

# Semiquantitative mRNA Measurements of Osteoinductive Growth Factors in Human Iliac-Crest Bone: Expression of LMP Splice Variants in Human Bone

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**Abstract.** Although osteotropic growth factors are known to play an important role in bone metabolism, knowledge about their expression in relation to age, sex and smoking remains limited. In this study we report mRNA levels of the recently discovered Lim mineralization protein splice variants (LMP-1, LMP-2, LMP-3) and the established osteotropic growth factors BMP-2, BMP-6, BMP-7, TGF- $\beta$ , IGF-I, IGF-II and b-FGF in human iliac crest bone.

Standardized bone biopsy specimens were obtained from the iliac crest during graft harvesting in 62 patients (38 males, 24 females, mean age 44.7 years, range 13–78 years) undergoing spinal surgery. Samples were immediately stored in liquid nitrogen for PCR analysis. Semiquantitative RT-PCR was performed for TGF- $\beta$ , IGF-I, IGF-II, BMP2, BMP-6, BMP7, bFGF, LMP-1, LMP-2 and LMP-3 using  $\beta$ -actin as internal standard. Triplicate measurements were made of each growth factor and  $\beta$ -actin.

mRNA for all examined growth factors was detected in 69% of the specimens. The lowest degree of detection was present for b-FGF and BMP-2, both of which were found in 85% of the specimens. LMP-1 was detected in 98% of the specimens. LMP-2 in 94% and LMP-3 in 27%, respectively. LMP-1 was generally expressed in higher amounts than LMP-2 and LMP-3. Nondetectable levels of the growth factors were more frequent in the >60-year-old males compared with >60-year-old females ( $P < 0.05$ ) and <60-year-old males ( $P < 0.01$ ). LMP-1 expression was more variable among young individuals, but mean values were similar between age groups. TGF- $\beta$ , BMP-2 and BMP-7 values did not differ between age groups, but generally a higher variation was found among older patients. IGF-I values were significantly higher ( $P < 0.05$ ) in males over 60 years, whereas the highest level of bFGF mRNA was present in males younger than 20 years ( $P < 0.05$ ). In addition, regression analysis revealed correlation between BMP-2 and BMP-7 ( $R^2 = 0.74$ ,  $P < 0.0005$ ), LMP-2 and BMP-2 ( $R^2 = 0.27$ ,  $P < 0.0005$ ) and LMP-2 and bFGF ( $R^2 = 0.40$ ,  $P < 0.0005$ ).

In conclusion, we have demonstrated expression of LMP-1 and LMP-2 in human bone. LMP-1 was ex-

pressed in higher amounts and showed a higher degree of variation among young individuals. LMP-2 was correlated to a number of other growth factors, suggesting that LMPs may also play a role in human bone metabolism. Higher variation in the expression of TGF- $\beta$ , BMP-2 and BMP-7 was found in the older age groups, but whether or not this can be correlated to age-related changes in bone turnover requires further studies.

**Key words:** Lim mineralization protein — Osteotropic growth factors — RT-PCR — Human, bone

Osteotropic growth factors with bone-inductive effects have been recognized for almost two decades and are generally considered to be significant mediators of cellular actions during bone remodeling and fracture healing [1–3]. However, growth factor expression in humans is poorly described and knowledge about human growth factor expression in relation to age, sex and smoking remains limited [4, 5].

In 1998 Boden et al. [6] discovered a novel intracellular protein in rats called LIM mineralization protein-1 (LMP-1). They found that glucocorticoids induced LMP-1 through the action of bone morphogenetic protein-6 (BMP-6). Blocking the expression of LMP-1 using antisense oligonucleotides prevented osteoblast differentiation *in vitro*. Over-expression of LMP-1 using a mammalian expression vector was sufficient to initiate *de novo* bone nodule formation *in vitro* and in subcutaneous implants *in vivo* [7]. It has therefore been speculated that LMP was an intracellular regulator of osteoblast differentiation. Work by the same group later identified the human analog and two human splice variants of LMP-1, i.e., LMP-2 and LMP-3. In comparison to LMP-1, LMP-2 was characterized by a 119 basepair deletion and a 17 basepair insertion, while LMP-3 only contained the 17 basepair insertion. Over-expression experiments in rat osteoblast cultures showed that LMP-3 had the same osteogenic potential as LMP-

