Osteoporosis is accompanied by reduced CD274 expression in human bone marrow derived mesenchymal stem cells

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<td>Keywords:</td>
<td>Osteoporosis, Mesenchymal Stem Cells, CD274, Osteoblasts, Gene Expression</td>
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<td>Abstract:</td>
<td>Underlying pathomechanisms of osteoporosis are still not fully elucidated. Cell based therapy approaches pose a new group of possibilities to treat osteoporosis and its complications. Aim of this study was to quantify differences of human bone marrow derived mesenchymal stem cells (hBMSC) from healthy donors and those suffering from clinically manifest osteoporosis. Cell samples of seven donors for each group were selected retrospectively from the cell bank of human bone marrow-derived mesenchymal stem cells of the Trauma Department of Hannover Medical School. Cells were evaluated for their adipogenic, osteogenic and chondrogenic differentiation potential, for their proliferation potential and expression of surface antigens. Furthermore, a RT2 Osteoporosis Profiler PCR Array, as well as quantitative real-time PCR of interesting marker genes were carried out to evaluate changes in gene expression. Cultivated hBMSCs from osteoporotic donors showed significantly lower cell surface expression of CD274 (PD-L1) (4.98 % ± 2.38 %) than those from the control group (26.03 % ± 13.39 %; p = 0.007) in flow cytometry analysis. In osteoporotic patients, genes involved in inhibition of the anabolic WNT signaling pathway and those associated with stimulation of bone resorption were significantly upregulated. Apart from these differences, no significant differences were found for the other cell surface antigens, adipogenic, osteogenic and chondrogenic differentiation and their proliferation potential. These findings support theories of an influence of CD274 on the regulation of bone metabolism. CD274 might be a promising target for further investigations regarding the pathogenesis of osteoporosis and into cell-based therapies involving MSCs.</td>
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</table>
Subject: Re-submission of a manuscript for publication

Dear Professor Richards,

we wish to re-submit the manuscript entitled "Osteoporosis is accompanied by reduced CD274 expression in human bone marrow derived mesenchymal stem cells" for the upcoming Bone Healing Special Issue in eCM. The manuscript ID is eCM-Oct-2020-BHSI-0085. We thank you and the reviewers for your helpful suggestions and insights and believe, that the manuscript has improved significantly from the revision.

As we have added more data, several minor changes and a rearrangement of the discussion were necessary to keep the manuscript well readable. Due to the additional information, the abstract and some parts of the discussion had to be rewritten. Relevant Changes have been highlighted in bold and red in the revised manuscript, those made as a response to the reviewers’ comments are explained in detail within this document. As the tables seem not to have been included within the prior PDF, we have added them at the end of the manuscript as well.

Thank you for your consideration. I look forward to hearing from you.

Alexander-N. Zeller M.D., D.D.S
Reviewer 1:

1. **Concern of the reviewer (Aim):** The goal of this work is to identify differences in MSCs obtained from healthy and osteoporotic individuals. This is not the first investigation of this topic. The authors have cited and addressed many of these. However, several papers with similar aims have recently appeared in print in 2020. The authors should consider the papers:
   - *Int J Mol Sci.* 2020 Nov 5;21(21):8309 (supports the current paper's conclusions)
   - *Stem Cell Res Ther.* 2020 Apr 3;11(1):146. (results with respect to differentiation differ from present results).
   - *Int Immunopharmacol.* 2015 Nov;29(1):119-26 (not as clean of study design, and more variables in the outcomes may make this paper hard to put in the context of the current paper).

   **Our response:** We thank the reviewer for this valuable comment. The contents of the publications listed by the reviewer have been added to the introduction. The article by Čamernik has been added to the discussion section of our manuscript as well. The third article by Zablotni et al., would have been extremely difficult to put in context to our manuscript, as it differs largely from our setting. Especially its focus on AChR is not given in our manuscript. Thus, we have refrained from adding it to avoid the manuscript to look artificial.

   **Revised text (Introduction):** Yet, osteoporosis itself seems not to be associated with a reduced osteogenic potential of MSCs (Haddouti et al., 2020). On the other hand, it has furthermore been...

   **Revised text (Introduction):** Regarding chondrogenic potential, some differences between MSCs of osteoporotic patients and non-osteoporotic patients have been described (Čamernik et al., 2020).

   **Revised text (Discussion):** …decreased osteogenic differentiation potential for BMSCs from osteoporotic patients. This is contrary to newer, methodically more robust studies (Haddouti et al., 2020), describing, that MSCs from osteoporotic and healthy patients possess similar differentiation properties. In our study, osteogenesis did not seem to be impaired on a cellular level in vitro. In...

2. **Concerns of the reviewer (Materials and Methods):** Please provide more details on the donors:
   - Are all donors female?
   - What is the mean/median age?
   - What is the mean/median T-score for DEXA for case and control populations?
   - How many patients had osteoporotic fractures?
   - Did the authors consider any interventions that might upregulate or downregulate CD274 expression in the cells in order to determine their responsiveness?

   **Our response:** We thank the reviewer for this comment. Data regarding gender and age (and its SD) is already available in the first paragraph of the results section. Due to the ethical restrictions, we were not able to perform a DEXA scan on patients solely for study purposes. Thus, the number of patients having T-scores available was low and these were also of different actuality. As this and the information about osteoporotic fractures would not have been useful in terms of an additional statistical analysis, we did not include them in the manuscript.

   **Revised text (Discussion / Limitations):** It cannot be completely excluded, that some drugs or interventions might have an effect on regulation of CD274. Even though it is mainly known for chemotherapeutics, which were a criterion for exclusion, a bias regarding this point cannot be completely ruled out.
3. **Concerns of the reviewer (Results):**

- page 9, line 25: Please give the exact p-value rather than stating p>0.05. It looks likely that p>0.5 based on the means and standard deviations given.
- The CD274 results should be included in table 1
- It is unclear why table 1 does not appear in the assembled PDF (not the author's error).

**Our response:** The reviewer is right, exact p-values do enhance the clarity of the manuscript. We have therefore included these values within the results section. This is also true for the CD274 results. We hope, that the re-uploaded table 1 has now been automatically included within the assembled PDF file.

**Revised text (Results):**

**CFU-F assay**

Colony forming unit assays were carried out for both groups in P1. Cells from osteoporotic donors formed 3.74 (± 3.13) colonies per 100 cells seeded on average, those in the control group 4.40 (± 4.86), respectively. The results were not statistically significant (p =1.000).

**Revised text (Tbl 1):**

<table>
<thead>
<tr>
<th>CD274</th>
<th>26.03 % ± 13.39 %</th>
<th>4.98 % ± 2.38 %</th>
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**Tbl. 1:** Basic parameters within both groups measured by FC analysis. Mean expression and standard deviation in percent. **CD274 expression was significantly decreased in patients with osteoporosis compared to control group (p = 0.007). No significant differences were found for all other antigens between the groups.**

4. **Concerns of the reviewer (Discussion):**

Page 12, line 46: Some more analysis of this outcome is warranted. Without knowing how PD1 is involved in osteoporotic/osteopetrotic phenotypes, it can't be directly concluded that lower expression of its ligand in a single cell type in the bone marrow can affect the progression of the disease. It seems that the level of PD-L1 expression is related to MAPK and NF-kappa-beta pathways, and hence the decreased expression in these MSCs may be a sequela of osteoporosis rather than a cause. The reviewer could not find a strong body of literature regarding PD-1/PD-L1 function in bone metabolism and physiology. It would be beneficial for the authors to probe more deeply in this area.

**Our response:** We thank the reviewer for this valuable comment. The idea of PD-L1 expression levels being a sequela of osteoporosis rather than a cause of has now been mentioned within the manuscript in more detail.

We therefore analyzed the gene expression of CD274 and its receptor CD279. It was found that CD279 is not expressed in hBMSCs. CD274, however, at the protein level, showed a significantly lower mRNA expression in patients with osteoporosis compared to cells from healthy controls.

