Increased osteoclastogenesis and bone resorption by peripheral blood mononuclear cells in chronic liver disease patients with osteopenia

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Abstract

Osteoporosis is a common complication of chronic liver disease and the underlying mechanisms are not completely understood. In this study we aimed to determine if peripheral blood mononuclear cells (PBMCs) from chronic liver disease patients with osteopenia contain more activated T lymphocytes and whether osteoclast formation is favoured compared to chronic liver disease patients without osteopenia or healthy controls. The activated T lymphocyte and monocyte populations were comparable for all three groups and RANKL expression was not detectable by FACS analysis. However, peripheral blood mononuclear cells from chronic liver disease patients with osteopenia formed more osteoclast–like cells which, when cultured in the presence of M-CSF and RANKL resorbed more bone than matched controls. Both the number of osteoclast-like cells and the amount of bone resorbed by these cells showed a correlation with the lumbar bone densities of the patients included in the study. Addition of M-CSF increased numbers of osteoclast-like cells formed in healthy controls however this was not observed in either of the chronic liver disease groups. Plasma levels of M-CSF were increased in both patients groups compared to healthy controls, suggesting that osteoclast precursors were primed with higher levels of M-CSF.

Conclusion: Our results show that circulating mononuclear cells from chronic liver disease patients with osteopenia have a higher capacity to become osteoclasts. This could partially be due to priming with higher levels of M-CSF in the circulation.

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Metabolic bone disease is a common complication observed in chronic liver disorders. It occurs in both parenchymal and cholestatic diseases as well as in a post-transplantation setting. Indeed osteoporosis characterized by a T-score of < -2.5 occurs in 20-53% of patients with chronic liver disease underscoring the need to better understand the pathogenesis of this condition [1].

The process of bone turnover is primarily controlled by osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation, while multinucleated osteoclasts are responsible for bone resorption. Normal bone turnover is largely controlled by three key-regulatory molecules including macrophage colony stimulating factor (M-CSF), receptor activator of nuclear factor κβ ligand (RANKL) and its soluble decoy receptor osteoprotegerin (OPG) which are produced by osteoblasts at the bone surface [2,3]. The binding of M-CSF and RANKL to their respective receptors c-fms and RANK, on mononuclear osteoclast precursor cells triggers the process of osteoclastogenesis giving rise to multinucleated osteoclasts [2,3].

The expression of RANKL by activated T lymphocytes, in combination with the presence of M-CSF, is sufficient to support osteoclast formation *in vitro* [4,5]. Mice, which have spontaneously activated T lymphocytes ($ctla^{-/-}$), develop severe osteoporosis which can be prevented by the administration of OPG, the soluble decoy receptor for RANKL, indicating a RANKL dependent process which implicates the involvement of the immune system in bone turnover [6].

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Osteoclast precursor cells which express RANK, originate from the monocyte/macrophage lineage and are also present in human peripheral blood [7,8,9]. Increased expression of RANKL by activated T lymphocytes within the circulation can increase the formation of osteoclasts from circulating osteoclast precursor cells [9].

Peripheral blood mononuclear cells (PBMCs) obtained in inflammatory conditions such as in multiple myeloma and psoriatic arthritis, cultured in the presence of M-CSF and RANKL, form osteoclast-like cells [10, 11]. Interestingly, the increased formation of these osteoclast-like cells also occurs when culturing PBMCs from patients with inflammatory diseases without the addition of cytokines. This "spontaneous" formation of osteoclast-like cells is associated with increased levels of RANKL and TNF-α in these inflammatory states [11]. TNF-α has been shown to directly increase osteoclastogenesis through a mechanism synergistic with RANKL [12]. We have previously demonstrated that rats suffering from hepatic osteodystrophy have significantly increased systemic levels of TNF-α [13]; a phenomenon that has also been documented for patients with chronic liver disease [14].

In remains unclear why certain individuals with chronic liver disease develop osteoporosis where others do not. Our group has recently shown that the administration of rapamycin, a general immune system suppressant, can partially ameliorate the bone loss observed in chronic liver disease rat models indicating immune system involvement [15]. The aim of the present study was twofold: (1) to establish if there was an increase in the circulating activated T

lymphocyte population and if this cell subset produced more RANKL in patients with chronic liver disease and osteopenia compared to controls and (2) to determine the extent to which osteoclast precursor cells obtained from the circulation of patients with chronic liver disease and controls, form osteoclasts in culture.

Materials and methods

Study population

Chronic liver disease is a heterogeneous disease with diverse causes. Many chronic liver disease patients also have other risk factors for osteoporosis related to their disease such as advanced age, low levels of 1,25-OH vitamin D, estrogen, testosterone, excess alcohol consumption and corticosteroid use [1]. This makes it difficult to establish the underlying molecular mechanism of bone loss and therefore extreme care was taken when recruiting patients for the study.

Patients with chronic cholestatic liver disease or auto-immune hepatitis were identified form the database of the Liver clinic in the department of Gastroenterology, Free University Medical Centre, Amsterdam. All patients included in the study were Dutch Caucasians. A patient was considered potentially eligible to participate in the study if a recent DEXA scan (within 1 year) was available, if no immunosuppressive drugs including high dose corticosteroids were used and if females were premenoupausal. Patients with an alcohol intake > 40g/day in men and > 20g/day in women and patients diagnosed with other chronic diseases were excluded from the study.

Patients were then screened for secondary causes of bone loss including hyperthyroidism, hypogonadism and osteomalasia by appropriate biochemical evaluation before inclusion in the study.

Patients were then divided into two groups: chronic liver disease without osteopenia (CLD -) and chronic liver disease with osteopenia (CLD +) according to the results of their DEXA scans where osteopenia was defined as T-score < -1.0. Four patients included (2 from CLD - group and 2 from CLD + group) in this study were on low doses of cortisone (< 5mg/day). Healthy controls (HC) were included as a third group and all individuals were processed in age and gender matched groups. In two of the ten paired chronic liver disease comparisons made the age difference between CLD – and CLD + exceeded 20 years thus each CLD patient was compared to their respective HC. Thus all results are reported as a comparison made between CLD – and their relevant HC, CLD + and their relevant HC and finally CLD – versus CLD +. Samples were obtained from matched groups and were immediately processed. Informed consent was obtained from all individuals included in the study and ethical committee approval was granted by the ethics review board of the Free University of Amsterdam.

