 0.8	3.2 ± 1.3	5.5 ± 1.4
VAT [L]	2.1 ± 1.0	0.9 ± 0.4	3.4 ± 2.1	3.8 ± 1.3	3.5 ± 1.1	1.4 ± 0.7	6.2 ± 2.7	5.0 ± 2.3
IHL [%]	1.2 ± 0.8	0.7 ± 0.6	1.9 ± 2.6	4.1 ± 5.6	2.1 ± 1.5	1.0 ± 0.7	7.6 ± 8.8	6.7 ± 5.2

Bone marrow adipose tissue: Factors of sex-specific modulation of bone turnover

METHODS

MRI. Proton density fat fraction (PDFF) ~ Bone marrow adipose tissue (BMAT)

Biomarkers. Plasma analysis of DPP4, CTX, and P1NP

Stratification.

Sex, Age, BMI

↓
Analysis of variance
Spearman correlations

CONCLUSION

Women: Age and BMI modulate relationship between BMAT, DPP4 and bone turnover

Men: Age is main determinant of relationship between BMAT, metabolic health and bone turnover