Furthermore, osteoporosis-associated genes were investigated with the help of the RT2 PCR Array. Here it was shown that inhibitors of the anabolic Wnt signaling pathway are increased, as well as the genes for bone resorption are significantly increased in osteoporotic patients. The exact investigation of the interaction of all these genes with each other is part of future research.

**Revised text (Discussion):**

As CD274 (PD-L1) is the ligand of CD279 all samples in our study were assessed for their CD274 expression by flow cytometry and via Real-Time PCR. We expected an increase in CD274 concentration in osteoporotic patients, as a deficiency of the receptor CD279 leads to osteopetrosis, as already written above. However, contrary to our expectations, we found a 5-fold reduction in CD274 concentration at protein and a 50% decrease on gene level in osteoporotic patients.

Furthermore, it is known that an IL-6 receptor blockade prevents the upregulation of CD274 (Eriksson et al., 2019) and that the IL-6 signal pathway is necessary for the
CD274 stability (Chan et al., 2019), as well as a maximum expression of CD274 is only possible in the presence of IL6 (Jin et al., 2013). As we detected significantly downregulated IL6R values and increased IL6 concentrations in the osteoporosis Gene Array, we believe that CD274 could be an interesting candidate in the field of bone and osteoporosis research. The underlying mechanisms and the signaling pathways involved in this setting are not yet known to us and are the subject of further research.

5. Additional questions of the reviewer:

1. Can the authors propose a pathway by which decreased CD274 expression might affect immune cells and consequently affect bone formation or resorption?
2. Do the results point toward a potential pathway of increased osteoclastogenesis and bone loss in the endosteal niche? Is this consistent with the results of Nagahama et al. 2004?

Our response: These are interesting questions brought up by the reviewer. Unfortunately, we are not able to answer these questions satisfactorily. After observing that CD274 is significantly downregulated on hBMSC of osteoporotic patients, we initially thought that this was in line with the findings of Nagahama et al. On closer inspection, however, it turned out that a deficiency of CD274 leads to an osteoporotic phenotype and a deficiency of CD276 leads to an osteopetrotic phenotype. In order to clarify this factual situation, more precise molecular biological investigations are required, which we want to carry out in the future.

Reviewer 2:

1. Concerns of the reviewer (Materials and Methods):

- Given that this is a paper about osteoporosis, it is a pity that only one criterion of osteogenesis was explored: mineral deposition/alizarin red. The authors missed the opportunity to measure expression of a number of important genes associated with osteogenesis.
- It is a pity that similar assays were not run with MSCs from younger patients. According to the discussion, expression of CD274 declines with age.

Our response: We thank the reviewer for these valuable comments. We have taken the opportunity and have carried out further measurements using a RT2 Profiler PCR Osteoporosis Array and Real Time PCR for gene expression of RNA isolated from stem cells in P3 from three patients of each group. This is now described in detail within the appropriate sections. Regarding the change of CD274 expression by age, we would like to mention, that our study group and control group did have very similar mean ages. Yet we are very much considering this point for further investigations if we have younger osteoporotic patients in the future.

Revised text (Abstract): … Furthermore, a RT2 Osteoporosis Profiler PCR Array, as well as quantitative real-time PCR of interesting marker genes were carried out to evaluate changes in gene expression. Statistical analysis of non-parametric data was carried out by a Mann-Whitney U-test.

Cultivated hBMSCs from osteoporotic donors showed significantly lower cell surface expression of CD274 (PD-L1) (4.98 % ± 2.38 %) than those from the control group (26.03 % ± 13.39 %; p = 0.007) in flow cytometry analysis. In osteoporotic patients, genes involved in inhibition of the anabolic WNT signaling pathway and those associated with stimulation of bone resorption were significantly upregulated. Apart from these differences in expression of this immunologically relevant antigen, no significant differences were found for the other cell surface antigens, adipogenic, osteogenic and chondrogenic differentiation and their proliferation potential.
Added text (Materials and Methods):

Gene expression

RT2 PCR Array Osteoporosis was carried out as described earlier (Bundkirchen et al., 2018). Summarized, RNA was isolated out of bone marrow derived mesenchymal stem cell pellets from three healthy and three osteoporotic donors using the direct-zol RNA extraction kit (Zymo Research Europe GmbH, Freiburg, Germany), according to the manufacturer’s instructions. RNA quality was determined using a using Epoch-Reader (BioTek Instruments, Bad Friedrichshall, Germany). cDNA was synthesized out of 1 μg RNA using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystem, Foster City, USA) regarding manufacturer's protocol.

For determination of involved signaling pathways the RT2 Profiler PCR Array Osteoporosis (Gene Globe ID: PAHS-170ZC-2, Qiagen) was performed using the Step-OnePlus Real – Time PCR Systeme (Applied Biosystems). For all samples, the threshold was set at 0.075 ΔRn. CT values were exported to an Excel file. This table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to control (healthy donors) and test group (osteoporotic donors). CT values were normalized based on the reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). The data analysis web portal calculates fold change/regulation using delta delta CT method, in which delta CT is calculated between gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta CT calculations (delta CT (Test Group)-delta CT (Control Group)). Fold Change is then calculated using 2^(-delta delta CT) formula. A fold change of 0.5 or less was considered as down-regulation and 2.0 or greater as up-regulation.

Determination of gene expression was performed with TaqMan probes (Applied Biosystems) for the following genes Programmed cell death 1 ligand (PD-L1; also known as CD274; Hs00204257_m1, Applied Biosystems), Programmed cell death 1 (PD-1; also known as CD279; Hs01550088_m1; Applied Biosystems) and cytotoxic T-lymphocyte associated protein 4 (CTLA4; also known as CD152; Hs00175480_m1; Applied Biosystems). As housekeeping gene eukaryotic 18S rRNA (Hs9999901 s1, Applied Biosystems) was used. Experiments were carried out with the Step-OnePlus Real – Time PCR System and the corresponding Mastermix (Applied Biosystems). The threshold was set at 0.2 ΔRn for all samples. The evaluation of the CT values was performed using the ΔΔCT method. The statistical analysis was performed with fold change values (2^-ΔΔCT).

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Added text (Results):

Gene regulation (RT2 PCR array)
In table 2 the abbreviation, gene name and gene function of the up- and downregulated genes of the RT2 Profiler PCR Array Osteoporosis are listed. The analysis resulted in 23 upregulated and one downregulated gene in the RNA of hBMSC from osteoporotic patients compared to healthy ones. The most expressed gene is Wnt5a (Fold change: 15.05).
Interestingly, also many inhibitors of the anabolic Wnt signaling pathway as Dickkopf (WNT signaling pathway inhibitor 1 (DKK1; fold change: 2.41), Secreted frizzled related protein 1 (SFRP1; fold change: 2.30) and SFRP4 (fold change: 2.32), as well as Sclerostin (SOST; fold change: 5.12) were significantly upregulated in samples from osteoporotic patients compared to healthy controls. Furthermore, known markers which stimulate bone resorption as Arachidonate 15-lipoxygenase (ALOX15; fold change: 3.12), Growth hormone releasing hormone (GHRH; fold change: 5.12), Interleukin – 6 (IL6; fold change: 3.09), Nuclear factor of activated T cells 1 (NFATC1; fold change: 2.36), Prolactin (PRL; fold change: 4.79), Parathyroid hormone (PTH; fold change: 2.13) and Parathyroid hormone like hormone (PTHLH; fold change: 6.42) are significantly increased in osteoporotic patients. The only downregulated gene was the Interleukin 6 receptor (IL6R) with a fold change of 0.05.