**Table 1.** Description of PCR conditions and product sizes

| Gene           | Sense primer                          | Antisense primer                      | Annealing temp. | Cycles | Product size/Bp |
|----------------|---------------------------------------|---------------------------------------|-----------------|--------|-----------------|
| $\beta$ -actin | 5'-TGT GCC CAT CTA<br>CGA GGG GTA TGC | 5'-GGT ACA TGG TGG<br>TGC CGC CAG ACA | 65.5            | 30     | 439             |
| TGF- $\beta$   | 5'-GGA CCT CGG CTG<br>GAA GTG GAT C   | 5'-CCT CGC GTG CTA<br>GTA CAA CCT G   | 65.5            | 33     | 248             |
| IGF-I          | 5'-GTA TTG CGC ACC<br>CCT CAA         | 5'-TTG TTT CCT GCA<br>CTC CCT CT      | 60.0            | 36     | 126             |
| IGF-II         | 5'-GAG GAG TGC TGT<br>TTC CGC AGC     | 5'-CGG GGT CTT GGG<br>TGG GTA GAG C   | 58.0            | 31     | 290             |
| BMP-2          | 5'-TCA AGC CAA ACA<br>CAA ACA GC      | 5'-ACG TCT GAA CAA<br>TGG CAT GA      | 60.0            | 35     | 200             |
| BMP-6          | 5'-GCG ACA CCA CAA<br>AGA GTT CA      | 5'-CCC ATA CTA CAC<br>GGG TGT CC      | 60.0            | 38     | 203             |
| BMP-7          | 5'-CCA ACG TCA TCC<br>TGA AGA AAT AC  | 5'-GCT TGT AGG ATC<br>TTG TTC ATT GG  | 60.0            | 37     | 271             |
| b-FGF          | 5'-GGA AGA TGG AAG<br>ATT ACT GGC TT  | 5'-GCT CTT AGC AGA<br>CAT TGG AAG AA  | 60.0            | 37     | 208             |
| LMPs           | 5'-CAG CCG GTT CAG<br>AGC AAA C       | 5'-GCC AGT CCT CTG<br>TGT TCT CC      | 60.0            | 40     |                 |
| LMP-1          |                                       |                                       |                 |        | 214             |
| LMP-2          |                                       |                                       |                 |        | 112             |
| LMP-3          |                                       |                                       |                 |        | 231             |

1, whereas LMP-2 was physiologically inert [8]. The precise intracellular mechanism of LMPs is unknown, but it has been speculated that LMPs work in conjunction with BMPs other than BMP-6. However, the importance of LMPs in humans has yet to be established.

BMPs play a crucial role in cell growth and differentiation in a variety of cell types [9], and are the only growth factors with a known ability to stimulate differentiation of bone marrow stromal cells into a chondro- or osteoblastic direction [10, 11]. BMP-2 and BMP-7 have both been shown to stimulate bone formation in various animal models [12–15].

Transforming growth factor- $\beta$  (TGF- $\beta$ ), insulin-like growth factor-I (IGF-I) and IGF-II all mediate osteoblastic cell proliferation and differentiated functions such as type I collagen expression [1, 16, 17]. In addition, TGF- $\beta$  has a chemotactic effect on osteoblastic cells and participates in the inhibition of bone resorption [18]. TGF- $\beta$ , IGF-I and IGF-II are produced and secreted by osteoblasts and are incorporated into bone matrix from where they subsequently exert their effects when liberated during bone resorption.

Basic-fibroblast growth factor (b-FGF), which is produced by both endothelial and bone cells and exerts potent mitogenic and chemotactic effects on endothelial cells, is also known to have a proliferative effect on osteoblasts [1]. Therefore, while b-FGF is considered important for bone vascularization [19–21], bFGF mRNA expression in human bone has not been comprehensively described in a larger patient material.

It is well known that the bone mass and bone turnover are influenced by age and gender. It is therefore our

hypothesis that age- and gender-related differences in bone properties can be at least partly explained by variation in growth and transcription factors. Furthermore, knowledge of the expression pattern of growth and transcription factors in different age groups might provide a rational basis for clinical interventional therapy in bone repair and remodeling. Therefore, the aim of this study was to examine the influence of age and sex on the mRNA expression of osteotropic growth factors BMP-2, BMP-6, BMP-7, TGF- $\beta$ , IGF-I, IGF-II, b-FGF and variants of the intracellular signaling factor LMP in human bone tissue.

## Material and Methods

### Patients

The study design was cross-sectional and comprised 62 patients undergoing spinal surgery. The study group consisted of 38 men (mean age 47 years, range 14–78 years), 14 premenopausal (mean age 27 years, range 13–47 years) and 10 postmenopausal women (mean age 62 years, range 50–73 years). Seven of the postmenopausal women received hormone replacement therapy. Nineteen patients were daily smokers 3 months preoperative. The younger patients were mainly surgically treated for scoliosis, and the older ones for degenerative spinal disorders. The patients had no known malignant diseases or bone metabolic diseases. Bone metabolic diseases were excluded after extensive examination. Smoking and menopause/menarche data were collected retrospectively. Following a standardized procedure [22] a cylindrical 8 mm unicortical bone biopsy was obtained from the posterior iliac crest of each patient prior to graft harvesting. Biopsy specimens were immediately stored in liquid nitrogen. This study is part of a prospective study that examines the impact of local growth

**Table 2.** Detection of growth factors in different age groups

|              | < 20 years        |                     | 20–40 years       |                     | 40–60 years        |                     | > 60 years         |                     |
|--------------|-------------------|---------------------|-------------------|---------------------|--------------------|---------------------|--------------------|---------------------|
|              | Male %<br>(n = 5) | Female %<br>(n = 7) | Male %<br>(n = 9) | Female %<br>(n = 3) | Male %<br>(n = 14) | Female %<br>(n = 8) | Male %<br>(n = 10) | Female %<br>(n = 6) |
| TGF- $\beta$ | 100               | 100                 | 89                | 100                 | 100                | 88                  | 100                | 100                 |
| IGF-I        | 100               | 100                 | 100               | 100                 | 100                | 88                  | 100                | 100                 |
| IGF-II       | 100               | 100                 | 100               | 100                 | 100                | 100                 | 80                 | 100                 |
| b-FGF        | 100               | 71                  | 89                | 100                 | 79                 | 88                  | 80                 | 100                 |
| BMP-2        | 100               | 71                  | 89                | 100                 | 86                 | 100                 | 70                 | 83                  |
| BMP-6        | 80                | 86                  | 89                | 100                 | 93                 | 88                  | 90                 | 83                  |
| BMP-7        | 100               | 100                 | 100               | 66                  | 100                | 100                 | 80                 | 100                 |
| LMP-1        | 100               | 100                 | 89                | 100                 | 100                | 100                 | 100                | 100                 |
| LMP-2        | 80                | 86                  | 89                | 100                 | 93                 | 100                 | 100                | 100                 |

