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Possible link between FSH and RANKL release from adipocytes in men with impaired gonadal function including Klinefelter syndrome

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ABSTRACT

Introduction: The FSH receptor (FSHR) has been found to be expressed in human bone cells and bone marrow-adipocytes, and highly-debated mouse studies have suggested extra-gonadal effects of gonadotropins on glucose, adipocyte and bone homeostasis. These putative effects could be direct or indirectly mediated by endocrine factors released from bone-cells or adipocytes. Here, we investigated whether gonadotropins are linked with glucose- and lipid-metabolism in hypergonadotropic men.

Methods: Single centre, cross-sectional study of 307 men with idiopathic infertility and 28 men with Klinefelter syndrome (KS). Outcome: associations between serum LH and FSH with soluble-RANKL (sRANKL), osteoprotegerin (OPG), osteocalcin, fasting glucose and insulin, sex steroids, and body composition. Expression of FSHR was studied in human-derived adipocyte-cell-models (hMADS, TERT-hWA) and FSH stimulation of RANKL expression and secretion in hMADS in vitro.

Results: Serum FSH was not directly linked with glucose- and lipid-metabolism. However, FSH was inversely associated with sRANKL in both infertile men and KS men ($p = .023$ and $p = .012$). Infertile men with elevated FSH (> 11 U/L) had significantly lower sRANKL ($p = .015$). sRANKL was positively associated with fat percentage, fasting insulin, and glucose (all $p < .05$). Men with prediabetes had higher sRANKL ($p = .021$), but lower testosterone ($p < .0001$) and Inhibin B ($p = .005$). The FSHR was expressed in the investigated human derived adipocytes, and 3–6 h treatment with FSH markedly increased RANKL release ($p < .05$).

Conclusion: KS and infertile men with prediabetes have low Inhibin B, and testosterone but elevated RANKL compared with non-prediabetic men despite comparable levels of serum gonadotropins. Serum FSH and sRANKL was inversely associated in both infertile and KS men, but the increased release of RANKL from FSH treated adipocytes suggest a direct effect of FSH on RANKL production in some tissues. Further studies are required to clarify whether FSH targets RANKL in the skeleton.

ClinicalTrial_ID:NCT01304927

1. Introduction

Gonadotropins refer to follicle stimulating hormone (FSH) and luteinizing hormone (LH) produced by the anterior part of the pituitary gland. Both hormones are essential for gonadal function in both men and women [1,2] because they control the two main functions of the gonads: production of gametes and sex steroids (testosterone and estradiol). FSH is essential for gametogenesis, while LH is a key regulator

Abbreviations: CV, inter-assay coefficients of variation; LH, luteinizing hormone; FSH, follicle stimulating hormone; FT, calculated free testosterone ad modum Vermeulen; FT%, calculated free testosterone in percent; SHBG, sex hormone binding globulin; AMH, anti-müllerian hormone; RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin; DXA, whole body dual-energy X-ray absorptiometry scan; BF%, body fat percentage, by DXA; AF%, android fat percentage, by DXA; GF%, gynoid fat percentage, by DXA; MAP, mean arterial blood pressure; BMD, bone mineral density; PTH, parathyroid hormone; KS, Klinefelter syndrome

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of steroidogenesis [2–4]. The kinetics of gonadotropin production is strikingly different between sexes although similarities exist such as the FSH and LH stimulation of inhibin B and LH of sex steroids in both men and women. Sex steroids and inhibins exert a negative feedback on the hypothalamus and pituitary gland that suppress production of gonadotropins [2,5]. The endocrine crosstalk between hypothalamus, pituitary and gonad (HPG axis) is maintained throughout life and at advanced age the declining gonadal function triggers a compensatory increase in serum concentrations of gonadotropins [6]. The increase in serum gonadotropins with age occurs gradually in men [7] and happens more abruptly after menopause in women [8].

Estradiol is known to be a central regulator of bone remodelling [9]. The decrease in circulating concentrations of sex steroids following menopause in women and in older men have clinical consequences for the skeleton with impaired bone formation and increased risk of developing osteoporosis [10,11]. The aging related skeletal changes have largely been attributed to reduced gonadal function and particularly low serum estradiol levels. However, preclinical investigations suggest an existing crosslink between glucose and bone metabolism and this endocrine crosslink supposedly also influence the HPG axis [12]. More than a decade ago it has been suggested that elevated FSH levels in the presence of declining serum concentrations of sex steroids following menopause were responsible for the development of osteoporosis [13]. This hypothesis was challenged by studies in transgenic mice overexpressing FSH, proposing that the effect was merely mediated indirectly through inhibins and testosterone [14,15]. A prospective human intervention study elegantly demonstrated that FSH had no major effect on bone resorption [16] although other preclinical studies reported direct effects of FSH on osteoclasts [17]. A recent rodent study, which due to the previous controversy was validated by an independent group, suggested that FSH also may be implicated in the postmenopausal changes in lipid distribution, adiposity and metabolism [18]. Interestingly, the effects of FSH in mice were apparent in both sexes, which indicate that men with impaired gonadal function, characterized by elevated FSH levels, also may be prone to a higher risk for osteoporosis and adverse metabolic changes.

The skeleton is an endocrine organ that releases bone specific proteins into circulation such as osteocalcin, fibroblast growth factor 23 (FGF23), osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL). Some of these factors have been suggested to exert functions in organs distant from the skeleton through presence of their respective receptors. Despite many controversies, several publications have proposed these factors as biomarkers for food intake, testosterone production, insulin production and sensitivity and development of type 2 diabetes [19,20]. The main source of RANKL in circulation and putative role as an endocrine factor remain to be determined. Normally, RANKL is a transmembrane protein residing in osteoblasts that binds and activates a specific receptor RANK in osteoclasts, which induces osteoclastogenesis [21]. This signalling is controlled by OPG that binds the ligand domain of RANKL and inhibits RANK activation and thereby prevents bone resorption [22]. Several studies have shown that RANKL and OPG are detectable in circulation and serum concentrations have in some studies been associated with bone mineral density (BMD), but also with several inflammatory diseases including type 2 diabetes. This indicates that origin and release of RANKL into circulation may be regulated differently than for instance FGF23 and osteocalcin. A novel study showed that RANKL is produced by fat cells residing in the bone marrow under influence of parathyroid hormone (PTH), and release of RANKL from these adipocytes was mirrored by changes in serum RANKL concentrations [23]. The link with adipocytes is of particular interest because RANKL and OPG have been suggested to be important for insulin production and pancreatic beta cell function. In this study, we investigated the link between bone mineral density, body composition, serum concentrations of gonadotropins, endocrine bone factors, serum lipids and glucose metabolism in men with impaired gonadal function and tested whether gonadotropins were able to influence production of RANKL from human-derived adipocyte cell lines in vitro.