Gene Expression
Further the mRNA expression of CD274, CD279 and CD152were examined. While CD279 is not expressed on hBMSCs, the mRNA expression of CD152showed no significant difference in cells from osteoporotic patients (0.833 ± 0.623%) compared to controls (1.042 ± 0.441%). In contrast, the mRNA expression of CD274 was significantly lower in hBMSCs from osteoporotic patients (0.648 ± 0.209) compared to controls (1.443 ± 0.745).

Additional text (Discussion): Therefore, we performed an osteoporosis array to examine differences between osteoporotic and healthy patients on the gene expression level. The results showed on the one hand a significant increase in genes associated with osteoclast activation and on the other hand an inhibition of the anabolic Wnt signaling pathway. These results are in line with the findings of other working groups who showed that inhibitors of the Wnt signaling as DKK1 or SOST play an important role in the development of osteoporosis (Baron and Gori, 2018; Baron and Kneissel, 2013). In addition, it is well known that in osteoporotic patients the balance between bone formation by osteoblasts and bone degradation by osteoclasts is shifted in favor of osteoclasts, wherefore many therapeutic strategies have been developed which aimed at inhibiting the excessive bone resorption (Rachner et al., 2011).

2. Concern of the reviewer (Results):

The decline in CD274 is dramatic - 5-fold - and there is little or no overlap between the two groups. This must mean something important, although this is not followed up in the paper.

Our response: The reviewer is right. As described within the paragraph before, we have now taken the opportunity and have measured the expression of several genes which are related to osteoporosis or CD274 in stem cells from P4 from osteoporotic or healthy donors. We hope, that the provided results and their discussion will help to sufficiently extend the manuscripts focus the concern stated by the reviewer

Revised text (Technique): see Concern 1, Reviewer 2.

Added text (Results): see Concern 1, Reviewer 2.

Added text (Discussion):
As CD274 (PD-L1) is the ligand of PD-1 all samples in our study were assessed for their CD274 expression by flow cytometry and via Real-Time PCR. We expected an increase in CD274 concentration in osteoporotic patients, as a deficiency of the receptor CD279 leads to osteopetrosis, as already written above. However, contrary to our expectations, we found a 5-fold reduction in CD274 concentration at protein and a 50% decrease on gene level in osteoporotic patients.
Furthermore, it is known that an IL-6 receptor blockade prevents the upregulation of CD274 (Eriksson et al., 2019) and that the IL-6 signal pathway is necessary for the CD274 stability (Chan et al., 2019), as well as a maximum expression of CD274 is only possible in the presence of IL6 (Jin et al., 2013). As we detected significantly downregulated IL6R values and increased IL6 concentrations in the osteoporosis Gene Array, we believe that CD274 could be an interesting candidate in the field of bone and osteoporosis research. The underlying mechanisms and the signaling pathways involved in this setting are not yet known to us and are the subject of further research.

3. **Concern of the reviewer (General):** This is a bit of a flimsy paper - just one experiment in essence - but the single finding is very intriguing and quite dramatic

**Our response:** We thank the reviewer for their comment on finding our results intriguing. The overall aim of this study was to find out differences in the stem cell properties of osteoporotic vs. healthy patients. In order to give this paper more weight and basis, we added in this revision the results of the gene expression of genes, which are associated with osteoporosis and CD274. However, more precise analyzes are only possible in follow-up studies.

4. **Additional question of the reviewer:**

There is much talk of using MSCs to treat osteoporosis. How do you envisage this happening, given that MSCs do not engraft into bone very well and, contrary to earlier expectations, do not allograft well. Do you think autologous cells engineered to express more CD274 might be useful?

**Our response:** This is an interesting question brought up by the reviewer. Please see the answer below.

**Answer:**

*We show here for the first time a lower expression of CD274 in hBMSCs of osteoporotic patients and this both at the mRNA and protein level. The exact connection between this observation and the clinical picture of osteoporosis has to be analyzed in more detail in further studies. However, this is a first indication on which further research should be carried out. Even though MSCs do not engraft into bone very well, stem cell therapy can still be successful, e.g. due to secreted factors that in turn activate surrounding cells and intervene in bone remodeling.*
Osteoporosis is accompanied by reduced CD274 expression in human bone marrow derived mesenchymal stem cells
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† These authors contributed equally to the study

Running Title: Reduced CD274 expression in osteoporotic hBMSCs

Abstract
Osteoporosis is a disease of increasing importance in an aging society. Underlying pathomechanisms of osteoporosis have been subject to current research but are still considered to be not fully elucidated. Cell based therapy approaches pose a new group of possibilities to treat osteoporosis and its complications. Aim of this study was to quantify differences of human bone marrow derived mesenchymal stem cells (hBMSC) from healthy donors and those suffering from clinically manifest osteoporosis. Cell samples of seven donors for each group were selected retrospectively from the cell bank of human bone marrow-derived mesenchymal stem cells of the Trauma Department of Hannover Medical School containing cryoconserved cells harvested by iliac crest aspiration from volunteers during elective surgical procedures. Cells were evaluated for their adipogenic, osteogenic and chondrogenic differentiation potential, for their proliferation potential and expression of surface antigens. Furthermore, a RT2 Osteoporosis Profiler PCR Array, as well as quantitative real-time PCR of interesting marker genes were carried out to evaluate changes in gene expression. Statistical analysis of non-parametric data was carried out by a Mann-Whitney-U-test.

Cultivated hBMSCs from osteoporotic donors showed significantly lower cell surface expression of CD274 (PD-L1) (4.98 % ± 2.38 %) than those from the control group (26.03 % ± 13.39 %; p = 0.007) in flow cytometry analysis. In osteoporotic patients, genes involved in inhibition of the anabolic WNT signaling pathway and those associated with stimulation of bone resorption were significantly upregulated. Apart from these differences in expression of this immunologically relevant antigen, no significant differences were found for the other cell surface antigens, adipogenic, osteogenic and chondrogenic differentiation and their proliferation potential.

These findings support theories of an influence of CD274 on the regulation of bone metabolism. CD274 might be a promising target for further investigations regarding the pathogenesis of osteoporosis and into cell-based therapies involving MSCs.
Key words: Osteoporosis, Mesenchymal Stem Cells, CD274, Osteoblasts, Gene Expression

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Introduction

Although the incidence of osteoporosis increases in our aging society (Burge et al., 2007; Johnell and Kanis, 2005), the underlying pathomechanisms have not been fully elucidated. Immunological factors (Arron and Choi, 2000; Nagahama et al., 2004) and altered mechanisms of osteogenic cell differentiation have been postulated (Rodríguez et al., 1999).

As mesenchymal stem cells (MSCs) have the ability of constant self-renewal and are able to differentiate into several cell types *in vivo* and *in vitro*, they are of immense interest for research in the fields of regenerative medicine and tissue engineering (Docheva et al., 2007; Kassem et al., 2004). MSCs can be found in most tissues of the human body. Besides aspirates from liposuction, they can be harvested safely by minimal invasive aspiration biopsy from bone marrow stroma (Chamberlain et al., 2007). They can be identified *in vitro* by growing plastic adherent, their differentiation potential and a set of surface markers (Bara et al., 2014; Dominici et al., 2006; Machado et al., 2013). Especially regarding their surface marker expression, no final consensus exists.

It is well known that MSCs can differentiate into osteoblasts, adipocytes, chondrocytes and other non-mesoderm-type cells *in vitro* (Banas et al., 2007; Docheva et al., 2007; Kassem et al., 2004). Therefore, their immense differentiation potential has raised hope for future use in standardized, cell based therapy approaches (Ayatollahi et al., 2012; Kassem et al., 2004; Wang et al., 2012). As MSCs are able to differentiate into osteoblasts, they have the potential to perform intramembranous bone formation, which make their therapeutic use applicable especially in reconstructive and regenerative therapies for bone defects (Gibon et al., 2017). Furthermore, MSC do not express T and B cell markers and may inhibit T cell proliferation through direct contact and cytokine secretion (Zhao et al., 2004). These immune-modulatory and immune evasive properties might facilitate the use of MSCs for cell-based therapies, especially for degenerative conditions. Cell therapies for osteoporosis involving MSCs (Aghebati-Maleki et al., 2019; Antebi et al., 2014) have been discussed, but have so far not been established in clinical use.