\* Nondetectable levels of the growth factors were more frequent in the > 60-year-old males compared with > 60-year-old-females ( $P = 0.04$ ;  $\chi^2$ -test) and < 60-year-old males ( $P = 0.003$ ;  $\chi^2$ -test)

factor expression in auto-graft on spinal fusion after surgery. The study was conducted in accordance with the Helsinki II declaration and approved by the local ethical committee.

#### RNA Isolation [23]

mRNA was isolated using a standard procedure [24]. In short, specimens were crushed using a metal bone-crushing device semisubmerged in liquid nitrogen and transferred into homogenization glasses containing 1 ml Trizol<sup>®</sup> (Invitrogen A/S, Taastrup, Denmark) per 100 mg of bone tissue. After 5 min homogenization and 5 min incubation at room temperature, the solutions were centrifuged in 15 ml Sarsted<sup>®</sup> tubes (Hounisens Laboratorie Udstyr, Aarhus, Denmark) for 10 min, 2,000 g at 4°C. The Trizol solution was then distributed into 1.5 ml Eppendorf<sup>®</sup> tubes (Hounisens Laboratorie Udstyr, Aarhus, Denmark) with 1 ml in each, and 0.2 ml of chloroform per ml Trizol was added for phase separation. Following centrifugation at 12,000 g, 15 min, 4°C, the aqueous phase containing RNA was then transferred into new 1.5 ml tubes. Precipitation and RNA wash were performed using isopropanol (Merch Eurolab, Copenhagen, Denmark) and 80% ethanol (Merch Eurolab, Copenhagen, Denmark), respectively. The washed RNA pellet was redissolved in 20  $\mu$ l of Tris-EDTA-buffer (Aarhus Kommunehospital Pharmacy, Aarhus, Denmark). The yield and quality of extracted RNA were assessed by the 260/280 nm optical density ratio and by electrophoresis under denaturing conditions on 0.8% agarose SeaKem<sup>®</sup> gels (Medinova Scientific, Hellerup, Denmark).

#### Reverse Transcription

Reverse transcription was performed according to a standard protocol [25]: 25 ng/ $\mu$ l RNA, 5 mM MgCl<sub>2</sub>, 1  $\times$  PCR-buffer II, 1 mM dNTPs, 1 U/ $\mu$ l RNase inhibitors, 2.5 U/ $\mu$ l Murine Leukemia Virus (MuLV) reverse transcriptase and 2.5  $\mu$ M random hexamer primer. All reagents were purchased from Perkin Elmer<sup>®</sup> (AB Applied Biosystems, Naerum, Denmark). The reverse transcription was performed using a 9700 Perkin Elmer thermal cycler<sup>®</sup> (AB Applied Biosystems, Naerum, Denmark) and the following temperature sequence: 10 min, 23°C; 60 min, 42°C; 10 min, 95°C.

#### Semiquantitative PCR

DNA sequences for  $\beta$ -actin, TGF $\beta$ , IGF-I, IGF-II, BMP2, BMP-6, BMP7, LMP-1, LMP-2, LMP-3 and bFGF were obtained on NIH's homepage [www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank). Primers were designed using Primer3<sup>®</sup> web-interface

([www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi](http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi)) and supplied from Genetechnology (Aarhus, Denmark), (Table 1). For detection of LMP-1, LMP-2 and LMP-3, one set of primers was used. The primers were designed to span the sequence that varies among the three LMPs. For each gene, PCR was performed according to standard procedures [25] after optimization, so that PCR-reactions were within the exponential range of amplification. The specific conditions are shown in Table 1. For every PCR-mix, a positive control of pooled cDNA from all samples and PCR-mix were made. Two separate negative controls without cDNA and reverse transcriptase, respectively, were systematically performed. Triplicate measurements were made of all genes in each patient, with  $\beta$ -actin used as internal standard.

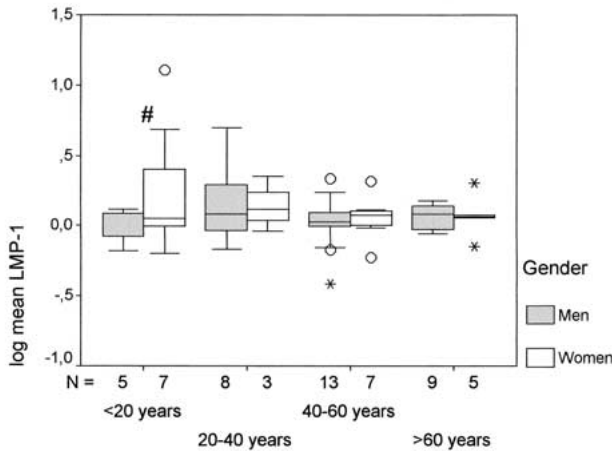
#### Gel Electrophoresis and PCR-Product Quantification