2. Methods

2.1. Copenhagen-bone-gonadal study

The cohort consist of 307 men without serious co-morbidities, included from a total of 1427 men referred from 2011 to 2015 to our andrological outpatient clinic with male infertility due to impaired semen quality [24,25]. All men delivered semen samples, detailed patient history, had a physical examination, ultrasound examination of testicles and full body Dual-energy X-ray absorptiometry (DXA) scan performed and blood samples drawn including analysis of karyotype and men with aberrant sex-chromosomes were excluded. All the presented data are from their initial visit, but the men were afterwards followed for 150 days and treated with either vitamin D or placebo as described previously [24]. DXA scan was done using GE Medical System Lunar, Prodigy Series X-Ray scanner with GE Healthcare, enCore Version 16 softwear. BMD was evaluated using Total T-score, Femoral neck T-score and L1-L4 T score, while whole body scan was used to calculate full body fat percentage (BF%), android fat percentage (AF%) and gynoid fat percentage (GF%). Anthropometric measurements (abdominal circumference, weight and height, body mass index (BMI)) were conducted by a nurse. All included variables were predefined (Clinical Trials: NCT01304927).

2.2. Klinefelter patients

We identified a total of 34 male patients with Klinefelter 47, XXY syndrome (KS) from our out-patient clinic, out of these we included 28 men above 14 years of age for measurements of RANKL concomitantly with reproductive- and sex hormones. Use of testosterone supplementation was registered.

2.3. Biochemical analyses

All blood samples were drawn fasting during morning hours (8.00 to 10.00 a.m.). To determine FSH and LH levels we used a time-resolved immuno-fluorometric assay (Delfia; Wallac, Turku, Finland) and for inhibin-B we used a specific two-sided enzyme linked immunoassay (inhibin B genII, Beckman Coulter, USA). The CVs were < 6%, < 4%, < 4% and < 11% for SHBG, FSH, LH and Inhibin B, respectively. AMH was measured using the Beckman Coulter enzyme immunometric assay (Immunotech, Beckman Coulter, Marseilles, France) with CV < 8%. Testosterone and sex hormone binding globulin (SHBG) were analysed in chemiluminescent immune assays (Access, Beckman Coulter, USA), and estradiol levels were analysed by radioimmunoassay (Coat-a-Count, Siemens and Pantex, Santa Monica, CA, respectively) with CV of 6, 8 and 13%, respectively. Measurements of OPG and soluble RANKL in both serum and cell culture medium were performed using the same kit by Biomedica (CV 3%, CV 5%), while IGF1, IGF-BP3 and osteocalcin were analysed by chemiluminescence assay from IDS-iSYS immunodiagnostics with CV of 10, 9 and 8% respectively. Undercarboxylated osteocalcin was measured using the same osteocalcin assay following precipitation of carboxylated osteocalcin: 100 μL serum was incubated with 100 μL 10 mg/mL hydroxyapatite for 1 h. at room temperature and subsequently centrifuged 2 min at 10000 × *g*. The resulting supernatant was used for measurement of undercarboxylated osteocalcin. Measurements of 25-OH vitamin D (CV < 10%) and $1,25(OH)_2D_3$ (CV 18%) were performed by LC-MS as described previously [26]. HbA1c was measured on TOSOH G8 (CV 2%), fasting glucose on ABL800 Flex (CV 2.5%), while parathyroid hormone (CV < 4%), fasting insulin (CV 5%), total cholesterol (CV 5%), LDL (CV 4%), HDL (CV 4%) and triglyceride (CV 5%) were all measured using Cobas 8000. Derived variables were calculated using

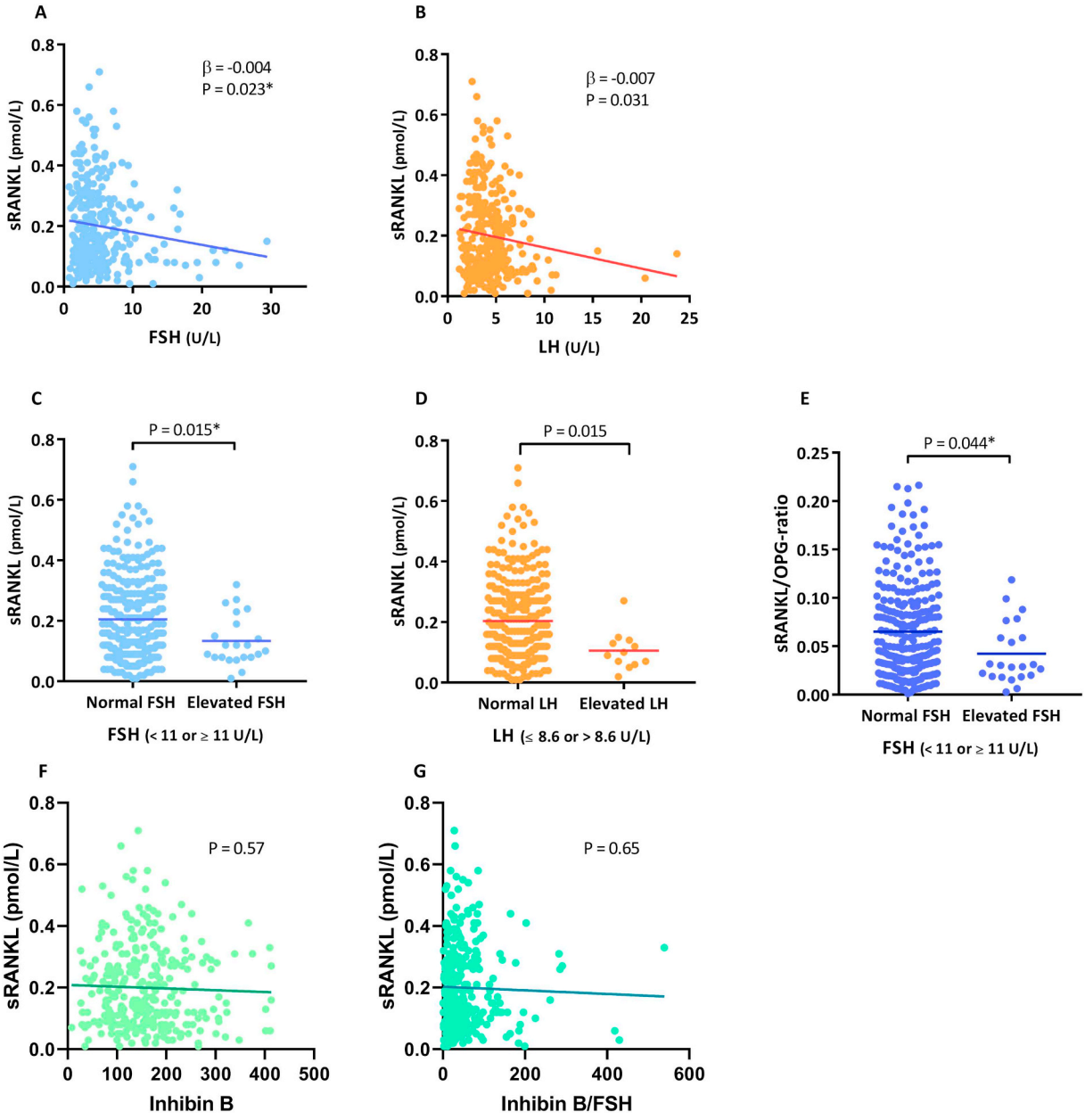

Fig. 1. Serum concentrations of gonadotropins and sRANKL. A: association between sRANKL and FSH B: Association between sRANKL and LH. C: sRANKL in men with normal or elevated FSH. D: sRANKL in men with normal or elevated LH. E: sRANKL/OPG ratio in men with normal or elevated FSH. F: association between sRANKL and Inhibin B. G: association between sRANKL and Inhibin B/FSH. * analyses with FSH are still significant (A: p 0.041; C: p 0.036) after adjusting for age, smoking and BF% (with or without estradiol).

the following formulas: Prediabetes [HOMA-IR: (fasting glucose x fasting insulin)/22.5], pancreatic β cell function [HOMA-β: (20 x fasting insulin)/(fasting glucose – 3.5)], quantitative insulin sensitivity index (QUICKI) [1/(log(fasting insulin) + log(fasting glucose)], mean arterial blood pressure [MAP: diastolic +0.33(systolic – diastolic)], free testosterone (FT) using the Vermeulen formula [27] and free estrogen by using the Mazer constant.