Routine biochemistry

Serum was obtained and analyzed for TSH, 1,25-OH vitamin D testosterone in men, estrogen in women to exclude secondary causes of bone loss. Routine liver function tests were performed including albumin, ALT, AST, ALP were measured as an indication of the severity of the chronic liver disease.

ELISA

Plasma samples from all individuals included in the study were collected and stored at –80 ℃. ELISA's were performed in duplicate as per manufacturer's instruction to quantify the levels of RANKL (Biovision, Mountain View, CA), OPG, M-CSF and TNF-α (R&D Systems, Minneapolis, MN) in plasma. The detection ranges for the ELISA kits were the following: RANKL (31.3 – 2000 pg/ml), OPG (62.5 – 4000 pg/ml), M-CSF (10 – 2000 pg/ml) and TNF-α (15.63 – 1000 pg/ml). When samples fell below the lower limit they were classed as not detectable and when they fell above the upper limit they were appropriately diluted so as to fall into the detectable range of the assay.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of individuals by means of Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation as per manufacturer's instruction. Heparinized whole blood (30ml) was drawn from each individual, and diluted 1:1 with phosphate buffered saline (PBS). Two parts of diluted blood were layered on top of one part Ficoll and centrifuged without a brake at 1000g for 30 minutes. The interface was transferred and washed 3 times with PBS.

Flow cytometry

1×10^{6} PBMC's were incubated with the following antibody combinations as per manufacturers instruction: anti- human CD3-FITC (BD PharMingen, San Diego, CA), anti-human CD25-APC (BD PharMingen, San Diego, CA) and

anti-human RANKL-PE (e-Bioscience, San Diego, CA). Anti-human CD14-PerCP (Beckton Dickinson, San Diego, CA) and anti-human CD11b-APC (BD PharMingen, San Diego, CA). Samples were analysed on the FACSCalibur and data was processed with CellQuest software, version 3.1F (Becton Dickinson, San Diego, CA). Anti–human RANKL-PE was tested by means of staining T lymphocytes activated *in vitro* for 40 hours in the presence of phytohemagglutinin and interleukin-2 as previously described by Kotake *et al.* [16].

Real time RT-PCR

RNA was isolated from $1x10^{6}$ total PBMCs by means of the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The concentration of RNA was assessed by means of the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesised by making use of a reverse transcriptase reaction which was performed according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers.

Real time PCR primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA) (Table 1) and have been previously validated and used in our laboratory [17]. To avoid amplification of genomic DNA, each amplicon spanned at least one intron. The external standard curve used in the PCR reactions is cDNA from the total PBMC's of a healthy donor previously assessed to express all the relevant cytokines.

Real time PCR was performed on the ABI PRISM 7000 (Applied Biosystems, Foster City, CA). The reactions were performed with 10 ng cDNA (RANK, TNF-α and M-CSF) and 45 ng cDNA (RANKL and OPG) in a total volume of 25 µl containing SYBR Green PCR Master Mix, consisting of SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP instead of dTTP, passive reference and buffer (Applied Biosystems, Foster City, CA) and 300 nM of each primer.

After an initial activation step of the AmpliTaq Gold DNA polymerase for 10 minutes at 94 °C, 40 cycles were run of a two step PCR consisting of a denaturation step at 95 °C for 30 seconds and annealing and extension step at 60 °C for 1 minute. Subsequently the PCR products were subjected to melting curve analysis to exclude that unspecific PCR products were generated.

The PCR reactions of the different amplicons had equal efficiencies. The relative quantity was calculated using the relative standard curve method. The target quantity was determined from the standard curve. Subsequently samples were normalized for the expression of porphobilinogen deaminase (PBGD), and the relative quantity of the different genes is expressed as the n-fold difference relative to a calibrator (average quantity obtained per gene of interest of the healthy control group).

Osteoclastogenesis assays

Osteoclasts are multinucleated cells which produce the enzyme tartrate resistant acid phosphatase (TRACP) and are capable of bone resorption. To investigate the formation of osteoclast like cells from peripheral blood, total PBMCs were seeded in 96 well plates at a density of $1x10^{6}$ cells per well on plastic and on 650 μm thick bovine cortical bone slices and analysed for the formation of TRACP+ multinucleated cells and their ability to resorb bone. Cells were cultured in four conditions: (1) α-MEM supplemented with 10% fetal calf serum (spontaneous formation of osteoclast-like cells), (2) M-CSF (for the propagation of monocytes), (3) M-CSF and OPG (osteoclast formation with an inhibitor for putatively present endogenous RANKL), (4) M-CSF and RANKL (propagation of monocytes, differentiation towards osteoclast-like cells). M-CSF (R&D Systems, Minneapolis, MN) was added at a concentration of 25ng/ml, OPG (PeproTech, Rocky Hill, NJ) was added at a concentration of 1000ng/ml and RANKL (PeproTech, Rocky Hill, NJ) was added at a concentration of 40ng/ml. Cells were seeded in duplicate and media with or without the relevant cytokines was replenished twice a week throughout the culture period. Bioactivity of OPG was assessed in a mouse osteoclastogenesis assay. One microgram per ml (but also a tenfold lower concentration) abolished the formation of TRACP+ cells in mouse bone marrow stimulated with 20 ng/ml RANKL and M-CSF.

TRACP staining

At the end of a three week culture period, cells were fixed in PBS buffered 4 % formaldehyde and stained for TRACP activity using the leukocyte acid

phosphatase kit (Sigma, St. Louis, MO). Nuclei were stained with 5µg/ml 2phenylindole dihydrochloride (DAPI) (Sigma, St. Louis, MO). Five micrographs from fixed positions per well were taken with a digital camera (Leica, Wetzlar, Germany) and analyzed for the number of multinucleated cells containing 3 or more nuclei.

Bone resorption

PBMCs cultured on bone slices were analyzed for bone resorption after a culture period of 4 weeks. After this period, the cells present on the bovine cortical bone slices were removed with 0.25 M NH_4OH. The slices were washed in distilled water, incubated in a water saturated alum ($KAl(SO_4)_2.12H_2O$) solution, washed in distilled water and stained with Coommassie Brilliant blue [18]. Five micrographs from fixed positions were taken with a digital camera (Leica, Wetzlar, Germany) and the percentage of bone area resorbed was quantified using Image-Pro plus (Media Cybernetics, Silver Spring, ML) software.