A heterogenic set of factors responsible for osteoporosis such as age, medication and genetics (Cummings and Melton, 2002) may be accountable for heterogeneous distribution of clinical manifestations. Donors' age has been described as a factor for reduced osteogenic and chondrogenic potential of MSCs in a murine model (Kretlow et al., 2008). The co-incidence of age-related decrease in osteoblastic differentiation (Zhou et al., 2008) suggests a possible link between BMSC differentiation potential and clinical manifestation of osteoporosis. Yet, osteoporosis itself seems not to be associated with a reduced osteogenic potential of MSCs (Haddouti et al., 2020). On the other hand, it has furthermore been described that MSCs derived from femoral heads of patients suffering from osteoporosis showed reduced migration ability upon bone morphogenetic protein (BMP)-2, BMP-7 or fetal calf serum (FCS) stimulation (Haasters et al., 2014) and reduced but qualitatively adequate ossification.
Clinical findings and in vitro studies showed increased bone marrow adiposity and a negative association between bone-marrow fat and rate of bone formation in osteoporotic patients (Kassem and Marie, 2011; Paccou et al., 2015; Rosen and Bouxsein, 2006). Therefore a shift from osteogenesis towards adipogenesis for MSCs from osteoporotic patients has been proposed (Kassem and Marie, 2011; Rosen and Bouxsein, 2006).

Furthermore a link between osteoporosis and decreased proliferation rates of MSCs harvested from iliac crest aspirates has been reported (Rodríguez et al., 1999). Moreover, there is evidence that anti-resorptive substances routinely used in the therapy of osteoporosis may interact beneficially with MSCs regarding bone formation. In vitro, alendronate had a positive effect on osteoblastic differentiation of MSCs of healthy donors (Duque and Rivas, 2007). For osteoporotic patients, cell therapy may also play a role in the treatment of side effects of anti-resorptive substances. In a rat model, beneficial effects of growth factors and cytokines secreted by MSCs were found for cases of medication related osteonecrosis of the jaw (Ogata et al., 2015).

In the publication of Čamernik et al. a differences between MSCs of osteoporotic patients and non-osteoporotic patients have been described. In their work, the chondrogenic pellet diameter of bone-derived MSCs in osteoporotic donors was significantly lower compared to controls (Čamernik et al., 2020). Also deficiency of the CD152 (Cytotoxic T-lymphocyte associated protein 4; CTLA-4) and CD279 (Programmed cell death-1;PD-1 ) gene, a receptor of CD274, have been linked to bone malformation in a murine model (Nagahama et al., 2004). Hypermethylation of the CD279 gene locus, PDCD1, has recently been linked to the occurrence of osteoporosis in postmenopausal women (Cheishvili et al., 2018).

As little is known about possible effects of altered MSCs on bone metabolism, the aim of the recent study was to investigate differences between hBMSCs from osteoporotic and non-osteoporotic donors. We especially focused on evaluating differences within the potential of osteogenic, adipogenic and chondrogenic differentiation, proliferation as well as alternations of surface antigen expression and gene expression.
Materials and methods

Criteria for inclusion, exclusion and donors’ consent
All tissue samples were acquired by bone marrow aspiration from volunteers undergoing elective surgical procedures at the Trauma Department at Hannover Medical School, Hannover, Germany. Donors suffering from clinically relevant osteoporosis were included into the study group. Criteria for inclusion were previous pathologic fractures, vertebral wedge fractures and T scores ≤ - 2.5 in dual-energy absorptiometry (DEXA scan). Immunosuppressed patients and those suffering from malignant diseases were excluded from the study. An anonymous questionnaire about pre-existing conditions and lifestyle and reports about secondary diagnoses was obtained from all donors. Written informed consent was obtained from all donors prior to inclusion in the study. The study protocol and process of sample donation comply with the Declaration of Helsinki and were approved by the institutional review board (Hannover Medical School - Votum No. 2562).

MSC purification and proliferation
After intraoperative bone marrow aspiration from the iliac crest, samples were transferred into a phosphate buffered saline (PBS) solution and subsequently separated using a synthetic polysaccharide-epichlorohydrin-copolymer (Biocoll®, Biochrom, Berlin, Germany) by centrifugation for 30 minutes at 500 xg without brake. Then, the mononuclear cell layer was extracted, washed with PBS and centrifuged for five minutes at 500 xg with brake. Afterwards, the generated pellet was re-suspended in MSC specific growth medium containing DMEM FG 0415 (Biochrom) with 10 % (v/v) FBS (Hyclone® Fetal Bovine Serum (FBS), Thermo Fisher Scientific, Schwerte, Germany), 20 mM HEPES, 1 % (100 U/mL / 100 μg/mL) penicillin/streptomycin (Biochrom), and 2 ng/mL human recombinant FGF2 (PeproTech, Hamburg, Germany).

To facilitate proliferation of BMSCs, cells were incubated at 37°C and 5 % CO₂ as described before (Schäck et al., 2013). In passage 1 (P1) of in vitro cultivation, cells were cryoconserved using freezing medium containing 95 % FBS and 5 % dimethyl sulfoxide (DMSO). Cells were stored at -152°C in a cell bank for human bone marrow-derived mesenchymal stem cells at the Trauma Department at Hannover Medical School for further analyses.

Colony forming unit-fibroblast assay (CFU-F assay)
During BMSC characterization, a CFU-F assay was carried out at P1 to investigate cells’ proliferation potential. Cells were seeded in duplicates in ascending concentrations of 125, 250 and 500 cells per well in a six-well plate. Cells were fixated with methanol (Merck, Darmstadt, Germany) after ten days of incubation at 37°C and 5 % CO₂. After dyeing in 1 % crystal violet solution (Merck) macroscopically
visible BMSC colonies were counted to calculate the number of colonies per 100 cells seeded.

**Flow Cytometry (FC)**

To assess CD274 expression and to confirm identifying criteria for MSCs, cell samples were analyzed via flow cytometry. In addition to the criteria defined by Dominici et al. (expression of CD105, CD73 and CD90, but not of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR), expression of CD29, CD44, CD166, CD11c, CD15 and CD31 was analyzed as postulated in the literature (Bara et al., 2014; Dominici et al., 2006; Machado et al., 2013).

After controlled thawing, cells were incubated as previously described (Schäck et al., 2013) in MSC specific growth medium. In P3 cells were detached by 0.05 % / 0.02 % trypsin-EDTA solution (Biochrom) and washed twice with FC buffer (2 % (v/v) FBS in PBS (Thermo Fisher / Biochrom). All centrifugation steps were performed at 400 xg and 4°C for two minutes. For each sample 1*10^5 cells were used and incubated with appropriate fluorochrome-conjugated antibodies for 60 minutes at 4°C in the dark. The following monoclonal antibodies (mouse anti-human) were used: CD11b APC, CD15 FITC, CD19 PerCP, CD29 APC, CD31 FITC, CD34 PE-Cy7, CD44 FITC, CD45 APC-Cy7, CD73 APC, CD90 PerCP Cy5.5, CD105 PE, CD166 FITC. All antibodies were purchased from BioLegend (San Diego, California, U.S.) apart from CD166 FITC, which was purchased from MBL (Woburn, Massachusetts, U.S.). CD274 expression was evaluated during FC analysis using PE/Cy7 anti-human CD274 (B7-H1, PD-L1) antibodies (Clone 29E.2A3, Biolegend).