2.4. FSHR, LHR and RANKL in human multipotent adipose-derived cells

The human-derived adipocyte-cell-models used were: hMADS [28] (from a subcutaneous depot) and TERT-hWA [29] (from a subcutaneous neck depot), they were propagated and differentiated essentially as described previously [29]. Briefly, all cells were cultured until two days post-confluence (designated day 0) where they were induced to differentiate in advanced DMEM/F12 supplemented with 2% fetal bovine serum (FBS) (Life Technologies), L-glutamine (2 mM) (Life Technologies), penicillin (62.5 µg/mL), streptomycin (100 µg/mL) Sigma-Aldrich), insulin (5 µg/mL) (Roche), dexamethasone (1 µM) (Sigma-Aldrich), 3-isobutyl-1-methylxanthine (0.5 mM) (Sigma-Aldrich), rosiglitazone (1 µM) (Cayman Chemical), human cortisol (1 µM) (Sigma-Aldrich) and T_3 (1 nM) (Sigma-Aldrich). At day 3, the medium was refreshed with the same medium used at day 0. At day 6 and 9, the cells received Advanced DMEM/F12 supplemented with 2% FBS. At day 12, the adipocytes were defined as mature, and the percentage of differentiated cells was 50–70% based on microscopic inspection, irrespective of cell model. RNA was extracted for cDNA synthesis from undifferentiated (designated day 0) and differentiated (day 12) cells and expression of RANKL (F: TGGATCACAGCACATCAGAGCAG, R: TGGG GCTCAATCTATATCTCGAAC) was investigated by qPCR. FSHR was

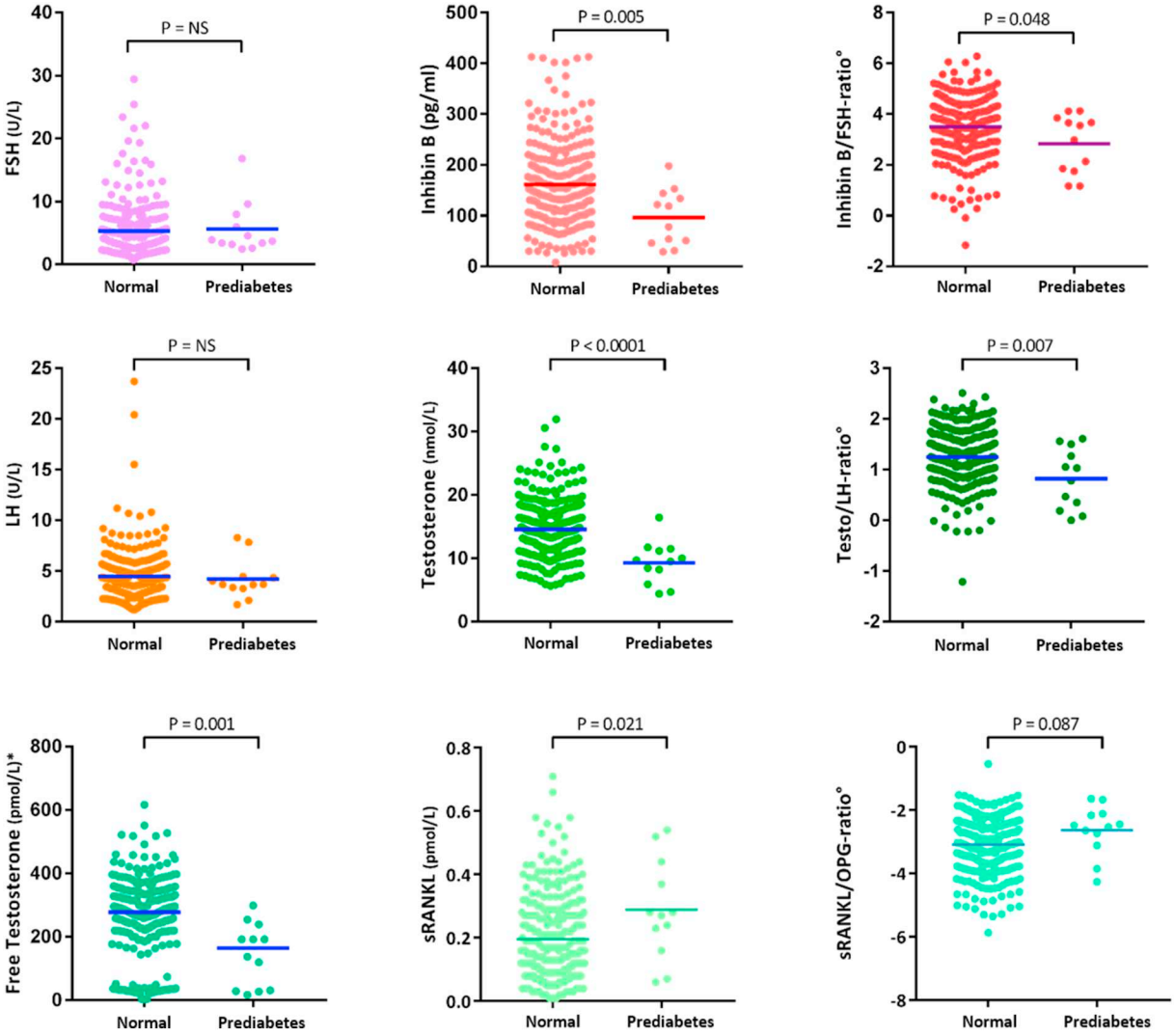

Fig. 2. Sex steroids, reproductive hormones and RANKL in infertile men with and without prediabetes (fasting glucose ≥ 6 mmol/L). Bar show mean and comparison with students T-test. * indicate variables with bar shown as median and comparison using Mann-whitney. ° indicate ln-transformed variables.

