

# **EMD/amelogenin stimulates resistin expression and secretion**

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## **Abstract**

Enamel matrix derivative (EMD: Emdogain®) is well recognized in periodontology where it is used as a local adjunct to periodontal surgery to stimulate regeneration of periodontal tissues lost to periodontal disease. The biological effect of EMD is through stimulation of local growth factor secretion and cytokine expression in the treated tissues, inducing a regenerative process that mimics odontogenesis.

In this study we want to identify the effect of EMD on the newly discovered adipokine/cytokine resistin, and try to identify the bioactive fraction of EMD.

Normal human osteoblasts (NHO), mesenchymal stem cells (MSC) and monocytes were incubated with EMD at various concentrations ranging from 10-100 µg/ml and harvested after 6 hours, 1, 2, 3, and 7 days. EMD were divided in 13 fractions on a size exclusion column. The effect of each fraction, containing material equivalent to the content found in 10 µg/ml EMD, were tested on NHO cells after 1, 2 and 3 days incubation and compared to the effect of EMD (10 µg/ml) and untreated cells.

2 mg EMD was injected intramuscular (gluteus maximus) of rats every 3th day for 14 days. Resistin levels in blood samples were measured after 1, 3, 6 and 14 days.

The levels of resistin in cell culture medium were measured by Luminex, an immunobased fluorescent technique, and the level of resistin in rat plasma was measured by the Rat resistin ELISA kit (UM-104101). Further, the total protein content and ALP activity in the cell culture medium were measured.

In NHO and MSC cells treated with EMD, the resistin concentration in cell culture medium was highly increased compare to untreated cells. We were not able to identify a specific EMD fraction causing the same increase in resistin as EMD. We observed no significant changes in resistin levels in monocytes treated with EMD.

In rats injected with EMD intramuscularly, we found a significant increase in circulating resistin levels, compared to rats receiving saline.

Our results indicate that cells of mesenchymal origin respond to EMD with increased resistin secretion, whereas cells of hematopoietic origin do not.

## **Introduction**

### ***EMD***

Emdogain, a mixture of porcine developing enamel proteins [Gestrelus S et al., 2000], composed mainly of amelogenins (90%)[Carinci et al., 2006], with a molecular mass distribution similar to that of the porcine secretory enamel, ranging from 10 to 40 kD were amelogenins with the majority of components at 20 kD [Maycock et al., 2002]. The remaining 10% is composed of non-amelogenin enamel matrix proteins such enamelin, tuftelin, amelin and ameloblastin [Carinci et al., 2006]. Proteolytic activity, both metalloendoprotease and serine protease activity were present within Emdogain [Maycock et al., 2002]. A previous report found that EMD prepared from developing porcine teeth possesses bone morphometric protein (BMP) - like and tumour growth factor (TGF)- $\beta$ -like activities. EMD contains, beside amelogenin, growth factors like BMP2/4, but not TGF- $\beta$  [Saito et al., 2008].

### ***EMD and periodontal regeneration***

EMD, marketed as Emdogain, is now used therapeutically to treat periodontal disease in humans by periodontal regeneration [Brookes et al., 2005]. It enhance bone formation and osteoblast cell proliferation, and promote the regeneration of non-cellular cementum, periodontal ligament, and alveolar bone in treating periodontal defects [Du et al., 2005]. EMD stimulate the regeneration of the mineralized tissues of the periodontal attachment apparatus in part though the upregulation of Cyclooxygenase-2 (COX-2) and possibly osteoprotegrin (OPG) and the downregulation of Receptor Activator for Nuclear Factor  $\kappa$ B Ligand (RANKL) [Takayanagi et al., 2006].

BMP2/4 induces differentiation of osteoblasts and belongs to the TGF- $\beta$  family of proteins that acts as a potent osteogenic morphogens capable of inducing calcification in animal models. BMP 2 induces the phosphorylation of Sma- and Mad related proteins (SMAD), proteins that regulate the activity of TGF- $\beta$  ligands and have an important signalling pathway in calcification. Saito et al., (2008) found that the phosphorylation of SMADs 1/5/8 was not induced by amelogenin alone, but rather by the whole EMD, indicating that amelogenin does not inhibit BMP signalling.

Recent studies are reporting that amelogenins also can interact directly with cell types other than cementoblasts, suggesting that these molecules have a more direct role in the regrowth of mesenchymal tissues [Lyngstadaas et al., 2009]. These studies also show that EMD and

amelogenin enhance the expression of tissue-specific maturation markers, such as alkaline phosphatase (ALP), collagen, and osteocalcin (OC) within osseous tissues. A secondary stimulation of osteoclast activity is also evident through increased secretion of proteins such as IL-6 and OPG [Lyngstadaas et al. 2009].