Statistics

To determine if there was a correlation between any of the general biochemistry parameters and the osteoclastogenesis assays use was made of a nonparametric correlation test. Due to the paired nonparametric nature of the results obtained in this study use was made of the Wilcoxon signed rank test to analyze the ELISA data, FACS data, real time RT-PCR, TRACP positive multinucleated cells and bone resorption. To compare different culture conditions within groups for TRACP+ multinucleated cells, use was

made of a nonparametric Mann-Whitney U test. Graphs were generated using Graph Pad Prism 4 (GraphPad Software, San Diego,CA).

All statistics were calculated accordingly by comparing the relevant healthy controls to chronic liver disease patients without osteopenia, the relevant healthy controls to chronic liver disease patients with osteopenia and finally chronic liver disease patients without osteopenia to chronic liver disease patients with osteopenia.

Results

Baseline Analysis

General parameters

The age and gender distributions along with the type of chronic liver disease for patients included in this study are given in Table 2. Routine biochemistry of the sex hormones showed a mean testosterone level for males of 16 +/- 1.7 nmol/l (range 15 –18 nmol/l) and 15 +/- 3.6 nmol/l (range 12 –19 nmol/l) for CLD – and CLD + groups respectively. Testosterone levels did not differ significantly between CLD – and CLD + groups (p = 0.7). Estradiol in female patients showed mean values of 73.3 +/- 71.5 pmol/l (range 23 – 171 pmol/l), and 126.3 +/- 166 pmol/l (range 19 – 426 pmol/l) for CLD- and CLD+ groups respectively. The estradiol levels also did not differ between goups (p = 0.6691). 25-OH-D3 levels in CLD- group was 53 +/- 19.2 nmol/l (range 21 – 77 nmol/l) and 76.3 +/- 17.38 nmol/l (range 59 – 109 nmol/l) for the CLD+ group. The normal range for 25-OH-D3 levels in healthy individuals is 25 –

100 nmol/l. 25-OH-D3 levels did differ significantly between CLD – and CLD+ groups included in the study (p = 0.014).

Flow cytometry

Activated T lymphocytes and RANKL Expression

In order to address whether there was a shift in the activated T lymphocyte population of chronic liver disease patients FACS analysis was performed. Total PBMCs were stained for CD3 and CD25 and the double positive population was quantified. There were no significant differences in the activated T-lymphocyte population between healthy controls and chronic liver disease patients without or with osteopenia. Activated T-lymphocytes could potentially serve as a source of increased RANKL within the peripheral blood. Expression of RANKL, as analyzed by means of FACS, was not found in any of the three groups (Table 3).

PBMC fraction containing osteoclast precursors

The phenotypic profile previously described for the subset of PBMCs which contain OCPs is the CD14+/CD11b+ subset [10,12]. None of groups differed significantly with regards to their CD14+/CD11b+ PBMC subset (Table 3).

Real time RT-PCR

The mRNA expression of RANKL, RANK, M-CSF and TNF-α on an RNA level reflected no significant differences between groups and OPG was not detectable in any of the samples (Table 4).

Spontaneous Osteoclastogenesis

TRACP staining was performed on PBMCs cultured without the addition of any cytokines (representative photographs are given in Figure 1A). Total PBMCs from chronic liver disease patients with osteopenia "spontaneously" formed more TRACP+ mutinucleated cells than healthy controls (Figure 1B). When taken together, the results from the four different culture conditions showed that total PBMCs isolated from healthy controls formed significantly more TRACP + multinucleated cells when cultured in the presence of M-CSF than without. This was observed for PBMCs cultured with M-CSF regardless of the exogenous addition of OPG or RANKL. The M-CSF effect on osteoclast formation was not observed when PBMCs from either of the chronic liver disease groups were cultured. The formation of TRACP + multinucleated cells was not inhibited by OPG nor enhanced with the addition of RANKL in any of the three groups (Figure 1C). This data hints to the fact that increased endogenous production of M-CSF, and not RANKL, by PBMCs of chronic liver disease patients could be responsible for the observed increase in the "spontaneous" formation of osteoclast-like cells. In an attempt to further clarify the underlying molecules responsible, ELISAs were performed on plasma samples. Plasma levels of M-CSF where significantly higher in the two chronic liver disease groups when compared to healthy controls (Figure 1D). These data taken together with the osteoclastogenesis culture data show that an increase in circulating M-CSF levels could be responsible for the increased "spontaneous" formation of osteoclast-like cells observed in patients with chronic liver disease. RANKL and OPG were detectable but showed no group variation and TNF-α was not detectable in any of the plasma samples.

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Osteoclastogenesis cultures with M-CSF and RANKL

Total PBMCs were also cultured with M-CSF and RANKL, a condition required for achieving bone resorbing osteoclasts. PBMCs from chronic liver disease patients with osteopenia formed significantly more osteoclast-like cells than either of the control groups (Figure 2A).

No correlations were found between the number of TRACP+ multinucleated cells spontaneously formed and the levels of testosterone, estrogen, 1,25-OH vitamin D, albumin or liver enzymes. There was also no correlation between the use of low levels of cortisone (< 5mg/day) and the spontaneous osteoclastogenesis observed in chronic liver disease patients (data not shown). However, there was a correlation between the number of TRACP+ multinucleated cells formed when culturing PBMCs with the addition of M-CSF and RANKL and the bone density of patients (Figure 2B).

Bone resorption

The ability of the TRACP+ multinucleated cells to resorb bone was assessed by means of bone resorption assays (representative micrographs are shown in Figure 3A). Bone resorption was only present when PBMCs were cultured in the presence of M-CSF and RANKL. PBMCs from chronic liver disease patients with osteopenia resorbed significantly more bone than PBMCs from healthy controls and chronic liver disease patients without osteopenia under the same culture conditions. PBMCs from chronic liver disease without osteopenia did not resorb significantly more bone than PBMCs from healthy controls (Figure 3B). Again, no correlations were found between the area of

bone resorbed and serum levels of testosterone, estrogen, 1,25-OH vitamin D, albumin, liver enzymes or the use of low levels of cortisone in chronic liver disease patients. A correlation was found between the bone resorption results and the bone density of patients (Figure 3C), indicative of the fact that OCPs in peripheral blood from chronic liver disease patients with osteopenia are functionally different when compared to chronic liver disease patients without osteopenia.

Discussion

Metabolic bone disease is a common complication of chronic liver disease. The underlying molecular mechanisms are not understood. In the present study we investigated whether differences exist in peripheral blood mononuclear cell constitution or priming in chronic liver disease patients with, compared to patients without osteopenia.