investigated with different primers targeting exon 1 (F: ATAAGGGCA CTGTGTGGAGC, R: TGAGCCCAAGCTCAGGAATG), exon 2 (F: CCTGG TCTCTTTGCTGGCAT, R: TTGGTGAGGACAAACCTCAGTT) and exon 10 (F: TCTGGCAGAAGACAATGAGTCC, R: TGAGGATGTTGTACCCCATG ATA). RT-PCR band of *FSHR* in adipocytes and human testis tissue was directly sequenced. The degree of differentiation in both TERT-hWA and hMADS cells was quantified by qPCR of the adipocyte-marker adiponectin (*ADIPOQ*) gene on both day 0 and day 12. FSHR and RANKL expression in differentiated hMADS and TERT-hWA cells (day 12) was investigated by immunocytochemistry of cytospins stained with FSHR antibody (ab150557) or two different RANKL antibodies from rabbit and goat targeting different RANKL epitopes (sc-9073 and sc-7628). Antibodies were validated in human testis tissue (FSHR) and human fetal bone tissue (RANKL) (suppl. method section for protocols). We selected only one cell line for exposure studies and hMADS was chosen due to the consistent expression of FSHR. Differentiated hMADS cells were treated for 3, 6, 24 or 48 h with recombinant FSH (Puregon) 0.01, 0.1, 1.0 or 10.0 IU/mL or vehicle (H_2O + 0.01% BSA) to compare RANKL and FSHR expression using qPCR. Moreover, media from the different treatments were collected and sRANKL from adipocytes was measured using the previously described ELISA platform. Furthermore, sRANKL was also measured in media from vehicle treated TCam2 and NTera2 cells (high expression of RANKL) for comparison with vehicle-treated adipocytes. Both cell lines were grown under standard conditions at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (NTera2) or RPMI 1640 (TCam-2) as previously described [30]. All cell experiments on FSHR were conducted in triplicates and preformed twice. All cells were cultured at 37 °C in 5% CO_2 atmosphere.

2.5. Statistical analysis

Descriptive statistics were calculated for all variables and presented as mean with standard deviation. Associations between gonadotropins and selected bone and glucose related outcome variables were conducted using multiple regression analyses. In all the regression models of infertile men, the following biologically relevant confounders were included: age, bodyfat-percentage and smoking. In addition, putative additional confounders were tested afterwards for instance estradiol. We had limited data on these confounders in the KS cohort and data from KS patients were therefore presented only age-adjusted. KS men were further stratified in two groups according to whether or not they received testosterone supplementation. Gaussian distribution of all numerical variables was evaluated by PP-plots of residuals to secure validity of the regression models. As a result, the following variables were transformed with natural logarithm: anti-müllerian hormone

Table 1
Baseline Characteristics of all men in the Copenhagen-Bone-Gonadal study. Mean values of metabolic parameters, reproductive hormones and semen quality, reference-values when available in the far-right column. Distribution of clinical groups. * presented as median and not mean.

Baseline characteristics of men in CBG study	Mean	(SD)	Ref.
Age (years)	34.8	(6.6)	
BMI (kg/m^2)	26.4	(4.4)	
Body fat percentage (%)	26	(8.7)	
Smoking (%)	24%		
25-OHD (nmol/L)	45.4	(20.0)	> 50
1,25$(OH)_2D_3$ (pmol/L)	84	(32.2)	37–216
Bone mineral density, Total T-score (SD)	0.822	(1.14)	
FSH (U/L)	5.3	(4.2)	1.5–11
LH (U/L)	4.4	(2.4)	1.8–8.6
Testosterone (nmol/L)	14.4	(4.6)	8.6–29
Estradiol (pmol/L)	100.3	(26.8)	90–220
SHBG (nmol/L)	30.68	(12.1)	18.3–54.1
Inhibin B	159	(78)	30–400
sRANKL (pmol/L)	0.199	(0.135)	
OPG (pmol/L)	3.5	(0.9)	
Osteocalcin (ug/L)	15.5	(5.9)	
Sperm volume (mL)	3.9	(1.8)	≥ 1.5
Total sperm count* (10^6)	96	(127)	≥ 40
Sperm concentration* (10^6/mL)	27	(33.9)	≥ 15
Morpholgical normal sperm (%)	3,3	(2.9)	≥ 4
Progressive motile sperm (%)	32	(19.8)	≥ 40

Clinical stratification	%	n
FSH above 11 U/L	7.3%	22
LH above 8.6 U/L	4%	12
Prediabetes (fasting glucose ≥6.0 mmol/L)	4%	12
Hypertension (systolic BP ≥140 and/or diastolic BP ≥90)	29%	87
Osteopenia (Total T-score between −1 and −2.5 SD)	6%	18
Waist circumference > 102 cm	23%	69

(AMH), insulin, insulin resistance, β cell function, triglyceride, PTH, sRANKL/OPG-ratio, T/LH-ratio and Inhibin B/FSH-ratio. It was not possible to obtain a Gaussian distribution of calculated free testosterone. Afterwards the men were stratified into clinical groups: hypergonadotropic and having prediabetes based on fasting glucose ≥6.0 and supported by QUICKI index < 0.357 [31,32]. Differences between clinical groups were performed using either one sided ANOVA or Students *t*-test depending on number of groups in comparison. Mann-Whitney *U* test was applied when non-Gaussian distribution was observed. One sided ANOVA or Students t-test were used to compare treatment effects on gene expression and secreted RANKL in human-derived adipocytes.

3. Results

All 307 infertile men had biochemical analyses performed, while 294 men had a full body and columnar/femoral DXA scan performed. Basic characteristics of all men are presented in Table 1. All men were stratified into clinically relevant binomial groups: prediabetes defined by fasting glucose and supported by HbA1C, QUICKI-index and insulin sensitivity (Suppl. Table 1), waist circumference > 102 cm (NIH indicator of High Risk for metabolic syndrome) osteopenia (total T score ≤ −1 SD but ≥ − 2.5 SD), hypergonadotropic men (FSH ≥ 11 U/L or LH > 8.6 U/L) and hypertension (≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg) (Table 1). Infertile men with osteopenia had a mean vitamin D, 25-OHD of 45.8 nmol/L (SD 19.8 nmol/L) and 1,25-OH_2D_3 of 96.3 pmol/L (SD 37.4 pmol/L), which did not differ from serum 25-OHD of 45.5 nmol/L (SD 19.9 nmol/L) and 1,25-OH_2D_3 of 83.5 pmol/L (SD 31.6 pmol/L) in men with normal BMD. FSH and LH were negatively associated with serum AMH and Inhibin B (both $p < .05$, Table 2), while LH was negatively associated with free testosterone ($p < .05$, Table 2).