All samples but LMPs from one individual were examined on the same 3% Sigma<sup>®</sup> agarose gel (Sigma-Aldrich, Copenhagen, Denmark). LMP-1, LMP-2 and LMP-3 were examined on 4% NuSieve<sup>®</sup> agarose gels (Medinova Scientific, Hellerup, Denmark). Ten microliter of PCR product were loaded together with 5  $\mu$ l of orange-G (BHD Becton Dickenson, Broendby, Denmark). Electrophoresis was run for 48 min at 85 V and 1.4 hours at 100 V for the LMPs. Detection of LMP-2 and LMP-3 were confirmed by cleavage of the PCR product by the restriction enzyme BSG1 (Medinova Scientific, Hellerup, Denmark), which recognizes the sequence 5'gtgcag3' only found in the 17 basepair insertion. The gels were stained with ethidium bromide [26] (Sigma-Aldrich, Copenhagen, Denmark). Quantification was conducted using Gel Doc 1000<sup>®</sup> densitometric scanner (Bio-rad Laboratories, Herlev, Denmark) and corresponding computer software Molecular Analyst 1.4.1<sup>®</sup> and Multianalyst<sup>®</sup>, measuring band intensity under UV illumination. The relative level of growth factor mRNA was calculated as the average of the 3 growth factor/ $\beta$ -actin levels.

#### Statistics

Distributions were tested for normality using Q-Q plots. When appropriate, data were transformed using log<sub>10</sub>. Differences between more than two groups were evaluated by ANOVA. Post-hoc testing was done using Bonferroni or Dunnett's T3. Equality of variances was tested by the F-test or Levene's test. Comparisons between the two groups were done using independent sample T-test. Linear relationships were examined with scatter plots. Linear regression analysis was done with untransformed and unstandardized values. Assumptions for linear regression were checked by plotting the residuals against the x-value and by Q-Q plots of the residuals. The  $\chi^2$  test was

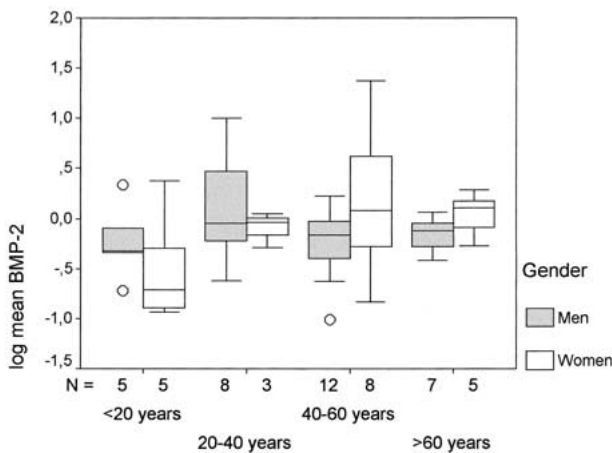
## LMP-1



## A Age groups

# A greater variation was observed in patients younger than 20 years old compared to patients older than 20 years old for LMP-1 ( $p < 0.01$ ).

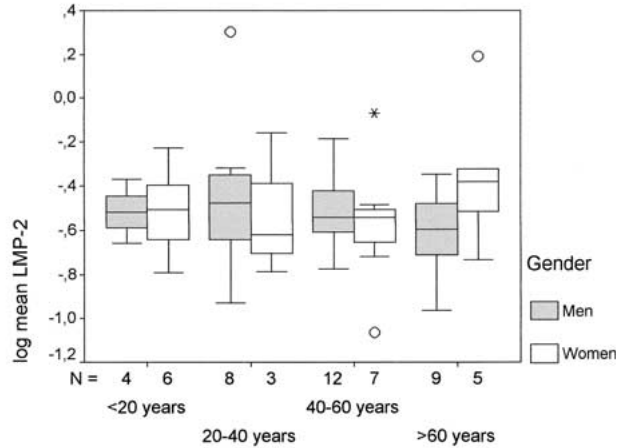
## BMP-2



## C Age groups

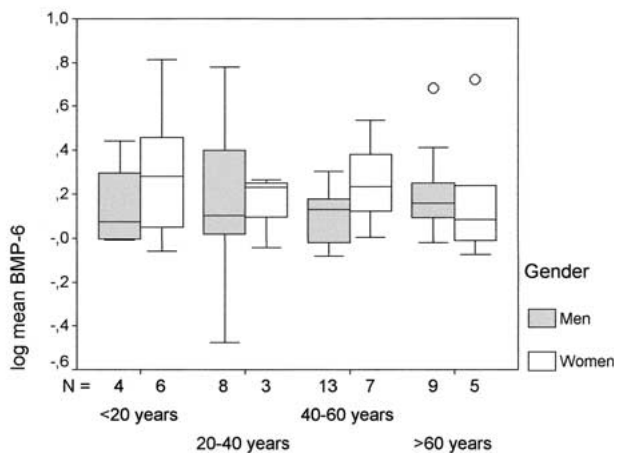
**Fig. 1. (A-I).** Growth factor expression relative to  $\beta$ -actin in 4 age groups, subdivided into men and women. The box represents the interquartile range which contains 50% of the values. The whiskers extend from the box to the lowest and highest

## LMP-2



## B Age groups

## BMP-6



## D Age groups

value excluding outliers and extremes. Outliers<sup>o</sup> and extreme\* are defined as cases with values 1.5–3 box-lengths and more than 3 box-lengths from the interquartile range.

applied to test differences in frequency of missing values between men and women in the same age groups and between individuals of the same gender but different age groups. For calculation of the variance within samples from the same individual and variances between individuals, random effect ANOVA was used.  $P$  values  $< 0.05$  were considered significant. The analyses were performed using SPSS 10.0<sup>®</sup> software (SPSS, Holte, Denmark).