### ***Amelogenin***

Amelogenin comprise a family of highly conserved extracellular matrix protein derived from one gene [Lau et al., 1989]. Amelogenin and ameloblastin were also detected in Hertwig's epithelial root sheath, an enamel free tissue, during rat molar formation [Brookes et al., 2005]. The amelogenins are known to self-assemble into supramolecular aggregates that form an insoluble extracellular matrix [Fincham et al. 1994] with high affinity for hydroxyl apatite and collagens [Gestrelus et al. 1997].

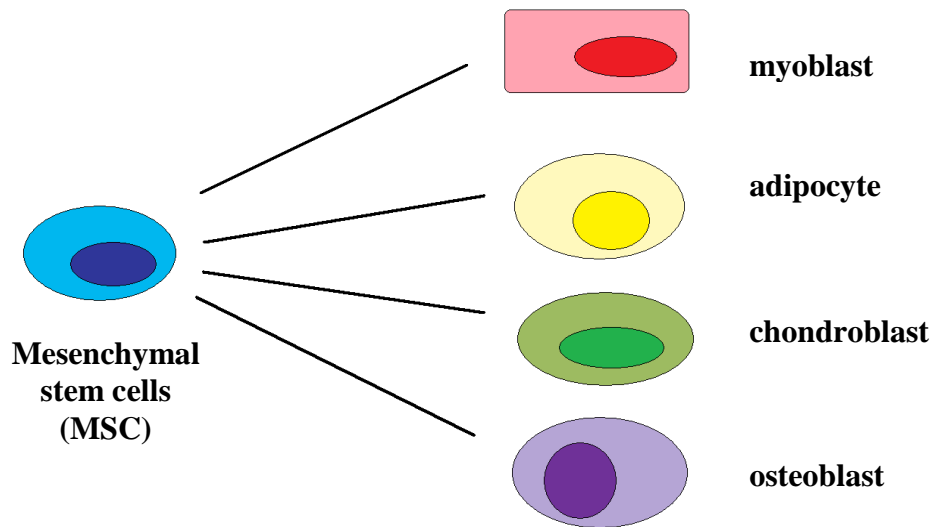
The potential biological activity of amelogenins is not just restricted to dentally related tissues. Alternatively spliced amelogenin variants placed in intramuscular implants have been shown to induce mineralization. The splice variants in question are commonly referred to as leucine rich amelogenin peptides (LARPs) [Brookes et al., 2005]. LARPs have been shown to be osteogenic and chondrogenic in vitro [Brookes et al., 2005].

Amelogenin has also been shown to suppress cultured osteoclast formation and the expression of RANKL, which is a pivotal factor for bone resorption [Yagi et al., 2009]. Amelogenin peptides have been shown to be osteoinductive in other systems. In addition, the self-aggregating properties of the amelogenins under physiological conditions present a possible slow-release vehicle for any potential active component in vivo. [Maycock et al., 2002]

### ***Bone modelling and remodelling***

The skeleton is a metabolically active organ that undergoes continuously remodelling throughout life [Tenta et al., 2010]. Remodelling of bone is important not only for maintaining bone mass, but also to repair microdamage and for mineral homeostasis [Tenta et al., 2010]. Bone remodelling and metabolism are based on self-regulating cellular events that occur through the coupling of bone formation by osteoblasts with bone resorption by osteoclasts [Livshits, 2006]. Osteoblasts origin from pluripotent mesenchymal stem cells (MSC) which are able to differentiate into a number of cell types, including chondroblasts, adipocytes, myoblasts, fibroblasts and  $\beta$ -pancreatic cells. Osteoblasts produce and secrete the major part of the organic bone matrix, which in a well-regulated process becomes calcified to form mineralized bone [Aubin, 2001]. Osteoblasts express receptors for number of cytokines

and hormones which exert their effects on bone metabolism by regulating osteoblast production of OPG and RANKL [Greenfield et al., 1999].



*Mesenchymal progenitor cells (MSC) have the ability to differentiate to odontoblasts, myocytes, adipocytes and chondrocytes. (From Harada & Rodan Nature 2003; 423, 349-355)*

Osteoclasts are multinuclear cells originating from granulocyte hematopoietic stem cells in bone marrow [Parfitt, 1984]. Osteoclasts are responsible for bone resorption by dissolving the mineral and the bone matrix [Teitelbaum, 2000].