3.1. Associations between gonadotropins and endocrine bone factors, bone mineral density and metabolic parameters in infertile men

FSH and LH were inversely associated with serum concentrations of sRANKL (β −0.004 pmol/L: p0.023, β −0.01 pmol/L:p0.031, respectively). However only FSH remained significantly associated after adjustment of relevant confounders (β −0.004 pmol/L:p0.041) (Fig. 1) and the association remained significant after adjustment of serum estradiol (β – 0.004 pmol/L:p 0.050). Serum Inhibin B levels and Inhibin B/FSH ratio were not associated with sRANKL (Fig. 1). LH was inversely associated with osteocalcin (β −0.021 U/L p 0.017) but weakened after adjustment for relevant confounders (Table 2). Neither FSH nor LH was associated with any of the other investigated skeletal proteins or BMD. Of all evaluated metabolic parameters, FSH was positively associated with HDL cholesterol (β 0.010 U/L: p0.040), while LH was positively associated with β-cell function (β 0.03 U/L p 0.044). No difference in serum gonadotropin levels was found after stratifying the men into clinically relevant groups of osteopenia, prediabetes, hypertension or increased waist circumference. However, stratification into men with normal or elevated (hypergonadotropic) FSH and LH levels (FSH ≥ 11 U/L or LH > 8.6 U/L) showed that men with elevated FSH levels had significantly lower sRANKL levels (Fig. 1). Men with elevated FSH had on average sRANKL of 0.13 pmol/L and thus markedly lower than 0.21 pmol/L in men with normal FSH levels after adjusting for age, smoking, and BF% (p0.018), which remained after adjustment of serum estradiol. Men with elevated serum LH also had lower sRANKL levels (0.11 pmol/L vs. 0.20 pmol/L, p0.015) but the difference disappeared after adjustment for age, smoking and BF% (Fig. 1). Men with low versus high FSH did not differ in age or BF% (Suppl. Fig. 1). sRANKL was inversely associated with testosterone, SHBG and T/*E*-ratio (all,$p < .05$), but positively associated with FT% (Table 2). In contrast, osteocalcin was positively associated to SHBG and inversely associated with FT% (all,$p < .05$) (Table 2). IGF-1 and IGF-BP3 were positively associated with sRANKL and osteocalcin ($p < .05$).

Men with prediabetes ($n = 12$), differed markedly from the rest of the group of infertile men (Fig. 2, Suppl. Table 1). They had significantly higher HbA1c, insulin, insulin resistance and lower QUICKI-index. Moreover, they had higher RANKL (0.29 pmol/L vs 0.20 pmol/L:p0.021) and borderline higher RANKL/OPG-ratio (0.071 vs. 0.045:p 0.087) but they also had markedly lower inhibin B (97 ng/L vs. 161 ng/L:p0.005) and inhibin B/FSH-ratio (7.7 vs. 9.5: p0.048) as well as lower total and free testosterone (9.3 nmol/L vs. 14.6 nmol/L:p7.4×10^{-5} and 164 pmol/L vs. 277 pmol/L:p0.001) (Suppl. Table 1). However, FSH (5.64 U/L vs. 5.32 U/L: p0.80) and LH (4.20 U/L vs. 4.46 U/L: p0.74) in these prediabetic men did not differ compared with the infertile men having normal glucose level (Fig. 2). Interestingly PTH-levels were higher in men with prediabetes, however the associations between sRANKL and FSH remained significant also after adjusting for PTH level and/or activated vitamin D (1,25OH2D3) (β −0.004 p 0.052 and p0.043, respectively). Stratification of the men based on insulin resistance based on low QUICKI-index showed similarly higher RANKL (0.22 pmol/L vs 0.18 pmol/L:p0.014), and higher RANKL/OPG-ratio (0.071 vs. 0.056:p 0.010), lower total and free testosterone (12.7 nmol/L vs. 16.1 nmol/L:p1.5×10^{-11} and 236 pmol/L vs. 270 pmol/L:p0.003). Inhibin B, inhibin B/FSH-ratio, while FSH and LH did not differ (data not shown). Undercarboxylated osteocalcin was higher than expected using our methodological setup and was not correlated with metabolic variables, testosterone, gonadotropins or sRANKL and did not differ between men having prediabetes or normal glucose.

3.2. Endocrine bone markers, BMD and metabolic parameters

None of the bone factors measured in serum were associated with BMD, however, 1,25$(OH)_2D_3$ was negatively associated with BMD (β −4.3 SD, p 0.018). sRANKL was associated with mean arterial pressure (MAP) (β −11.8 p 0.014), BF% (β 9.51 p 0.016) and IGF-BP3 (β 659.6

Table 2
Linear Regression showing associations between FSH, LH sRANKL, OPG and Osteocalcin and respectively investigated bone factors, BMD, reproductive hormones and metabolic parameters. Investigated variables in columns set as independent. *P* values in bold represent findings of interest. na: not applicable. ns: not significant. * adjusted for smoking, age and body fat % (analyses with body fat % only adjusted for age and smoking).

Linear regression analyses										
		FSH				LH				sRANKL
		Unadjusted		Adjusted*		Unadjusted		Adjusted*		Unadjusted
		β	p value	β	p value	β	p value	β	p value	β
Bone factors	sRANKL	**−0.004**	**0.023**	**−0.004**	**0.041**	**−0.007**	**0.031**	–	ns	*na*
	RANKL/OPG-ratio	–	ns	–	ns	–	ns	–	ns	5.79
	OPG	–	ns	–	ns	–	ns	–	ns	−2.28
	PTH	–	ns	–	ns	–	ns	–	ns	–
	Osteocalcin	–	ns	–	ns	**−0.021**	**0.017**	–	ns	5.99
BMD	Total T-score	–	ns	–	ns	–	ns	–	ns	–
	Femoral neck T-score	–	ns	–	ns	–	ns	–	ns	–
	L1-L4 T-score	–	ns	–	ns	–	ns	–	ns	–
Reproductive hormones	Testosterone	–	ns	–	ns		ns	–	ns	**−4,00**
	Estradiol	–	ns	–	ns	1.81	0.004	–	ns	–
	SHBG	–	ns	–	ns	0.72	0.012	0.893	0.004	**−22.03**
	AMH	−0.058	< 0.0001	−1.691	< 0.0001	−0.034	0.03	−0.046	0.012	–
	Inhibin B	−11.15	< 0.0001	−11.969	< 0.0001	−8.23	< 0.0001	−9.408	< 0.0001	–
	Free testosterone %	–	ns	–	ns	−0.022	0.036	−0.029	0.014	**0.641**
Metabolic parameters	HbA1c	–	ns			–	ns	–	ns	–
	Fasting insulin	–	ns	–	ns	–	ns	–	ns	**0.636**
	Fasting glucose	–	ns	–	ns	–	ns	–	ns	**0.685**
	Insulin resistance (HOMA)	–	ns	–	ns	–	ns	–	ns	**15.17**
	β cell function (HOMA)	–	ns	–	ns	–	ns	**0.029**	**0.044**	–
	Total cholesterol	–	ns	–	ns	–	ns	–	ns	–
	HDL cholesterol	**0.012**	**0.014**	**0.01**	**0.04**	–	ns	–	ns	–
	LDL cholesterol	–	ns	–	ns	–	ns	–	ns	–
	Triglyceride	–	ns	–	ns	–	ns	–	ns	**0.56**
	MAP	–	ns	–	ns	–	ns	–	ns	**−10.16**
	Waist circumference	–	ns	–	ns	–	ns	–	ns	–
	Body fat percentage	–	ns	–	ns	–	ns	–	ns	–
	Gynoid fat percentage	–	ns	–	ns	–	ns	–	ns	**7.72**
	Android fat percentage	–	ns	–	ns	–	ns	–	ns	–
	BMI	–	ns	–	ns	–	ns	–	ns	**5.01**
	IGF-1	–	ns	–	ns	–	ns	–	ns	**49.46**
	IGF-BP3	–	ns	–	ns	–	ns	–	ns	**1.142,00**