After two washing steps with FC buffer, cells were analyzed on a FACS Canto (BD Biosciences, Heidelberg, Germany) as described before (Schäck et al., 2013). Briefly, for each flow cytometric analysis 3*10^4 cells were recorded. Cell debris was excluded by using scatter parameters in BD FACSDiva Software and Flowing Software version 2.5.0. Within the analyzed surface antigens, minimal standards regarding expression according to those commonly described in literature (Bara et al., 2014; Dominici et al., 2006; Machado et al., 2013) were expected to be met.

**Gene expression**

RT2 PCR Array Osteoporosis was carried out as described earlier (Bundkirchen et al., 2018). Summarized, RNA was isolated out of bone marrow derived mesenchymal stem cell pellets from three healthy and three osteoporotic donors using the direct-zol RNA extraction kit (Zymo Research Europe GmbH, Freiburg, Germany), according to the manufacturer’s instructions. RNA quality was determined using a using Epoch-Reader (BioTek Instruments, Bad Friedrichshall, Germany). cDNA was synthesized out of 1 μg RNA using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystem, Foster City, USA) regarding manufacturer’s protocol.

For determination of involved signaling pathways the RT2 Profiler PCR Array Osteoporosis (Gene Globe ID: PAHS-170ZC-2, Qiagen) was performed using the Step-OnePlus Real – Time PCR Systeme (Applied Biosystems). For all
samples, the threshold was set at 0.075 ΔRn. CT values were exported to an Excel file. This table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to control (healthy donors) and test group (osteoporotic donors). CT values were normalized based on the reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). The data analysis web portal calculates fold change/regulation using delta delta CT method, in which delta CT is calculated between gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta CT calculations (delta CT (Test Group)-delta CT (Control Group)). Fold Change is then calculated using $2^{-\Delta \Delta CT}$ formula. A fold change of 0.5 or less was considered as down-regulation and 2.0 or greater as up-regulation.

Determination of gene expression was performed with TaqMan probes (Applied Biosystems) for the following genes CD274 (also known as Programmed cell death 1 ligand; PD-L1; Hs00204257_m1, Applied Biosystems), CD279 (also known as Programmed cell death 1; PD-1; Hs01550088_m1; Applied Biosystems) and CD152 (also known as cytotoxic T-lymphocyte associated protein 4; CTLA4; Hs00175480_m1; Applied Biosystems). As housekeeping gene eukaryotic 18S rRNA (Hs9999901 s1, Applied Biosystems) was used. Experiments were carried out with the Step-OnePlus Real-Time PCR System and the corresponding Mastermix (Applied Biosystems). The threshold was set at 0.2 ΔRn for all samples. The evaluation of the CT values was performed using the ΔΔCT method. The statistical analysis was performed with fold change values ($2^{-\Delta \Delta CT}$).

**Osteogenic differentiation**

To assess the osteogenic potential of each specimen, BMSCs from each donor were transferred onto six-well plates with each well containing a total of 150,000 cells. Samples were incubated either in 3 ml control medium containing DMEM FG0415 (Biochrom, Berlin, Germany) with 20 mM HEPES-Buffer (Biochrom), 10 % FBS-solution (Thermo Fisher, Waltham, Massachusetts, U.S.) and 1 % penicillin/streptomycin 100 U/mL / 100 µg/mL (Biochrom) or 3 ml induction medium for osteogenic differentiation with 10 nM Dexamethsone (Merck, Darmstadt, Germany), 50 µM ascorbate-2-phosphate (Merck) and 3 mM Na$_2$H$_{3-x}$PO$_4$ at pH 7.4 (Merck) added to the control medium. Each medium was changed on a weekly basis. Histologic specimens were harvested at days 28 and 42, fixated for 30 min in 4 % formalin solution and consequently dyed with 0.5 % Alizarin Red S (Carl Roth, Karlsruhe, Germany) at pH 4.5.

**Adipogenic differentiation**

Apart from a different medium to induce adipogenic differentiation, all specimens underwent the identical procedure as described for osteogenic differentiation. The induction medium for adipogenic differentiation contained 1 µM dexamethasone (Merck), 500 µM IBMX (Merck), 60 µM indomethacin (Merck) and 172 nM insulin in addition to the aforementioned control medium. Each medium was changed on a weekly basis. Histologic specimens were harvested at day 28, fixated in 4 % formalin
solution for 30 min and consequently dyed with Oil Red O (5 g/l in 60 % isopropanol; Merck).

**Chondrogenic differentiation**

To assess the chondrogenic potential of each specimen, 2.5 x 10^5 BMSCs from each donor were formed into pellets by centrifugation at 200 x g for 5 minutes. The pellets were incubated in 0.5 ml control medium containing DMEM FG0435 (Biochrom) with 20 mM HEPES-buffer (Biochrom), 1 % penicillin/streptomycin 100 U/mL / 100 µg/mL (Biochrom), 0.1 µM dexamethasone (Merck) 10 µl/ml ITS (Sigma Aldrich), 170 µM ascorbate-2-phosphate (Sigma Aldrich), 1 mM Na-pyruvate (Biochrom) and 350 µM proline (Carl Roth, Karlsruhe, Germany). After one day, the control medium was replaced by induction medium for chondrogenic differentiation including 10 ng/ml TGF-β3 in addition to the control medium. Each medium was changed on a weekly basis. Histologic specimens were harvested at day 28. They were fixated in 4% formalin solution for 30 min and embedded in Tissue-Tek. O.C.T. (Sakura Finetek, Tokyo, Japan) under the use of liquid nitrogen. 5 µm frozen sections were dyed in 0.1 % Safranin O solution and covered in Vitro-Clud (Langenbrinck, Freiburg, Germany). At 40x magnification, the quality of chondrogenic differentiation was assessed and the area covered by the pellet in relation to the total area was evaluated, to assess the pellet growth.

**Digital processing and Statistics**

All samples were assessed by light microscopy as described. Photographs were evaluated by automatic threshold-based segmentation with an in-house coded, Java-based software. Results were collected in Excel for Mac 14.7.7 (Microsoft, Richmond, Washington, U.S.) and processed with Wizard Statistics 1.9.16 (by Evan Miller, Chicago, Illinois, U.S.). Normal distribution of the data was evaluated by Kolmogorov-Smirnov-test. Non-normally distributed groups were compared by their median using Mann-Whitney-U-test. A p-value of ≤ 0.05 was considered to be significant. Further statistical analyses were carried out in SPSS Statistics 24 (IBM, Armonk, New York, U.S.).
Results

Cells from seven donors suffering from symptomatic osteoporosis were compared to seven samples from patients not suffering from osteoporosis in the control group. Groups were matched by age and gender. Donors suffering from cancer and autoimmune disorders were excluded from the study. Immunotherapy, chemotherapy und regularly intake of steroidal drugs were further criteria for exclusion. Previous intake of anti-resorptive substances was not taken into account. By gender, distribution within the groups was equivalent with 5 female : 2 male donors. Average ages were 80.00 years (± 7.24 years) and 80.14 years (± 3.52 years), for the osteoporosis and control group, respectively.

CFU-F assay
Colony forming unit assays were carried out for both groups in P1. Cells from osteoporotic donors formed 3.74 (± 3.13) colonies per 100 cells seeded on average, those in the control group 4.40 (± 4.86), respectively. The results were not statistically significant (p = 1.000).

Expression of MSC specific surface antigens
Surface antigen expression of CD11b, CD11c, CD15, CD19, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD166, CD274 were measured in P3 by flow cytometry. The results are displayed in Table 1. The cells displayed a typical pattern of MSC specific surface antigens. No significant differences were observed between the surface antigens in the osteoporosis and control group, except for CD274 (Programmed cell death 1 ligand; PD-L1). MSC from osteoporotic donors had significantly less CD274 surface expression (4.98 % ± 2.38 %) than those from the control group (26.03 % ± 13.39 %, see Fig. 1). Differences between both groups were statistically significant (p = 0.007).