When peripheral blood mononuclear cells were cultured from osteopenic chronic liver disease patients in the presence of M-CSF and RANKL, more osteoclast-like cells (Figure 2A), which resorbed more bone (Figure 3B) were cultured, compared to non-osteopenic chronic liver disease patients or healthy controls. Importantly, the increased osteoclast number (Figure 2B) and activity (Figure 3C) correlated inversely with the lumbar bone density (g/cm^2), indicating that the circulating osteoclast precursors could be responsible for increased bone loss.

Previous studies have found an imbalance of RANKL and OPG serum levels in chronic liver disease patients [19, 20]. In the present study we could not confirm this finding. Instead mRNA expression of RANKL and OPG in total peripheral blood mononuclear cells did not differ between the groups. RANKL and OPG detection by Elisa in serum was similar between the groups confirming no differences for either of these molecules on a circulating protein level. Importantly, no RANKL was detectable by means of FACS analysis on T lymphocytes,and,no significant differences in the activated T lymphocyte populations between the three groups were found. This could suggest that liver disease associated osteopenia, in contrast to other immune mediated bone loss states [6,11], is not associated with RANKL expression on activated T-cells. TNF-α which has also been associated bone loss in chronic inflammatory diseases was also not found to be different at the mRNA or circulating protein level between the groups.

M-CSF is needed for the survival, proliferation and differentiation of monocytes and macrophages [21]. When M-CSF was added to peripheral blood mononuclear cells collected from chronic liver disease patients, no further increase in osteoclast-like cell formation was observed, however the addition of M-CSF to peripheral blood mononuclear cells obtained from healthy controls increased the formation of osteoclast-like cells. These results taken together with the increase in circulating plasma levels of M-CSF observed in both chronic liver disease groups suggest that priming with higher levels of M-CSF in the circulation may be responsible for the "spontaneous" formation of osteoclast-like cells observed. The increased M-CSF plasma

levels observed in the peripheral circulation is not produced by the peripheral blood mononuclear cell population as reflected by the results of the RT-PCR. Instead we speculate that M-CSF produced by the inflamed liver environment in chronic liver disease states and in particular the hepatic stellate cell population, may be responsible for the priming of the peripheral blood mononuclear cells [22]. Why this phenomenon selectively occurs in some individuals needs to be further elucidated by looking for candidate genes in larger population co-horts.

There was no significant increase in the spontaneous formation of osteoclast-like cells when comparing chronic liver disease patients without osteopenia to healthy controls (Figure 1B). Also, peripheral blood mononuclear cells from chronic liver disease patients without osteopenia did not form significantly more osteoclast-like cells with the exogenous addition of M-CSF compared to peripheral blood mononuclear cells from healthy controls (Figure 1C). It appears that upon culturing of peripheral blood mononuclear cells without the addition of any cytokines (Figure 1B) that two distinct populations of patients can be recognised within the chronic liver disease without osteopenia group. Some individuals clearly show more osteoclasts-like cell formation, whereas others show a lower level of osteoclastogenesis. This suggests that patients with a higher inclination to develop osteoclasts-like cells may be at higher risk to develop osteopenia in future. This parameter may have the potential to serve as a marker to identify chronic liver disease patients at risk to develop osteopenia in future.

The "spontaneous" formation of osteoclast-like cells from human peripheral blood mononuclear cells in individuals suffering from diseases associated with chronic inflammation and bone loss, which appears to be independent of exogenous RANKL, is not uncommon. Numerous studies from various groups have reported similar findings [11,14,23,24,25]. Ritchlen *et al.* showed a "spontaneous" increase in the formation of osteoclasts from peripheral blood mononuclear cells in patients with psoriatic arthritis [11] and Roato *et al.* showed similar findings with peripheral blood mononuclear cells from cancer patients with bone involvement [24]. The regulation of bone turnover is a complex process which is not yet completely understood and involves numerous cytokines. An alteration in cytokine production in patients with chronic inflammatory conditions can have a major impact on bone. The fact that there is an increase in the "spontaneous" formation of osteoclast-like cells of chronic liver disease both with and without osteopenia and the fact that both these patient groups have increased levels of circulating M-CSF suggests that this cytokine may play an important role in bone loss associated with chronic inflammatory conditions. To our knowledge this is the first study to show that M-CSF could play an important role in the priming of osteoclast precursor cells within the circulation.

The "spontaneously" formed osteoclast-like cells in our study did not appear to be capable of bone resorption in the four week culture week period. Only peripheral blood mononuclear cells, cultured with the exogenous addition of M-CSF and RANKL, resorbed bone. Peripheral blood mononuclear cells from chronic liver disease patients with osteopenia formed significantly more

osteoclast-like cells and resorbed more bone than control groups. These results show a striking correlation with the bone density of the patients included in the study (Figure 4B). These results together with the "spontaneous" osteoclastogenesis data seem to indicate that there is at least one (M-CSF), if not more altered cytokine pathways not covered by our study responsible for osteopenia observed in chronic liver disease patients.

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Table 1: Primers.

Primer	Sequence 5' --> 3'	Amplicon length (bp)	Ensembl Gene ID
PBGD:		84	ENSG00000113721
Forward	TgCAgTTTgAAATCATTgCTATgTC		
Reverse	AACAggCTTTTCTCTCCAATCTTAgA		
RANKL:		60	ENSG00000120659
Forward	CATCCCATCTggTTCCCATAA		
Reverse	GCCCAACCCCgATCATg		
RANK:		67	ENSG00000141655
Forward	CCTggACCAACTgTACCTTCCT		
Reverse	ACCgCATCggATTTCTCTgT		
OPG:		100	ENSG00000164761
Forward	CTgCgCgCTCgTgTTTC		
Reverse	ACAgCTgATgAgAggTTTCTTCgT		
M-CSF:		100	ENSG00000184371
Forward	CCgAggAggTgTCggAgTAC		
Reverse	AATTTggCACgAggTCTCCAT		
TNF-α:		103	ENSG00000111956
Forward	CCCAgggACCTCTCTCTAATCA		
Resverse	GCTTgAgggTTTgCTACAACATg		

Table 2: Patient characteristics.

	CLD - :	HC - :	CLD + :	HC + :
Gender & Age Distribution:				
Number of Females	7	7	7	7
Number of Males	3	3	3	3
Mean Age	44	44	50	49
Range	27 - 69	18 - 67	18 - 64	18 - 67
Type of Liver Disease:				
Primary Biliary Cirrhosis	4	0	5	0
Primary Sclerosing Cholengitis	3	0	1	0
Autoimmune Hepatitis	3	0	4	0

Table 3 : Percentage of activated T lymphocytes and monocytes in patients and controls.