Linear regression analyses											
	sRANKL			OPG				Osteocalcin			
	Unadjusted	Adjusted*		Unadjusted		Adjusted*		Unadjusted		Adjusted*	
	p value	β	p value	β	p value	β	p value	β	p value	β	p value
Bone factors	*na*	*na*	*na*	−0.052	< 0.0001	−0.05	< 0.0001	0.003	0.018	0.004	0.024
	< 0.0001	5.59	< 0.0001	−0.625	< 0.0001	−0.609	< 0.0001	–	ns	–	ns
	< 0.0001	−2.02	< 0.0001	na	na	na	na	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	0.012	0.003
	0.018	5.51	0.024	–	ns	–	ns	*na*	*na*	*na*	*na*

(*continued on next page*)

Table 2 (*continued*)

Linear regression analyses											
	sRANKL			OPG				Osteocalcin			
	Unadjusted	Adjusted*		Unadjusted		Adjusted*		Unadjusted		Adjusted*	
	p value	β	p value	β	p value	β	p value	β	p value	β	p value
BMD	ns	–	ns	–	ns	–	ns	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	–	ns
Reproductive hormones	**0.041**	–	ns	–	ns	–	ns	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	–	ns
	< 0.0001	**−12.94**	**0.009**	–	ns	–	ns	**0.263**	**0.025**	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	–	ns
	0.001	–	ns	–	ns	–	ns	**−0.011**	**0.012**	–	ns
Metabolic parameters	ns	–	ns	–	ns	–	ns	**−0.008**	**0.019**	–	ns
	0.014	–	ns	–	ns	–	ns	–	ns	–	ns
	0.004	**0.475**	**0.05**	–	ns	–	ns	**−0.014**	**0.009**	–	ns
	0.007	–	ns	–	ns	–	ns	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	**0.012**	**0.047**
	ns	–	ns	–	ns	–	ns	**−0.026**	**0.007**	–	ns
	ns	–	ns	–	ns	–	ns	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	**−0.019**	**0.02**	–	ns
	0.013	–	ns	–	ns	–	ns	**−0.017**	**0.001**	–	ns
	0.027	**−11.79**	**0.014**	–	ns	–	ns	–	ns	–	ns
	ns	–	ns	–	ns	–	ns	**−0.269**	**0.024**	–	ns
	ns	**9.51**	**0.016**	–	ns	–	ns	**−0.295**	**0.001**	**−0.295**	**0.003**
	0.032	–	ns	–	ns	–	ns	**−0.261**	**0.001**	–	ns
	ns	–	ns	–	ns	–	ns	**−0.41**	**0.0002**	–	ns
	0.009	–	ns	–	ns	–	ns	**−0.129**	**0.003**	–	ns
	0.01	–	ns	**−8.03**	**0.007**	–	ns	**2.83**	**< 0.0001**	**1.79**	**< 0.000-1**
	0.0002	**659.63**	**0.038**	–	ns	–	ns	**19.77**	**0.005**	**18.1**	**0.028**

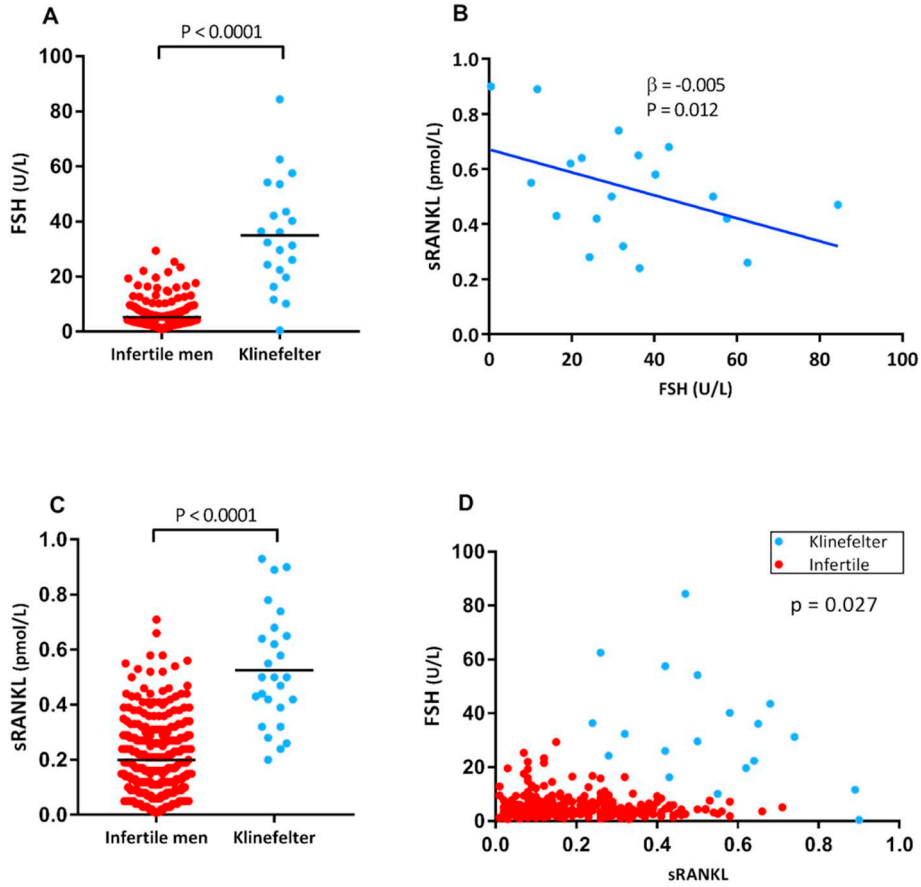

Fig. 3. sRANKL and FSH levels in infertile men vs. Klinefelter Syndrome men (KS), p values adjusted for age. A: FSH level in serum. B: linear regression of sRANKL and FSH in Klinefelter men. C: sRANKL level in serum (also adjusted for FSH), D: FSH/sRANKL-ratio in infertile vs Klinefelter men.

p 0.038) and fasting glucose prior to and after adjustment of age, smoking and BF% (β 0.48 p 0.050), while only unadjusted with QUICKI index (β −0.041 p 0.006). sRANKL/OPG-ratio was associated with fasting glucose (β 0.091 p 0.025), triglyceride (β 0.089 p 0.015) and MAP (β −1.56 p 0.050) after adjustment of confounders (Table 2 and Suppl. Figs. 2 and 3). OPG was inversely correlated with IGF-BP3 (R^2–0.160 p 0.007) but not after adjustment. All associations involving osteocalcin disappeared after adjustment of relevant confounders except for IGF-1 (β 1.8 $p < .0001$), IGF-BP3 (β 18.1 p 0.028), BF% (β −0.30 p 0.003) and β-cell function (β 0.01 p 0.047). sRANKL, OPG and osteocalcin were not different when comparing men with osteopenia, hypertension or increased waist circumference with men having normal levels.