## Results

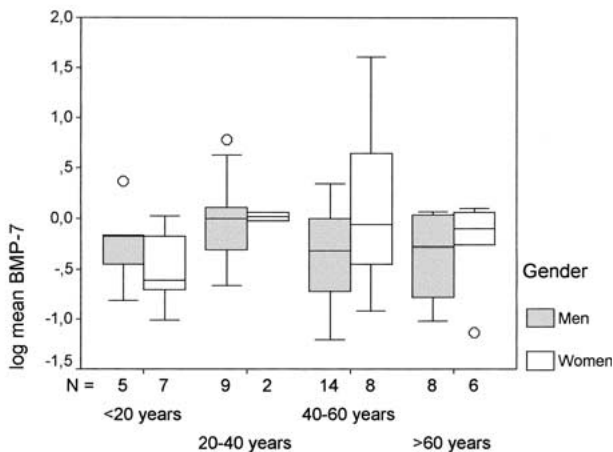
In 69% of the biopsies, mRNA for all tested growth factors was detected. The lowest degree of detection was present for bFGF and BMP-2: both were detected in 85% of the biopsies. Detectable levels of growth factors or  $\beta$ -actin were not correlated to the biopsy weight.

Furthermore, the level of  $\beta$ -actin was not correlated to age or gender.

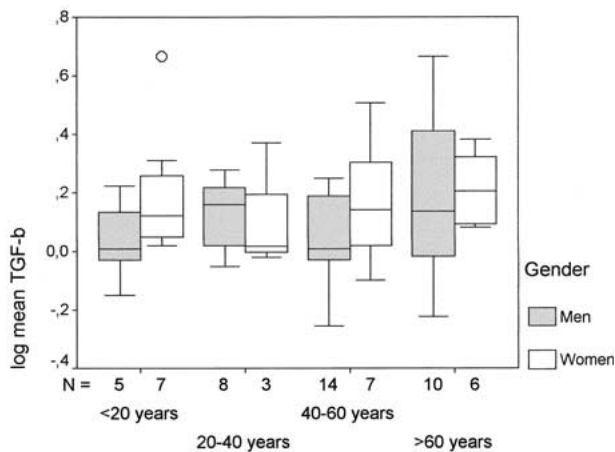
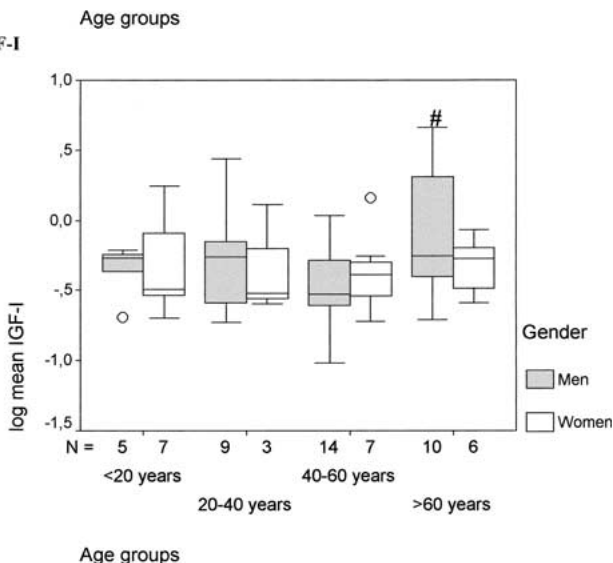
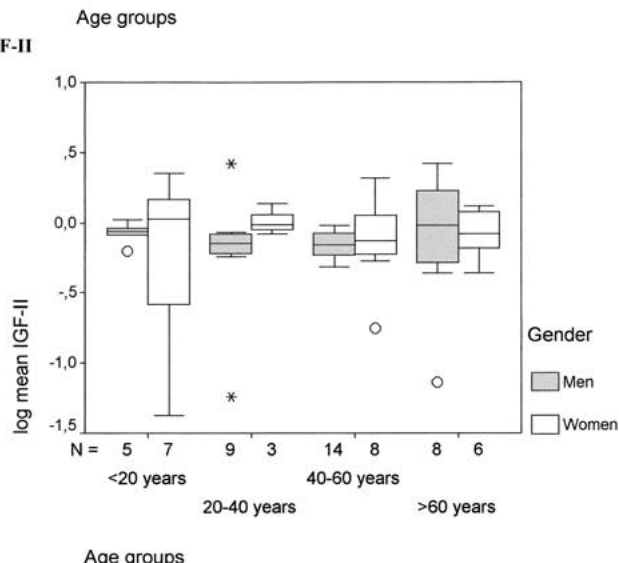
Nondetectable levels of the growth factors were more frequent in the  $> 60$ -year-old males compared with the  $> 60$ -year-old females ( $P < 0.05$ ) and  $< 60$ -year-old males ( $P < 0.01$ ), Table 2. The coefficient of variation of the triplicate measurements varied from 0.16 for LMP-1 to 0.38 for bFGF. LMP-1 was detected in 98% of the biopsies, LMP-2 in 94% and LMP-3 in 27%, respectively. Furthermore, LMP-1 was the highest expressed LMP in the majority of biopsies LMP-1 (98%) followed by LMP-2 and LMP-3. The LMP-3 bands were too weak to quantify.