Gene regulation (RT2 PCR Osteoporosis Array)
In table 2 the abbreviation, gene name and gene function of the up- and downregulated genes of the RT2 Profiler PCR Array Osteoporosis are listed. The analysis resulted in 23 upregulated and one downregulated gene in the RNA of hBMSC from osteoporotic patients compared to healthy ones. The most expressed gene is Wnt5a (Fold change: 15.05). Interestingly, also many inhibitors of the anabolic WNT signaling pathway as Dickkopf WNT signaling pathway inhibitor 1 (DKK1, fold change: 2.41), Secreted frizzled related protein 1 (SFRP1; fold change: 2.30) and SFRP4 (fold change: 2.32), as well as Sclerostin (SOST; fold change: 5.12) were significantly upregulated in samples from osteoporotic patients compared to healthy controls. Furthermore, known markers which stimulate bone resorption as Arachidonate 15-lipoxygenase (ALOX15; fold change: 3.12), Growth hormone releasing hormone (GHRH; fold change: 5.12), Interleukin – 6 (IL6; fold change: 3.09), Nuclear factor
of activated T cells 1 (NFATC1; fold change: 2.36), Prolactin (PRL; fold change: 4.79), Parathyroid hormone (PTH; fold change: 2.13) and Parathyroid hormone like hormone (PTHLH; fold change: 6.42) are significantly increased in osteoporotic patients. The only downregulated gene was the Interleukin 6 receptor (IL6R) with a fold change of 0.05.

**Gene Expression**

Further the mRNA expression of CD274, CD279 and CD152 were examined for 5 randomly chosen specimens per group. While CD279 is not expressed on hBMSCs, the mRNA expression of CD152 showed no significant difference (p > 0.05) in cells from osteoporotic patients (fold change: 0.833 ± 0.623) compared to controls (fold change: 1.053 ± 0.651). In contrast, the mRNA expression of CD274 was significantly (p = 0.016) lower in hBMSCs from osteoporotic patients (fold change: 0.648 ± 0.209) compared to controls (fold change: 1.443 ± 0.745).

**Adipogenesis**

Adipogenic differentiation was induced by insulin and dexamethasone. All of the specimens were capable of adipogenic differentiation. On day 28, within the osteoporosis group 35.02 % (± 22.71 %) of the investigated area was covered with lipid droplets (Fig. 2a), while 38.10 % (± 11.31 %) of the area had undergone adipogenic differentiation within the control group. Differences were not statistically significant (p > 0.05). The non-induced samples showed no signs of major spontaneous cell differentiation.

**Osteogenesis**

As observable signs of osteogenic differentiation become visible later than signs of adipogenic differentiation, measurements were carried out at day 28 and 42. The non-induced samples showed no signs of major spontaneous cell differentiation. On day 28, the area covered by stained calcium deposits was 85.39 % (± 10.74 %) in the osteoporosis group and 83.40 % (± 15.40 %) in the control group (Fig. 2b). By day 42, 84.09 % (± 5.71 %) of the area in the osteoporosis group and 94.09 % (± 5.71 %) of the area in the control group (Fig. 2c) were covered by histological signs of osteogenic differentiation. Differences between the groups were not statistically significant (p > 0.05).

**Chondrogenesis**

Chondrogenic differentiation was induced by TGF-β3. All induced samples in the osteoporosis or control group were capable of chondrogenic differentiation. The non-induced samples showed no signs of major spontaneous cell differentiation. By day 28, the relative area covered was 17.28 % (± 8.56 %) within the osteoporosis group versus 27.04 % (± 15.76 %) in der control group (Fig. 2d). Differences were not statistically significant (p > 0.05).
Discussion

Osteoporosis is a disease of increasing economic importance (Kling et al., 2014). Even though a variety of screening methods are available, osteoporosis is still considered to be under-diagnosed and under-treated (Golob and Laya, 2015). A variety of therapy approaches exists, some with severe side-effects (Kling et al., 2014). Drugs with direct long-term effect on bone metabolism such as bisphosphonates and monoclonal antibodies (i.e. Denosumab) are frequently administered. Nevertheless, they come with severe long-term side effects, e.g. medication induced necrosis of the jaw (MRONJ) (Fliefel et al., 2015; Kling et al., 2014; Lemound et al., 2017; Pichardo et al., 2013; Ruggiero et al., 2014). Cell based therapies might pose an alternative to current standard therapy (Antebi et al., 2014; Kassem et al., 2004; Mikami et al., 2014). As they may be able to provide a causal therapy approach, they could be especially useful for young patients to avoid long-term drug administration. To develop these specific therapy approaches for osteoporosis, complete and profound understanding of underlying pathomechanisms is essential. Therefore, the aim of our study was to identify differences in the stem cell characteristics of osteoporosis patients compared to healthy subjects.

Some studies in the field of bone metabolism (Rodríguez et al., 1999) propose decreased osteogenic differentiation potential for BMSCs from osteoporotic patients. This is contrary to newer, methodically more robust studies (Haddouti et al., 2020), describing, that MSCs from osteoporotic and healthy patients possess similar differentiation properties. In our study, osteogenesis did not seem to be impaired on a cellular level in vitro. In line with this observation, other studies suggest that MSCs from osteoporotic donors might have impaired signal transduction but sustained osteoinduction upon stimulation (Prall et al., 2013). Therefore, we performed an osteoporosis array to examine differences between osteoporotic and healthy patients on the gene expression level. The results showed on the one hand a significant increase in genes associated with osteoclast activation and on the other hand an inhibition of the anabolic Wnt signaling pathway. These results are in line with the findings of other working groups who showed that inhibitors of the Wnt signaling as DKK1 or SOST play an important role in the development of osteoporosis (Baron and Gori, 2018; Baron and Kneissel, 2013). In addition, it is well known that in osteoporotic patients the balance between bone formation by osteoblasts and bone degradation by osteoclasts is shifted in favor of osteoclasts, wherefore many therapeutic strategies have been developed which aimed at inhibiting the excessive bone resorption (Rachner et al., 2011).

Furthermore, clinical findings in MRI studies, proposing a connection between increased amounts of bone marrow fat and decreased bone density (Paccou et al., 2015) suggest possible differences in adipogenic and osteogenic differentiation potential. On a molecular level, different concentrations of pro-adipogenic and pro-inflammatory regulatory factors were reported for osteoporotic bone marrow by Pino et al. (Pino et al., 2012). In the current study, no significant differences in
adipogenic; osteogenic and chondrogenic differentiation potential of hBMSCs could be found in vitro, substantiating the assumption of a strong modulatory effect of the surrounding environment within the bone marrow in vivo.

The surface antigen expression on MSCs is still a subject of current research and several amendments have been made to the criteria postulated by Dominici et al. The meaning of expression of some antigens such as HLA-DR (Bocelli-Tyndall et al., 2010; Dominici et al., 2006; Larghero et al., 2008; Tarte et al., 2010) and CD34 (Dominici et al., 2006; Lin et al., 2012) remains unclear. Regarding HLA-DR expression it was shown, that an exposition of hBMSC to fibroblast growth factor (FGF) 2 is suspected to lead to its expression in vitro (Bocelli-tyndall et al., 2015). As cells in our study were exposed to FGF2 prior to flow cytometry analysis, HLA-DR expression was, as postulated by Tarte et al. (Tarte et al., 2010), excluded from the minimal criteria for MSCs (Dominici et al., 2006). Regarding CD34 it is highly controversial whether its non-expression should be considered to be a minimal criteria as postulated by Dominici et al. (Dominici et al., 2006). In our study more than 5% of the cells expressed CD34. As cells were propagated in culture, we did, concordant with Lin et al. (Lin et al., 2012), not consider CD34 non-expression as an appropriate minimal criteria for MSCs. The other surface antigens, which should be measured according to the ISCT criteria, were, as expected, positive (CD105, CD73 and CD90) or negative (CD45, CD11b, CD19) in our study in both patients groups without significant differences. Additionally, we extended the criteria defined by Dominici et al. by flow-cytometry surface antigen testing of CD29, CD44, CD166, CD11c, CD15 and CD31 as postulated in the literature (Bara et al., 2014; Dominici et al., 2006; Machado et al., 2013). These criteria were sufficiently met by the cells, thus supporting they represent sufficiently pure MSC populations.