3.3. Klinefelter syndrome patients

KS men had higher FSH levels compared with the infertile men (mean FSH 35.7 U/L vs 5.3 U/L $p = 3.4 \times 10^{-58}$, age adjusted, Fig. 3a). In the group of adult KS men, we found an inverse association between sRANKL and FSH β −0.005 p 0.012 (age adjusted), comparable to the group of infertile men (Fig. 3b). After stratification (testosterone supplementation yes/no) the inverse relationship existed only in the group receiving treatment (β −0.006 p 0.028, age adjusted). We found no association between sRANKL and inhibin B, Inhibin B/FSH-ratio, AMH, SHBG, estradiol or testosterone. sRANKL was immensely higher in KS men compared with infertile men also after adjusting for FSH level (sRANKL 0.59 pmol/L vs. 0.20 pmol/L, p $7{,}9 \times 10^{-15}$, FSH and age adjusted, Fig. 3c). However, the FSH/RANKL-ratio was significantly higher in KS men, (FSH/RANKL 105.3 vs 51.5 p 0.027, age adjusted, Fig. 3d).

3.4. FSHR, and RANKL expression in human derived adipocyte cell models

The degree of differentiation of hMADS and TERT-hWA cells was validated morphologically and by increased expression levels of *ADIPOQ* on day 12 compared with day 0 (Fig. 4A). Expression of *FSHR* was detectable with primers targeting exon 1 and exon 10 in differentiated hMADS cells (day 12) with stable CT values of approximately 33 (Fig. 4B and Suppl. Fig. 5B). *FSHR* expression was further validated by direct sequencing of RT-PCR bands targeting exon 10 in both human testis tissue and hMADS cells. Interestingly, no *FSHR* expression was detected in adipocytes when we specifically targeted exon 2, even though *FSHR* expression was detectable in human testis tissue (data not shown). Immunocytochemistry showed accordingly presence of membrane bound and cytoplasmic expression of FSHR and RANKL in both differentiated TERT-hWA and hMADS cells using antibodies validated in human testis and human fetal bone tissue respectively (Fig. 4C and Suppl. Fig. 4). Analysis of the media from differentiated hMADS cells treated with different doses of FSH or vehicle for 3,6,24 and 48 h demonstrated a marked increase in the concentration of sRANKL in the media following 3 h FSH treatment. Treatment with 1 and 10 IU/mL FSH resulted in a 3 and 5-fold increase in the release of RANKL into the media (both $p < .01$) (Fig. 4D). The same tendency was also found at later timepoints (Suppl. Fig. 5B) although after 48 h only 17% of the vehicle treated hMADS cells had detectable RANKL in the media compared with 33% of the FSH treated (data not shown). This indicates that release of RANKL can be exhausted although FSH treatment also induced a 43% upregulation of *RANKL* expression compared with vehicle treatment, however, this difference did not reach statistically significance (Fig. 4E). After 48 h of treatment we observed no difference in *RANKL* expression between FSH or vehicle treated cells. The validity of

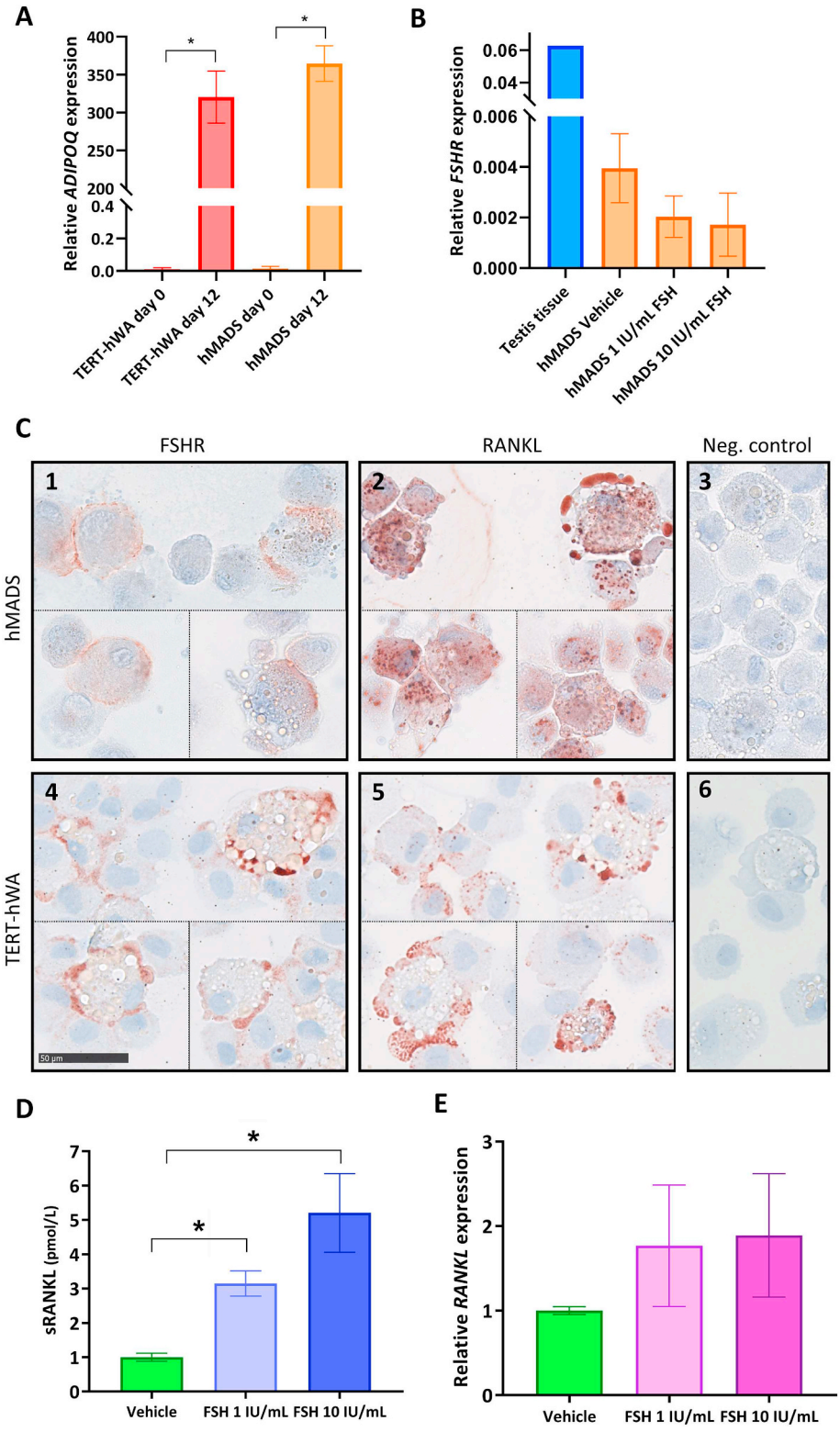

Fig. 4. Expression and activity of FSHR in adipocyte cell cultures in vitro. A: Degree of differentiation quantified by *ADIPOQ* expression in TERT-hWA and hMADS cells on day 0 and day 12 (normalized to *hTBP*). B: expression of *FSHR* targeting exon 10 in testis tissue and an adipocyte cell line (hMADS) after adipocyte differentiation (day 12) following treatment with vehicle, FSH 1 or 10 IU/mL (H_2O, BSA ± Puregon®) for 3 h (normalized to *hTBP*). C: immunocytochemistry of two differentiated human adipocyte cell-lines (day 12) expressing FSHR (ab-150,557) and RANKL (sc-76,628). All photos are enlarged x 40 and a selection of cells from each sample is displayed together, black ruler in C4 show 50 µm. 4C1: FSHR expression in hMADS. 4C2: RANKL expression in hMADS. 4C3: hMADS negative control. 4C4: FSHR expression in TERT-hWA. 4C5: RANKL expression in TERT-hWA. 4C6: TERT-hWA negative control. D: release of sRANKL to the media following treatment of vehicle or FSH 1 or 10 IU/mL (H_2O, BSA ± Puregon®) for 3 h in differentiated hMADS adipocytes (day 12). E: *RANKL* expression in hMADS adipocytes (day 12) 6 h after treatment with vehicle or FSH, vehicle is set to one (normalized to *RPS20*) Data presented as mean + SD, asterix $p < .01$.