No significant differences were found in expression of LMP-1 and LMP-2 with respect to age or gender, (Fig.

## BMP-7



## TGF-β

E  
IGF-IF  
IGF-II

## G

# The expression of IGF-I mRNA was significantly higher in males >60 years when compared to males <60 years ( $P < 0.05$ ).

## Fig. 1. Continued.

1A, B). However, a greater variation was observed in patients younger than 20 years old compared to patients older than 20 years for LMP-1 ( $P < 0.01$ ). No significant differences were found in the levels of BMP-2, BMP-6 and BMP-7 mRNA in relation to age or gender (Fig. 1C, D, E).

The level of TGF-β mRNA tended to be lower in men than in women ( $P = 0.08$ ). No significant differences were found among different age groups, but a higher variation was observed in the oldest age group among men ( $P < 0.05$ ) (Fig. 1F).

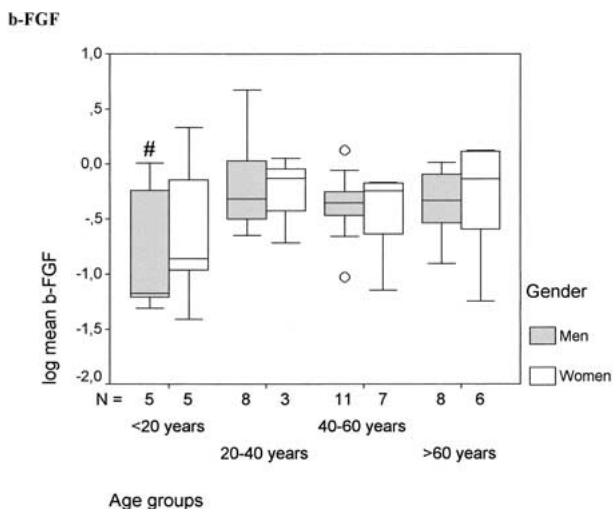
The expression of IGF-I mRNA was significantly higher in males >60 years when compared to males <60 years ( $P < 0.05$ ) (Fig. 1G). Comparison between pre- and postmenopausal females revealed a higher variation in the premenopausal group for IGF-I ( $P < 0.05$ ). Also, a higher variation was present in males >60

than in the corresponding female age group ( $P < 0.05$ ). IGF-II showed no significant differences among age groups, but in the age group younger than 20 years, females showed a higher variation than males ( $P < 0.01$ ) (Fig. 1H). No correlation between mRNA levels of IGF-I and IGF-II was observed.

For bFGF, the lowest levels of mRNA were found in patients younger than 20 years although the difference was only significant in males (compared with males >20 years:  $P < 0.05$ ) (Fig. 1I). Furthermore, a greater variation was found in the young male age group (<20 years old) compared with the older male age groups ( $P < 0.01$ ).

Regression analysis revealed significant associations between expressions of several growth factors (Table 3). Expression of LMP-1 and LMP-2 was correlated ( $R^2 = 0.134$ ,  $P < 0.01$ ). The expression of LMP-1 was

## H



**I**  
# The expression of bFGF was found to be significantly lower in males younger than 20 years old compared with males older than 20 years old,  $p < 0.05$ . Furthermore, a greater variation was found in the young male age group (<20 years old) compared with the older male age groups,  $p < 0.001$ .

**Fig. 1.** Continued.

associated with expression of IGF-I ( $R^2 = 0.116$ ,  $P < 0.01$ ) and LMP-2 expression was strongly associated with expression of bFGF ( $R^2 = 0.40$ ,  $P = 0.01$ ) and BMP-2 ( $R^2 = 0.27$ ,  $P < 0.0005$ ). Furthermore, the expression of BMP-2 was associated with expression of BMP-7 ( $R^2 = 0.741$ ,  $P < 0.0005$ ) and bFGF ( $R^2 = 0.296$ ,  $P < 0.0005$ ).

In this study, no association could be demonstrated between growth factor expression and preoperative tobacco consumption or hormone replacement therapy.

## Discussion

In this cross-sectional study we investigated the expression of growth factors with known stimulatory effects on bone formation in bone biopsies obtained during spinal surgery. BMPs have mainly been considered important during the development of the skeletal system. However, since BMP-2, BMP-6 and BMP-7 were expressed also in older age groups, the present study suggests that BMPs also may play an important role during adult life. Among the several different known BMPs, significant homology exists in amino acid sequences between BMP-2 and 4 and among BMP-5, 6 and 7, respectively [27]. By regression analysis we found a positive correlation between mRNA levels of BMP-2 and BMP-7, but none between BMP-6 and BMP-2 or BMP-7. This suggests a common pathway in terms of regulation and transcription of BMP-2 and BMP-7 in adult life and this regulation may be different from that of BMP-6.