	CD3+/CD25+ (% Total PBMC)	CD14+/CD11b+ (% Total PBMC)
HC	11.07 (+/- 7.10)	16.11 (+/- 7.64)
CLD-	10.91 (+/- 6.05)	15.66 (+/- 7.64)
HC	11.14 (+/- 7.29)	17.22 (+/- 7.60)
CLD+	12.18 (+/- 5.26)	18.89 (+/- 11.80)
CLD-	10.91 (+/- 6.05)	15.66 (+/- 7.64)
CLD+	12.18 (+/- 5.26)	18.89 (+/-11.80)

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Table 4: Base line expression of osteoclastogenesis genes.

	RANKL	RANK	OPG	M-CSF	TNF-α
HC	1.00 (+/-0.78)	1.00 (+/-0.32)	ND	1.00 (+/-0.51)	1.00 (+/-0.64)
CLD -	0.76 (+/-0.75)	0.96 (+/-0.71)	ND	1.22 (+/-1.32)	1.30 (1.24+/-)
HC	1.00 (+/-0.74)	1.00 (+/-0.23)	ND	1.00 (+/-0.50)	1.00 (+/-0.54)
CLD +	0.72 (+/-0.47)	0.91 (+/-0.47)	ND	0.82 (+/-0.48)	0.89 (+/-0.47)
CLD -	0.76 (+/-0.75)	0.96 (+/-0.71)	ND	1.22 (+/-1.32)	1.30 (1.24+/-)
CLD+	0.72 (+/-0.47)	0.91 (+/-0.47)	ND	0.82 (+/-0.48)	0.89 (+/-0.47)
CT Range	26.77 – 34.49	28.15 – 31.78	>40 cycles	26.29 – 30.04	26.54 – 32.83