the sRANKL ELISA was tested by measuring serum samples from patients before and after RANKL-inhibition with Denosumab treatment showing total suppression of sRANKL to undetectable levels 20–120 days after Denosumab injection (data not shown). Moreover, to exclude that all cell lines expressing RANKL also releases sRANKL into the media, we showed expression of *RANKL* in NTera2 and TCam2 cells but found undetectable sRANKL in the media from these cells (data not shown). Finally, we also the tested the effects of LH on hMADS cells and found no significant changes in *RANKL* expression or sRANKL release (data not shown).

4. Discussion

In this cohort of men with high serum levels of gonadotropins we found no direct link between FSH and BMD, glucose or lipid metabolism. A putative effect of FSH on the skeletal compartment is therefore

most likely indirectly mediated. Such an indirect link could be through RANKL as we show here that FSH stimulated shedding of RANKL in adipocytes and soluble RANKL in serum is linked with glucose homeostasis. The study group was selected because infertile men have higher FSH levels than normal men and rodent studies have shown that increased FSH particularly the marked increase in FSH levels following menopause had the largest effect on metabolism [13,18]. FSHR was expressed in the human-derived adipocyte cell models, while RANKL expression was detectable and released into the media in the investigated adipocytes at a basal low level that increased after FSH treatment of mature adipocytes. Interestingly, no transcripts were detected when using specific primers targeting *FSHR* exon 2 in any of the human cell lines. This finding is in line with previous work, showing that *FSHR* in adipocytes from mice is fully functional, but lacks exon 2 [18]. The low RANKL mRNA expression in peripheral adipocytes indicates that human visceral or subcutaneous adipocytes are unlikely to be main source for RANKL in circulation. Moreover, the rapid effect of FSH on sRANKL release, which was exhausted at later timepoints, indicates that the main effect is on cleavage of the transmembrane form of RANKL, for instance by induction of MMP activity, and subsequent shedding of sRANKL. This suggestion is supported by undetectable levels of sRANKL in media from non-adipocyte cell lines such NTera2 and TCam2 cells with high *RANKL* expression, which indicates that release of sRANKL into the media is cell-type dependent. It is reasonable to propose that FSH in theory also could influence *RANKL* expression and shedding in either osteoblasts, osteoclasts, marrow adipocytes or special subpopulations of peripheral adipocytes if these cells express FSHR and cleavage enzymes at a higher level than in peripheral adipocytes. Several studies have demonstrated FSHR in adipose tissue of chickens [33], sheep [34] and humans [18,35]. However, a recent study showed that RANKL in mice exclusively was expressed in bone marrow adipose tissue [23], which is in line with our human data showing that peripheral derived fat cells have a low RANKL expression.

Release of sRANKL into circulation is a complicated area because both mouse models and human studies have shown that the transcriptional level of RANKL in bone cells or bone marrow adipocytes are inversely linked with serum levels of RANKL [36]. RANKL can be cleaved by different proteases in the MMP-family to produce soluble RANKL [37], but the main human source is unknown. Post translational modifications of RANKL has been proposed as a potential explanation for the observed inverse relationship because activity of “shedding enzymes” may decrease when mRNA RANKL transcripts increases [38]. To complicate things even further a new study suggests reverse RANKL signalling and thus highlights a putative role of circulating RANK, which ultimately also could influence circulating RANKL levels [39]. This may at least in part explain why hypergonadotropic men with clinically elevated FSH had lower serum RANKL independent of sex steroid levels and Inhibin B. Another possibility is that RANKL in circulation may originate from the skeleton, while OPG may originate from other organs, which is in line with a new study showing similar levels of OPG in serum and bone marrow [40]. A recent mouse study showed that RANKL is highly expressed in marrow adipocytes and a marked increase in marrow fat resulted in high RANKL expression in these cells, which was mirrored by changes in serum RANKL concentration [23]. One may argue that sRANKL may differ from total RANKL, but a positive correlation between total RANKL and sRANKL has been shown previously [41]. Previous work in rodents and men have shown that RANKL expression in bone are highly dependent of age and indices of bone structure exclusively in males [36], which implies that there could be an interesting sex difference. The high RANKL level in infertile men with prediabetes was supported by a positive association between RANKL and fasting glucose. Previous work in wild type, diabetic mice and human pancreatic islet cells transplanted into mice have shown increased pancreatic β cell proliferation and improved glucose tolerance following RANKL inhibition [42]. In accordance, osteoporotic but non-diabetic women treated with Denosumab improved their glucose tolerance test but did not improve HbA1c [43,44]. Inhibins and activins are unlikely to explain our findings because there was no link between Inhibin B and sRANKL. This is in line with mouse studies where the effects on bone and adipocyte function were changed by using an antibody specifically blocking the effects of FSH [18]. Serum concentrations of FSH rarely reach postmenopausal levels in infertile men. However, men with Klinefelter syndrome have very high FSH levels but also a high degree of adiposity and prediabetes, which may explain why KS patients had higher RANKL than infertile men despite of high FSH levels. A recent study found higher baseline levels of RANKL in young women compared with young men [45], which in addition to a higher prevalence of prediabetes may explain the higher serum RANKL as KS men may have a more “feminine” phenotype. Another study has shown that KS adolescents have impaired bone mineral status with low osteocalcin and high PTH [46]; which indicates that the higher RANKL also could be due to higher PTH, although we found the same inverse relation between FSH and RANKL in KS men as in infertile men. Comparison of FSH/sRANKL ratio between KS and infertile showed that the KS men after all had suppressed RANKL level considering their high level of FSH.

Combined, these data highlights that metabolic factors, PTH and other unknown factors may be important for soluble RANKL levels and FSH is a potential regulator but not the main determinant.

To conclude, elevated FSH had no effect on bone and glucose homeostasis in men with impaired gonadal function. sRANKL levels were associated with glucose and lipid homeostasis and men with prediabetes or Klinefelter syndrome had higher sRANKL levels. FSH treatment leads to increased release of sRANKL from adipocytes in vitro, which suggests that part of the putative effect of FSH on bone, lipid and glucose homeostasis, besides the known actions of sex steroids and inhibin, may be mediated through RANKL.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.03.022.

Disclosure

Author states no conflicts of interest.

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