In rats, it has been documented that LMP-1 is essential for BMP-6 signaling and thereby differentiation of bone marrow stromal cells into a chondro- or osteoblastic direction [7], however, its role for osteoblast differentiation in humans is still not completely under-

stood [8]. For the first time our study documents the existence of transcription factor LMP-1, -2 and -3 in human cancellous bone. It has previously been demonstrated that LMPs are expressed in various tissues, including bone marrow. The predominant splice variant may vary with the origin of tissue, however, in all tissues formerly investigated, the amount of LMP-3 mRNA appeared limited [8]. In our study we also found LMP-3 to be less abundant than LMP-1 and LMP-2. However, in contrast to Liu et al. [8], who found LMP-2 in bone marrow to be expressed in higher amounts than in LMP-1, we found LMP-1 to be the most predominant variant. This could be explained by our biopsies containing more bone, and collaborates well with LMP-1 being the one with the highest osteogenic potential compared with LMP-2. The study provides no further information on the intracellular function of LMPs, but two points are to be drawn regarding LMPs' role in humans. First, the expression does vary, especially among younger individuals and more for LMP-1 than LMP-2, suggesting that the transcription of LMPs is regulated in relation to human growth. Secondly, regression analysis revealed correlation between expression of LMP-2 and a number of different growth factors, especially BMP-2 and bFGF, suggesting interaction with growth factors other than BMP-6. In addition, a threshold effect in the LMP-BMP axis would make the correlations incomplete, however, judging from the scatter-plot this does not seem to appear.

Our detection of higher IGF-I mRNA levels in older males deviates to some degree from a previous study that found an age-related linear decline of IGF-I-protein in cortical bone [28]. Though this may be explained by the composition of our biopsies, which contained both cortical and trabecular bone as well as bone marrow. Another explanation might be that the content of IGF differs between skeletal sites [29] and the cortical bone examined by Nicolas et al. [28] was obtained from femoral heads, whereas the bone biopsies examined in this study are from the posterior notch of the iliac crest.

Estrogen deficiency at menopause increases the rate of bone loss in women [30]. Moreover, studies show that individual variability in bone turnover markers such as osteocalcin and collagen 1 also increases after menopause, reflecting a variable skeletal response to estrogen deficiency among women [31, 32]. We were not able to detect any significant differences in growth factor levels between pre- and postmenopausal women, but for IGF-I we found a higher variation among the premenopausal women. In our study, 6 of 9 postmenopausal women received estrogen therapy, but no differences in growth factor expression were observed between the women who received HRT and those who did not. It has previously been demonstrated that postmenopausal women treated with hormone replacement therapy expressed lower levels of cytokines stimulating bone resorption

**Table 3.** Linear regression analysis of correlations between growth factor expressions

|              | IGF-I                        | IGF-II                             | BMP-2                        | BMP-6                        | BMP-7                           | bFGF                               | LMP-1                          | LMP-2                               |
|--------------|------------------------------|------------------------------------|------------------------------|------------------------------|---------------------------------|------------------------------------|--------------------------------|-------------------------------------|
| TGF- $\beta$ | $R^2 = 0.059$<br>$P = 0.061$ | $R^2 = 0.231$<br>$P < 0.0005^{**}$ | $R^2 = 0.066$<br>$P = 0.068$ | $R^2 = 0.005$<br>$P = 0.620$ | $R^2 < 0.0005$<br>$P = 0.912$   | $R^2 = 0.145$<br>$P = 0.005^*$     | $R^2 = 0.012$<br>$P = 0.425$   | $R^2 = 0.019$<br>$P = 0.390$        |
| IGF-I        |                              | $R^2 = 0.026$<br>$P = 0.224$       | $R^2 = 0.027$<br>$P = 0.243$ | $R^2 = 0.001$<br>$P = 0.833$ | $R^2 = 0.031$<br>$P = 0.188$    | $R^2 = 0.001$<br>$P = 0.791$       | $R^2 = 0.116^*$<br>$P = 0.009$ | $R^2 = 0.136^*$<br>$P = 0.006$      |
| IGF-II       |                              |                                    | $R^2 = 0.005$<br>$P = 0.608$ | $R^2 = 0.046$<br>$P = 0.125$ | $R^2 = 0.004$<br>$P = 0.634$    | $R^2 = 0.096$<br>$P = 0.026^*$     | $R^2 = 0.035$<br>$P = 0.172$   | $R^2 = 0.077^*$<br>$P = 0.047$      |
| BMP-2        |                              |                                    |                              | $R^2 = 0.001$<br>$P = 0.826$ | $R^2 = 0.741$<br>$P < 0.0005^*$ | $R^2 = 0.296$<br>$P < 0.0005^{**}$ | $R^2 = 0.024$<br>$P = 0.279$   | $R^2 = 0.272^{***}$<br>$P < 0.0005$ |
| BMP-6        |                              |                                    |                              |                              | $R^2 = 0.005$<br>$P = 0.631$    | $R^2 = 0.036$<br>$P = 0.194$       | $R^2 = 0.047$<br>$P = 0.113$   | $R^2 = 0.085^*$<br>$P = 0.033$      |
| BMP-7        |                              |                                    |                              |                              |                                 | $R^2 = 0.171$<br>$P = 0.002^*$     | $R^2 = 0.018$<br>$P = 0.337$   | $R^2 = 0.097^*$<br>$P = 0.026$      |
| bFGF         |                              |                                    |                              |                              |                                 |                                    | $R^2 = 0.012$<br>$P = 0.451$   | $R^2 = 0.397^{**}$<br>$P < 0.0005$  |
| LMP-1        |                              |                                    |                              |                              |                                 |                                    |                                | $R^2 = 0.134^*$<br>$P = 0.004$      |

$R^2$  = Regression coefficient

\*\* $P < 0.0005$ , \* $P < 0.05$

(IL-1 $\beta$ , TNF- $\alpha$ , IL-6) [5, 33]. One explanation for this difference could be that estrogen primarily affects bone resorption and only secondarily, bone formation [34]; it is therefore not surprising that the effect is more obvious on cytokines, which mediate resorption. Furthermore, the study was not aimed at detecting differences between postmenopausal women receiving hormone replacement therapy or not receiving it.