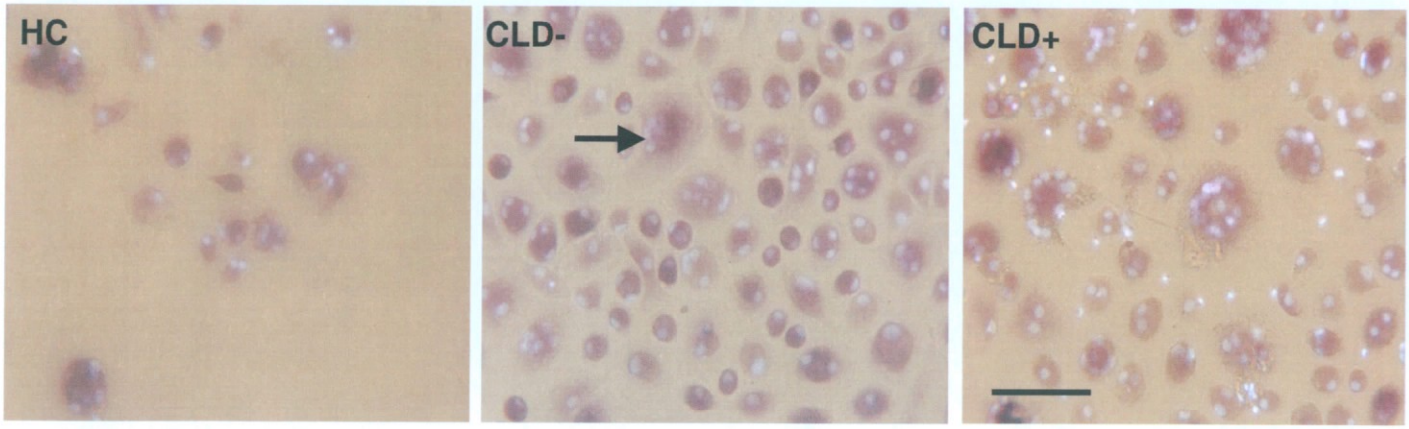

Figure 1A

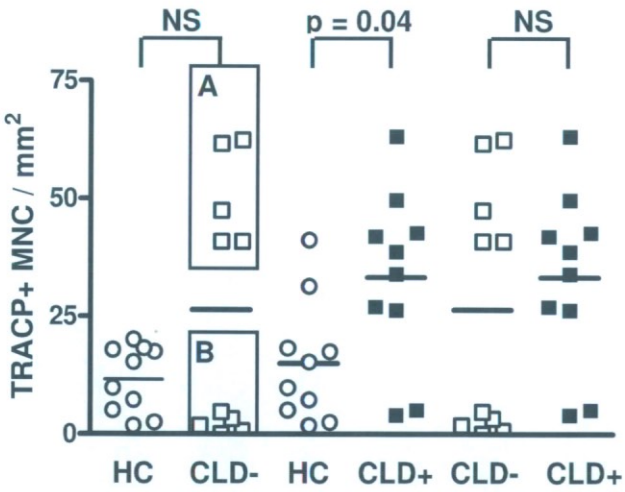

Figure 1B

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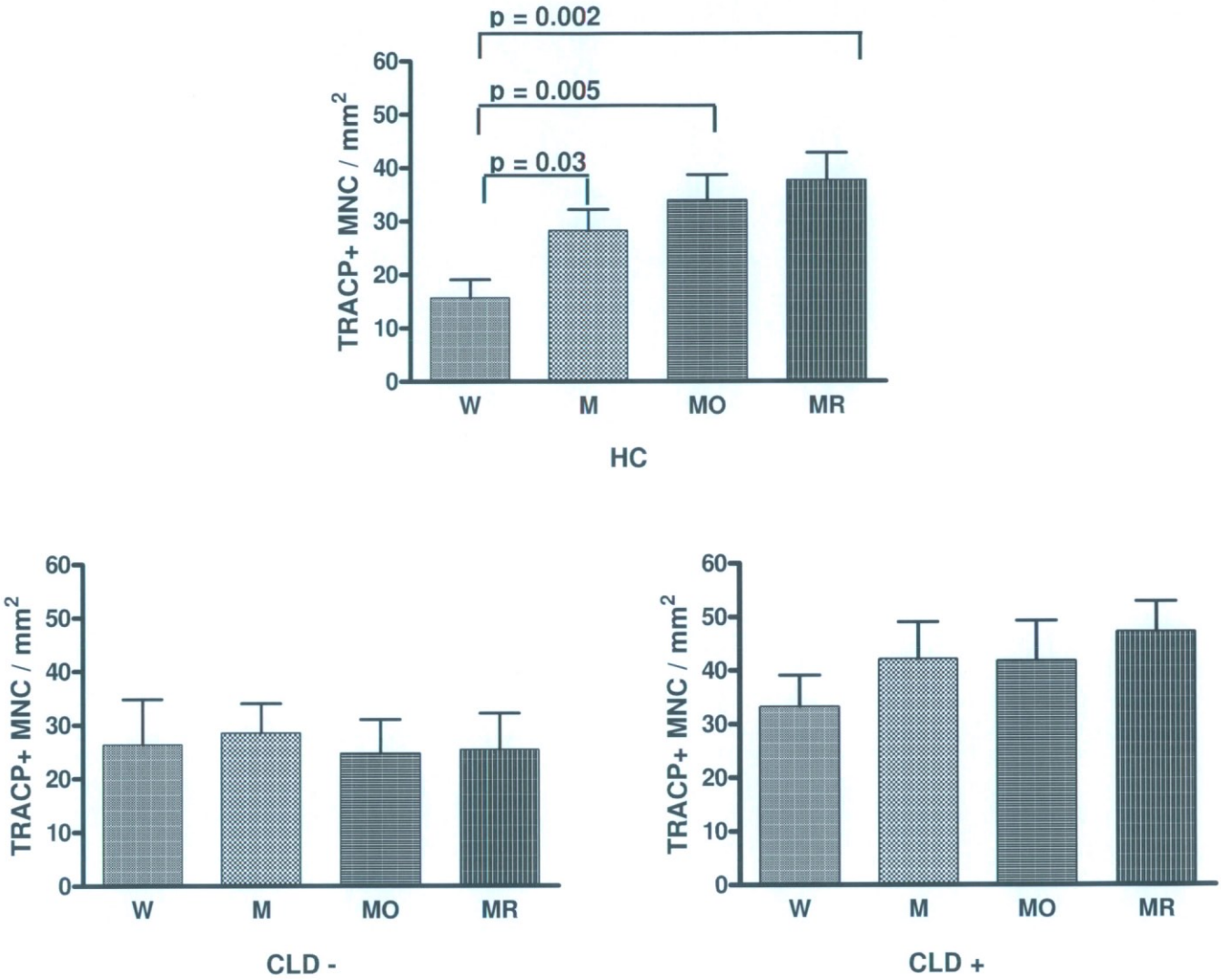

Figure 1C

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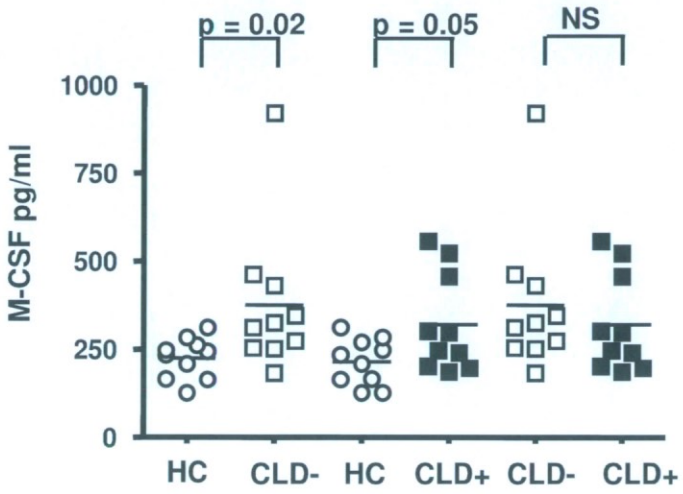