Besides degenerative factors, B cell and T cell influence on osteoclasts via RANKL-pathway and other immunological pathologies involving CD279 deficiency and CD40 expression have been discussed (Arron and Choi, 2000; Li et al., 2007; Nagahama et al., 2004; Pietschmann et al., 2016). CD274 expression rates have been described as highly heterogenic among different hBMSC samples and to correlate negatively with donors’ age (Siegel et al., 2013). To our knowledge, differences by gender have so far not been reported. Nagahama et al. were able to describe a link between deficiency of CD279 and bone malformation without effect on the rate of osteogenesis in vitro (Nagahama et al., 2004). They showed the occurrence of an osteopetrotic phenotype for CD279 deficient mice, suggesting an underlying mechanism mainly based on the modulation of the activation of osteoclast-progenitors.

As CD274 (PD-L1) is the ligand of CD279 all samples in our study were assessed for their CD274 expression by flow cytometry and via Real-Time PCR. We expected an increase in CD274 concentration in osteoporotic patients, as a deficiency of the receptor CD279 leads to osteopetrosis, as already written above. However, contrary to our expectations, we found a 5-fold reduction in CD274 concentration at protein and a 50% decrease on gene level in osteoporotic patients.
Additionally, Cheishvili et al. found the hypermethylation of PDCD1, the gene locus of CD279, to be associated with the occurrence of osteoporosis (Cheishvili et al., 2018). As the results of our study are showing an association of a CD274 deficiency with osteoporosis, they can be considered consistent with those by Cheishvili et al.

Furthermore, it is known that an IL-6 receptor blockade prevents the upregulation of CD274 (Eriksson et al., 2019) and that the IL-6 signal pathway is necessary for the CD274 stability (Chan et al., 2019), as well as a maximum expression of CD274 is only possible in the presence of IL6 (Jin et al., 2013). As we detected significantly downregulated IL6R values and increased IL6 concentrations in the osteoporosis Gene Array, we believe that CD274 could be an interesting candidate in the field of bone and osteoporosis research. The underlying mechanisms and the signaling pathways involved in this setting are not yet known to us and are the subject of further research.

Limitations

Some common drugs used in prophylactic treatment of patients at risk for or suffering from osteoporosis such as bisphosphonates have a long half-life (Lemound et al., 2017; Pichardo et al., 2013). Prior administrations, even decades ago, may therefore lead to effects of these substances onto the patient’s current bone metabolism. As this is frequently not remembered by the patient, prior intake of anti-resorptive substances was not taken into account for the current study. It cannot be completely excluded, that some drugs or interventions might have an effect on regulation of CD274. Even though it is mainly known for chemotherapeutics, which administration was a criterion for exclusion, a bias regarding this point cannot be completely ruled out.

The results presented are based on in vitro expanded hBMSCs. This was necessary due to the regulations of the institutional review board, as cells were obtained from a biobank containing samples from human donors undergoing standard surgical procedures. Yet, in vitro expansion may diminish the quality of cells, especially in aged individuals. Thus, further experiments should be conducted to verify the findings of our study in vivo.
Conclusions

CD274 expression on hBMSCs in vivo was found to be significantly reduced in patients suffering from osteoporosis. Interactions between CD274 (PD-L1), its receptor CD279 on T cells and bone metabolism have been described (Nagahama et al., 2004). The presented findings support theories of strong immunological components in the pathogenesis of osteoporosis (Arron and Choi, 2000). They may be a central starting point for further investigations regarding the pathogenesis of osteoporosis and into cell-based therapies involving MSCs.
Acknowledgements
We would like to thank the donors and surgeons of the Trauma Department at Hannover Medical School for providing us with bone marrow samples and Claudia Pütz, Melanie Weiß and Johanna Ehlers for their valuable assistance. The Institute of Functional and Applied Anatomy at Hannover Medical School generously made it possible for us to use their flow cytometer for this study.

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Conflicts of interests
The authors declare to have no potential conflicts of interest.

Data availability
The data used to support the findings of this study is available from the corresponding author upon request.
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Lin C-S, Ning H, Lin G, Lue TF (2012) Is CD34 Truly a Negative Marker for


Figure legends

**Figure 1.** Expression of CD274 was significantly lower on hBMSCs of the osteoporosis group than on those of the control group (*p = 0.007).

**Figure 2:** Representative images displaying the multipotent differentiation potential of hBMSCs from one donor of the osteoporosis group and one donor of the control group. All images display samples after incubation with the respective induction medium:

a) Adipogenic differentiation at day 28, Stained with Oil Red O. Images taken at 100x magnification.

b) Osteogenic differentiation at day 28: Stained with Alizarin Red. Images taken at 100x magnification.

c) Osteogenic differentiation at day 42: Stained with Alizarin Red. Images taken at 100x magnification.

d) Chondrogenic differentiation at day 28: Stained with Safranin O. Images taken at 40x magnification.
Tables

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Control Group</th>
<th>Osteoporosis Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>1.17 % ± 0.53 %</td>
<td>1.10 % ± 0.33 %</td>
</tr>
<tr>
<td>CD11c</td>
<td>4.22 % ± 1.92 %</td>
<td>3.23 % ± 1.54 %</td>
</tr>
<tr>
<td>CD15</td>
<td>0.78 % ± 0.34 %</td>
<td>0.87 % ± 0.18 %</td>
</tr>
<tr>
<td>CD19</td>
<td>5.19 % ± 4.61 %</td>
<td>2.66 % ± 0.65 %</td>
</tr>
<tr>
<td>CD29</td>
<td>89.72 % ± 19.44%</td>
<td>98.09 % ± 1.76 %</td>
</tr>
<tr>
<td>CD31</td>
<td>0.99 % ± 0.43 %</td>
<td>1.23 % ± 0.44 %</td>
</tr>
<tr>
<td>CD34</td>
<td>14.65 % ± 11.56%</td>
<td>14.53 % ± 4.78 %</td>
</tr>
<tr>
<td>CD44</td>
<td>81.72 % ± 28.83%</td>
<td>91.85 % ± 5.25 %</td>
</tr>
<tr>
<td>CD45</td>
<td>2.96 % ± 1.22 %</td>
<td>2.46 % ± 0.93 %</td>
</tr>
<tr>
<td>CD73</td>
<td>94.14 % ± 9.52 %</td>
<td>94.74 % ± 3.31 %</td>
</tr>
<tr>
<td>CD90</td>
<td>95.28 % ± 4.54 %</td>
<td>97.55 % ± 0.82 %</td>
</tr>
<tr>
<td>CD105</td>
<td>98.77 % ± 1.49 %</td>
<td>99.35 % ± 0.38 %</td>
</tr>
<tr>
<td>CD166</td>
<td>89.30 % ± 19.42 %</td>
<td>97.45 % ± 1.21 %</td>
</tr>
<tr>
<td>CD274</td>
<td>26.03 % ± 13.39 %</td>
<td>4.98 % ± 2.38 %</td>
</tr>
</tbody>
</table>