In addition to being important for angiogenesis and bone vascularization, bFGF acts as a modulator of cartilage and bone-cell function and is known to stimulate osteoblast proliferation [1]. Thus, it has been suggested that bFGF is a mediator of the coupling between chondrogenesis and osteogenesis [35] and is known to stimulate osteoclast formation and bone resorption [36]. Furthermore, whether application of bFGF in a fracture model leads to net bone formation seems to depend on interaction with other growth factors such as TGF- $\beta$  [37] and BMP-2 [38]. This study is the first to describe the quantitative *in vivo* expression of bFGF in age-wise broad human bone material. We found relatively stronger correlations between bFGF and growth factors with impact on bone cell differentiation rather than proliferation, e.g., BMP-2, -7 and LMP-2 vs. TGF- $\beta$  and IGF-I,-II. Furthermore, we found lower levels of bFGF mRNA in biopsies from young patients.

Changes in the intrinsic properties of the aging stromal cell system may also play a role in age-related bone loss. A decrease in the number of bone marrow stromal cells has been suggested as a possible cause of fewer osteoprogenitor cells and decreased bone formation capacity in the elderly [39]. Studies have shown a decline in osteoblasts progenitor cells after skeletal maturation up to the age of 30 years [40, 41]. After that, the number and capacity of proliferation and differentiation seem to

be stable [40, 42]. It might therefore be that the number of recruited osteoprogenitor cells is dependent on the availability of growth factors necessary for osteoblast commitment and not on the actual number of osteogenic stem cells. However, in our study, growth factor expression did not seem to attenuate with age but a greater variation was observed in the older age groups for several growth factors. The increased variation could also be caused by decreased responsiveness to growth factors in some elderly patients, leading to an up-regulation of the expression [43].

Smoking has a negative effect on bone mass [44], and different hypotheses have been suggested on the underlying mechanisms. One of these is that smoking may be related to decreased intestinal absorption of calcium and increased bone resorption as a result of secondary hyperparathyroidism [45]. Increased levels of PTH would be expected to increase cytokines involved in bone turnover [46, 47]. In our study, 70% were non-smokers and the remaining had a relatively low level of tobacco consumption. This might be why no differences in growth factor expression could be found in our study.

In this study we used the RT-PCR method for detection of growth factor expression. This method is capable of detecting very low levels of mRNA, but is therefore also sensitive to variation in the efficiency of reverse transcription and subsequent amplification, as well as pipetting errors. Diluting mRNA to a standardized concentration and using an internal standard minimize these errors. After extensive evaluation of different quantitative and semi-quantitative methods [48] we have chosen to determine mRNA expression of growth factors relative to a housekeeping enzyme ( $\beta$ -actin). The variation coefficient on the triple measurement varied from 0.16 for LMP-1 to 0.38 for bFGF. Therefore, interindividual variance explains between

60% (IGF-I) and 85% (BMP-6) of the total variation observed. The biopsies in the study are drill biopsies containing trabecular bone with bone marrow and only to a lesser extent, cortical bone. We have used a non-bone cell-specific internal standard. If an age-related change in the morphological composition of bone occurs and relatively fewer of the cells contained in the biopsy are osteoblast-like cells, the relative levels of growth factor mRNA would be reduced unproportionally. It could thereby act as a confounder for the measured growth factor levels. Despite the mentioned sources of variation, we find the method valuable for understanding the importance of growth factor expression in human bone.

Part of the bone biopsies examined in this study were obtained from patients with degenerative spinal disorders. Various age-related diseases affect bone mass differently, for example, osteoarthritis and osteoporosis are characterized by increased and decreased bone formation, respectively. This might be explained either by differences in number of osteoprogenitor cells, the levels of growth factors or other bone formation stimulating factors. Previously, Dequeker et al. [49] reported association of generalized osteoarthritis with increased levels of IGF-I, IGF-II and TGF- $\beta$ -protein in cortical bone from the iliac crest.

A number of different factors are known to have a modulating effect on growth factor functions. Numerous binding proteins have been described as well as receptor-mediated signaling pathways; in addition, differences in pre- and posttranslatory processing of mRNA cannot be excluded. It is possible that these factors together with actual protein content need to be included in the experiment in order to give a meaningful interpretation. Furthermore, factors with effect on bone resorption may also play an important role in changes of bone in relation to age, sex and smoking among patients with degenerative spinal disorders. Future studies will show whether the expression of growth factors can be correlated to age-related changes in the properties of the skeleton.

In conclusion, this study, comprising 62 human bone biopsies, confirmed the expression of LMP-1,-2 and -3 and that LMP-1 is the predominant transcript in human bone. Also, a strong correlation between expression of BMP-2 and BMP-7 was revealed. Furthermore, expression of extracellular osteoinductive growth factors generally showed a greater variation with increasing age.

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