Figure 1D

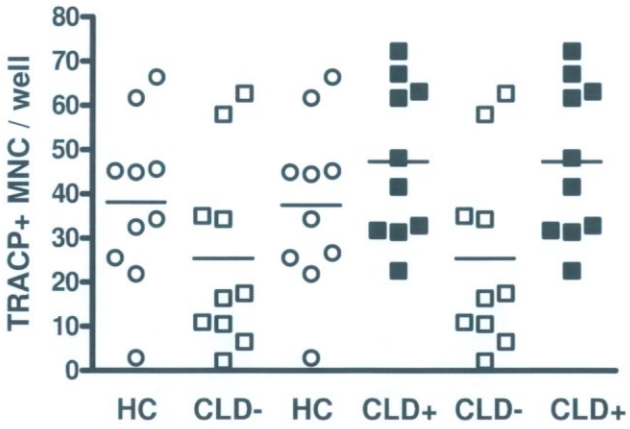

Figure 2A

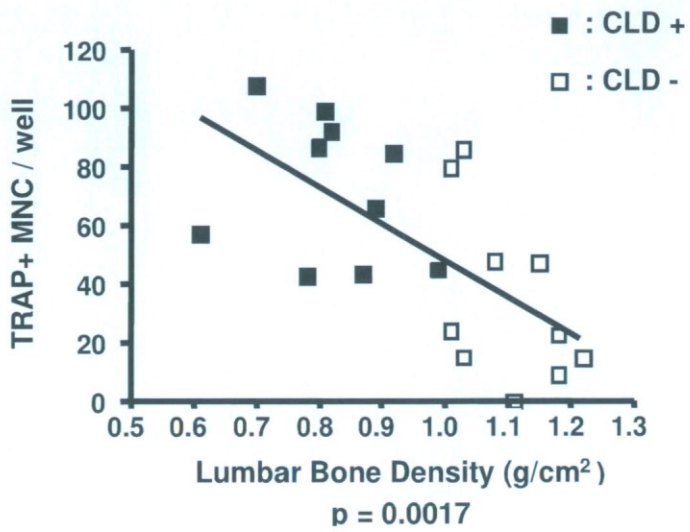

Figure 2B

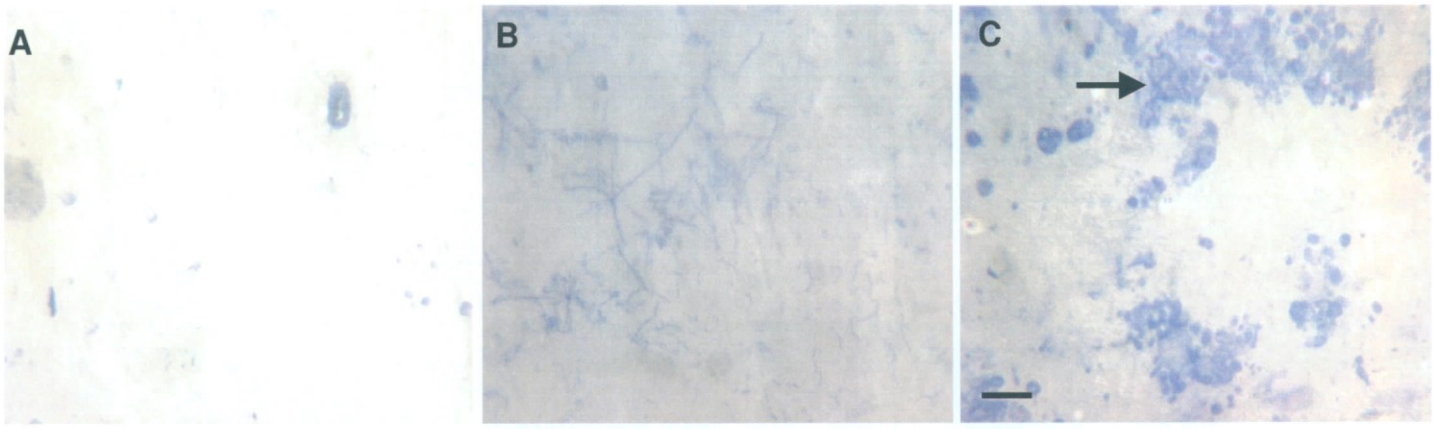

Figure 3A

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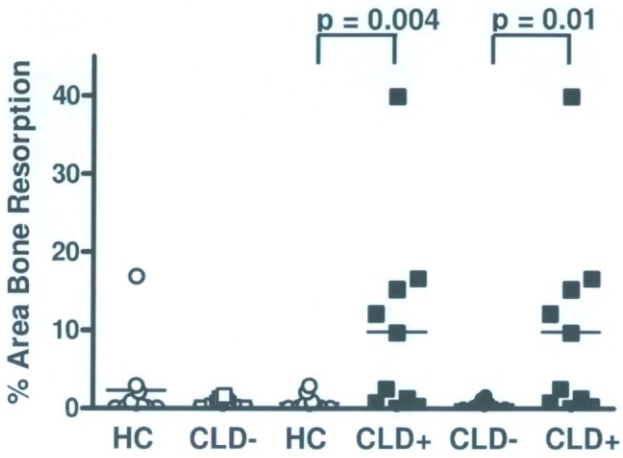

Figure 3B

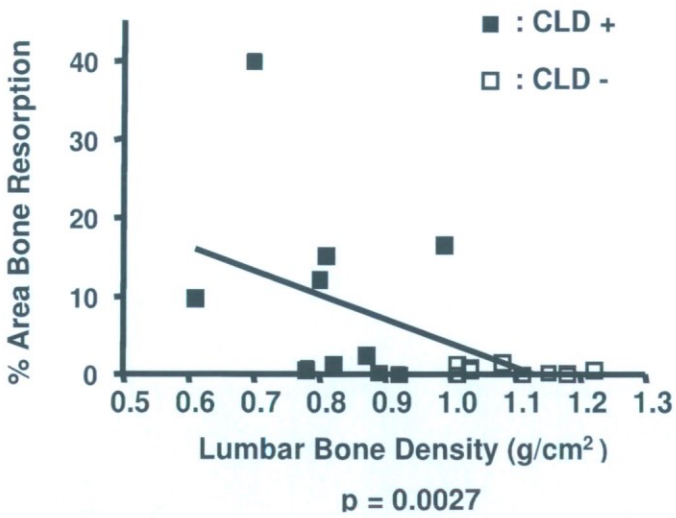

Figure 3C

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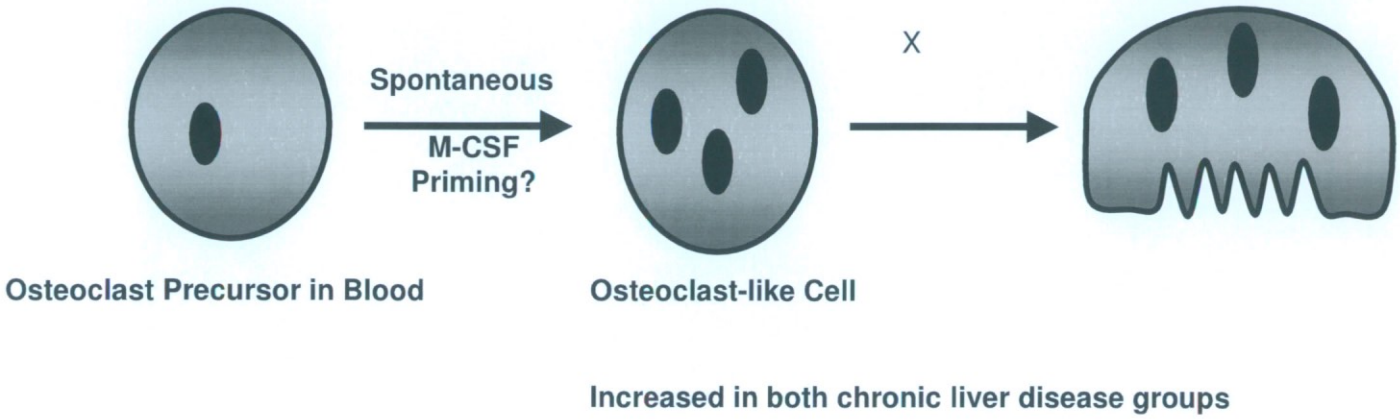