Tbl. 1: Basic parameters within both groups measured by FC analysis. Mean expression and standard deviation in percent. **CD274 expression was significantly decreased in patients with osteoporosis compared to control group (p = 0.007). No significant differences were found for all other antigens between the groups.**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6R</td>
<td>Interleukin 6 receptor</td>
<td>0.05</td>
<td>Receptor of IL6</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>TNF alpha induced protein 3</td>
<td>2.01</td>
<td>Inhibits osteoclastogenesis (Yan et al., 2020)</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
<td>2.13</td>
<td>Regulates the serum calcium concentration through its effects on bone (Wein and Kronenberg, 2018)</td>
</tr>
<tr>
<td>ITGA1</td>
<td>Integrin subunit alpha 1</td>
<td>2.24</td>
<td>Subunit of integrin receptors. Polymorphisms and haplotypes ITGA1 are associated with bone mineral density and fracture risk in postmenopausal woman (Lee et al., 2007)</td>
</tr>
<tr>
<td>NOG</td>
<td>Noggin</td>
<td>2.25</td>
<td>Extracellular BMP antagonist, decreases osteoblastogenesis (Moffett et al., 2009)</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
<td>Fold Change</td>
<td>Function</td>
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</tr>
<tr>
<td>TNFRSF11B/OPG</td>
<td>Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)</td>
<td>2.28</td>
<td>Antagonist of RANKL (Tella and Gallagher, 2014)</td>
</tr>
<tr>
<td>SFRP1</td>
<td>Secreted frizzled related protein 1</td>
<td>2.30</td>
<td>Inhibitor of the anabolic WNT signaling pathway (Wang et al., 2014)</td>
</tr>
<tr>
<td>SFRP4</td>
<td>Secreted frizzled related protein 4</td>
<td>2.32</td>
<td>Inhibitor of the anabolic WNT signaling pathway (Wang et al., 2014)</td>
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<tr>
<td>NFATC1</td>
<td>Nuclear factor of activated T cells 1</td>
<td>2.36</td>
<td>Key transcription factor contributes to osteoclast differentiation and bone resorption (Li et al., 2019)</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf WNT signaling pathway inhibitor 1</td>
<td>2.41</td>
<td>Inhibitor of the anabolic WNT signaling pathway (Ueland et al., 2019)</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin like growth factor 1</td>
<td>2.64</td>
<td>Critical growth factor to maintain bone mass (Crane and Cao, 2014)</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>Insulin like growth factor binding protein 2</td>
<td>2.92</td>
<td>The role of IGFBP-2 in bone homeostasis is somewhat conflicted with evidence to support agonistic and antagonistic actions depending on the model (DeMambro et al., 2015)</td>
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<tr>
<td>IL6</td>
<td>Interleukin 6</td>
<td>3.09</td>
<td>Mediates the upregulation of osteoclastogenesis (Fajar and Azharuddin, 2017)</td>
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<tr>
<td>ALOX15</td>
<td>Arachidonate 15-lipoxygenase</td>
<td>3.12</td>
<td>Negative regulator of peak bone mineral density (Klein et al., 2004)</td>
</tr>
<tr>
<td>ADCY10</td>
<td>Adenylate cyclase 10</td>
<td>3.24</td>
<td>Association with osteoporosis-related phenotypes (Xu et al., 2010)</td>
</tr>
<tr>
<td>LTA</td>
<td>Lymphotoxin alpha</td>
<td>3.26</td>
<td>LTA, formerly known as TNFβ, is the closest homolog to TNFα (Franki et al., 2005)</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>Cytochrome P450 family 17</td>
<td>3.44</td>
<td>Key factor in the synthesis of estrogen (Rodriguez-Sanz et al.,</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Value</td>
<td>Notes</td>
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<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
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</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
<td>4.79</td>
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Tbl. 2: Results of the RT2 PCR Array showing 23 upregulated and one downregulated gene.
Expression of CD274 was significantly lower on hBMSCs of the osteoporosis group than on those of the control group (*p = 0.007)

123x95mm (600 x 600 DPI)
Representative images displaying the multipotent differentiation potential of hBMSCs from one donor of the osteoporosis group and one donor of the control group. All images display samples after incubation with the respective induction medium:

a) Adipogenic differentiation at day 28, Stained with Oil Red O. Images taken at 100x magnification.
b) Osteogenic differentiation at day 28: Stained with Alizarin Red. Images taken at 100x magnification.
c) Osteogenic differentiation at day 42: Stained with Alizarin Red. Images taken at 100x magnification.
d) Chondrogenic differentiation at day 28: Stained with Safranin O. Images taken at 40x magnification.

200x100mm (400 x 400 DPI)
### Tbl. 1: Basic parameters within both groups measured by FC analysis. Mean expression and standard deviation in percent. CD274 expression was significantly decreased in patients with osteoporosis compared to control group (p = 0.007). No significant differences were found for all other antigens between the groups.
<table>
<thead>
<tr>
<th>Symbol</th>
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<th>Fold Change</th>
<th>Function</th>
</tr>
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<tr>
<td>IL6R</td>
<td>Interleukin 6 receptor</td>
<td>0.05</td>
<td>Receptor of IL6</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>TNF alpha induced protein 3</td>
<td>2.01</td>
<td>Inhibits osteoclastogenesis (Yan et al., 2020)</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
<td>2.13</td>
<td>Regulates the serum calcium concentration through its effects on bone (Wein and Kronenberg, 2018)</td>
</tr>
<tr>
<td>ITGA1</td>
<td>Integrin subunit alpha 1</td>
<td>2.24</td>
<td>Subunit of integrin receptors. Polymorphisms and haplotypes ITGA1 are associated with bone mineral density and fracture risk in postmenopausal woman (Lee et al., 2007)</td>
</tr>
<tr>
<td>NOG</td>
<td>Noggin</td>
<td>2.25</td>
<td>Extracellular BMP antagonist, decreases osteoblastogenesis (Moffett et al., 2009)</td>
</tr>
<tr>
<td>TNFRSF11B / OPG</td>
<td>Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)</td>
<td>2.28</td>
<td>Antagonist of RANKL (Tella and Gallagher, 2014)</td>
</tr>
<tr>
<td>SFRP1</td>
<td>Secreted frizzled related protein 1</td>
<td>2.30</td>
<td>Inhibitor of the anabolic WNT signaling pathway (Wang et al., 2014)</td>
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<tr>
<td>SFRP4</td>
<td>Secreted frizzled related protein 4</td>
<td>2.32</td>
<td>Inhibitor of the anabolic WNT signaling pathway (Wang et al., 2014)</td>
</tr>
<tr>
<td>NFATC1</td>
<td>Nuclear factor of activated T cells 1</td>
<td>2.36</td>
<td>Key transcription factor contributes to osteoclast differentiation and bone resorption (Li et al., 2019)</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf WNT signaling pathway inhibitor 1</td>
<td>2.41</td>
<td>Inhibitor of the anabolic WNT signaling pathway (Ueland et al., 2019)</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin like growth factor 1</td>
<td>2.64</td>
<td>Critical growth factor to maintain bone mass (Crane and Cao, 2014)</td>
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</tr>
<tr>
<td>IGFBP2</td>
<td>Insulin like growth factor binding protein 2</td>
<td>2.92</td>
<td>The role of IGFBP-2 in bone homeostasis is somewhat conflicted with evidence to support agonistic and antagonistic actions depending on the model (DeMambro et al., 2015)</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
<td>3.09</td>
<td>Mediates the upregulation of osteoclastogenesis (Fajar and Azharuddin, 2017)</td>
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<tr>
<td>ALOX15</td>
<td>Arachidonate 15-lipoxygenase</td>
<td>3.12</td>
<td>Negative regulator of peak bone mineral density (Klein et al., 2004)</td>
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<tr>
<td>ADCY10</td>
<td>Adenylate cyclase 10</td>
<td>3.24</td>
<td>Association with osteoporosis-related phenotypes (Xu et al., 2010)</td>
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<tr>
<td>LTA</td>
<td>Lymphotoxin alpha</td>
<td>3.26</td>
<td>LTα, formerly known as TNFβ, is the closest homolog to TNFα (Franki et al., 2005)</td>
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<td>CYP17A1</td>
<td>Cytochrome P450 family 17 subfamily A member 1</td>
<td>3.44</td>
<td>Key factor in the synthesis of estrogen (Rodriguez-Sanz et al., 2015)</td>
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