Figure 4A

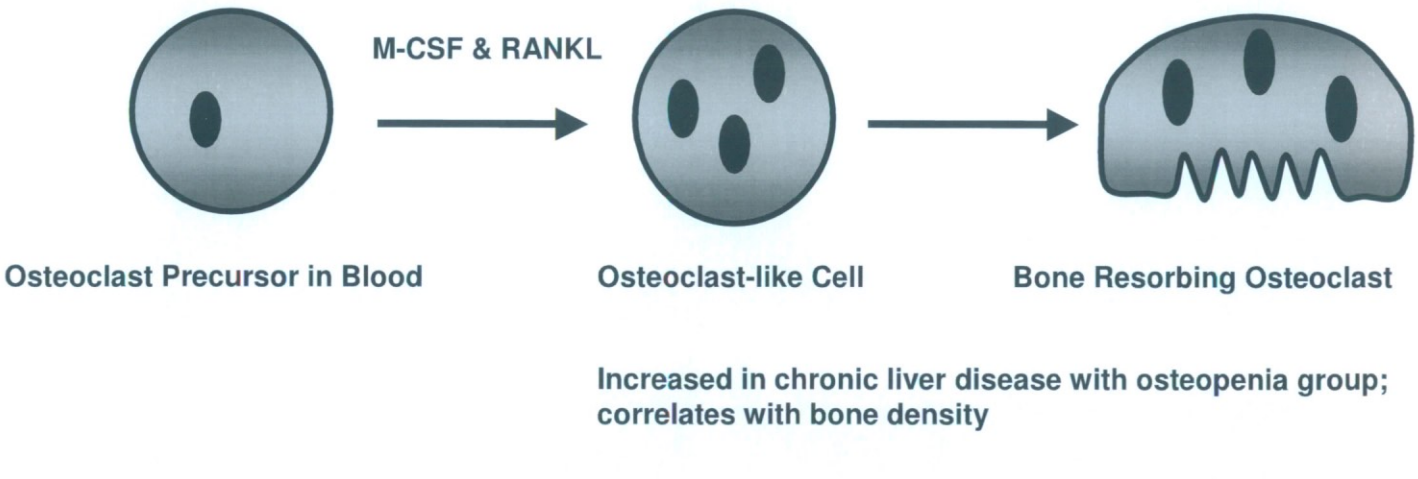

Figure 4B

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Table Legends:

Table 1 – Primers used for real time RT-PCR. Porphobilinogen deaminase PBDG was used as a housekeeping gene and Ensembl Gene ID (http://www.ensembl.org) is given for each gene.

Table 2 – Age and gender distributions for chronic liver disease patients without osteopenia (CLD -), their matched healthy controls (HC-) and chronic liver disease patients with osteopenia (CLD +) and corresponding HC+ included in the study, along with the type of cholestatic liver disease.

Table 3 – Results obtained from FACS analysis of total peripheral blood mononuclear cells (PBMCs), the mean of the group is given as a percentage of total PBMCs with the standard deviation given in brackets. None of the comparisons made were statistically significant ($p < 0.05$).

Table 4 - Expression of the relevant cytokines and their receptors as determined by real time RT-PCR performed on RNA isolated from total PBMCs at baseline. Results are given as a relative quantity with the mean per group and the standard deviation obtained indicated in brackets. None of the comparisons made were statistically significant ($p < 0.05$).

Figure Legends:

Figure 1A - Representative micrographs of tartrate resistant acid phosphatase (TRACP) staining when 1 x 10^6 total PBMCs were cultured in tissue culture plates without the addition of cytokines for three weeks. Arrows highlight typical examples of TRACP+ multinucleated cells. Micrographs clearly show the increased "spontaneous" formation of osteoclast-like cells in chronic liver disease patients. HC: healthy controls, CLD-: chronic liver disease patients without osteopenia and CLD+: chronic liver disease patients with osteopenia. Bar = 100 μm.

Spontaneous Osteoclastogenesis

Figure 1B – Higher number of osteoclast-like cells formed when culturing 1 x 10^6 total PBMCs of chronic liver disease patients with osteopenia without the addition of any cytokines for a three week culture period. The chronic liver disease group without osteopenia does not appear to form more osteoclast-like cells than healthy controls (NS = not significant). Possibly, the chronic liver disease without osteopenia consists of two subgroups with one of the subgroups having an increased potential to develop osteopenia over time. Subgroup A – individuals which form more TRAP+ multinucleated cells and could present with osteopenia in time and Subgroup B – individuals which form less TRAP+ multinucleated cells.

Figure 1C – Number of TRACP+ multinucleated cells formed from healthy controls (HC), chronic liver disease patients without osteoporosis (CLD-) and chronic liver disease patients with osteoporosis (CLD+) under different culture

conditions – without the addition of cytokines (W), M-CSF alone (M), M-CSF and OPG (MO), M-CSF and RANKL (MR). The number of osteoclast-like cells formed from 1 x 10^6 total PBMCs of healthy controls after a three week culture period significantly increased with the addition of M-CSF. This was not true for PBMCs from chronic liver disease patients.

Figure 1D - Plasma levels of M-CSF (pg/ml) from individuals included in the study as determined by means of ELISA. M-CSF levels of both chronic liver disease patients groups were higher than their healthy controls. NS = not significant.

Osteoclastogenesis after 3 week culture period with the addition of M-CSF and RANKL

Figure 2A - Number of TRACP+ multinucleated cells formed when culturing 1 x 10^6 total PBMCs in the presence of M-CSF and RANKL. PBMCs from chronic liver disease patients with osteopenia form more osteoclast-like cells than either control groups. NS = not significant.

Figure 2B - Correlation between the number of TRAP+ multinucleated cells formed (when culturing PBMCs with the addition of M-CSF and RANKL) and the bone densities of patients. Bone densities of patients correlated with the number of osteoclast-like cells formed in culture (r^2 = 0.4251, df = 18 and p = 0.0017).

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Bone Resorption

Figure 3A – Representative micrographs of bone resorption results obtained when culturing PBMCs with the addition of M-CSF and RANKL. Healthy controls (A), chronic liver disease patients without osteopenia (B) and chronic liver disease patients with osteopenia (C). Bar = 100 µm.

Figure 3B – Increased bone resorption by osteoclasts derived from PBMCs of chronic liver disease patients with osteopenia when cultured with M-CSF and RANKL. 1×10^{6} total PBMCs of chronic liver disease patients resorbed more bone than either of the control groups over a four week culture period. HC: healthy controls,

CLD -: chronic liver disease patients without osteopenia and CLD + : chronic liver disease patients with osteopenia. . NS = not significant.

Figure 3C – Correlation between the percentage of bone resorption and bone densities of chronic liver disease patients. Bone densities of patients correlated with the percentage area of bone resorbed by osteoclasts derived from PBMCs of chronic liver disease patients in culture ($r^2 = 0.2890$, df = 18 and $p = 0.0027$).

Summary

Figure 4A – Overview diagram illustrating the increased spontaneous formation of osteoclast-like cells from peripheral blood of chronic liver disease patients. This could be due to an increased priming of circulating osteoclast precursors in the periphery with higher levels of M-CSF compared to precursors from healthy controls. The spontaneously formed osteoclast-like cells are not capable of bone resorption.

Figure 4B – Overview diagram illustrating the increased formation of osteoclasts from PBMCs ,cultured with the addition of M-CSF and RANKL, of chronic liver disease patients with osteopenia . The osteoclast-like cells formed were capable of bone resorption, which was significantally increased when compared to control groups. Both the results obtained for the formation of osteoclasts and their functional capabilities i.e. bone resorbtion showed a correlation with the bone densities of the patients.

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