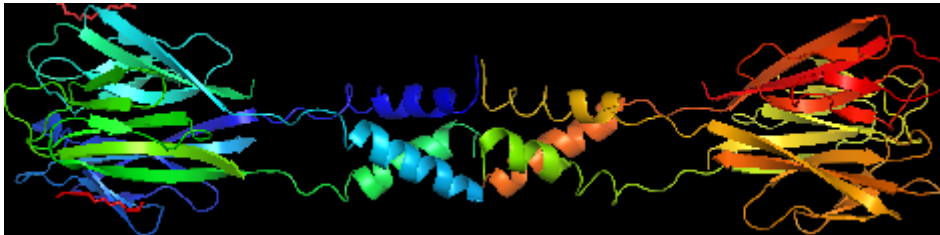
Molecular regulators of bone metabolism include OPG, RANKL, COX-2, and core binding factor alpha 1/Runt-related transcription factor 2 (Cbfa1/Runx2). COX-2 is thought to be an important mediator of both bone formation and resorption. COX-2-stimulated bone formation has been associated with the induction of Cbfa1. Runx2 is an important transcription factor for the initiation and modulation of osteoblastic differentiation [Takayanagi et al., 2006]. RANKL is expressed by osteoblasts and stromal cells, and is the vital signal for osteoclast formation and activation by binding to the RANK receptor on osteoclast/osteoclast precursors [Teitelbaum, 2000]. A decoy receptor for RANKL, OPG, is produced by osteoblastic/stromal cells and act by preventing ligand binding to RANK. Thereby, OPG exerts a negative regulation in osteoclastogenesis, promotes apoptosis of mature osteoclasts, and ultimately inhibits bone resorption [Ferrari-Lacraz and Ferrari, 2009].

RANKL/OPG mRNA ratio was decreased in PDL cells treated with EMD, which is consistent with decreased osteoclastic activity [Takayanagi et al., 2006].

Alkaline phosphatase (ALP) is a basic phosphatase, and it causes release of phosphate ions when activated. An increase in phosphate ions rise pH and it thus favourable for bone mineralization. Hence, ALP activity is used to evaluate bone matrix formation [Johnson et al., 2000]

### ***Resistin***

Resistin is a newly described 12.5-kDa adipokine that is a member of a cysteine-rich secretory protein family [Bokarewa et al., 2005].



*Crystallographic structure of a hexamer of mouse resistin (rainbow colored, N-terminus = blue, C-terminus = red). [Patel et al., 2004]*

Recent studies in humans suggest that very little resistin is expressed in adipocytes [Furugen et al., 2008], whereas high levels are expressed in mononuclear leukocytes, macrophages, spleen, and bone marrow cells. Low levels of resistin are also expressed in lung tissue, resting endothelial cells, and in placenta [Bokarewa et al., 2005].

### ***Resistin and inflammation***

An abundance of resistin in peripheral blood mononuclear cells and macrophages, suggest an important role of resistin in the process of inflammation [Furugen et al., 2008]. Release of resistin from the neutrophil granules probably serves the main source of resistin at the site of inflammation [Boström et al., 2009], there is associations between resistin and inflammatory factors, such as tumour necrosis factor (TNF)- $\alpha$ , Interleukin (IL)-6 and C-reactive protein [Zhang et al., 2010]. It has previously been shown that the Nuclear Factor-KappaB (NF- $\kappa$ B) transcription pathway mediates the stimulatory effect of resistin on cytokine release [Bokarewa et al., 2005], there both oligomeric and dimeric forms of resistin can promote the synthesis and secretion of TNF- $\alpha$  and IL-12 in macrophages, suggesting that the involvement of resistin in inflammation is independent of its conformation [Zhang et al., 2010]. It has also been demonstrated that resistin upregulates intracellular adhesion molecule-1 (ICAM1), vascular cell-adhesion molecule-1 (VCAM1) and Chemokine ligand 2 (CCL2), all of which are occupied in chemotactic pathways involved in leukocyte recruitment to sites of infection [Verma et al., 2003]. Localization of resistin in the neutrophils, the first cells engaged in inflammation, may provide an important mechanism of recruitment and activation of other

immunocompetent cells such as lymphocytes, monocytes and dendritic cells [Boström et al., 2009].

Similar to pro-inflammatory cytokines, resistin could significantly upregulate the expression of COX-2 when it was overexpressed in macrophage cells [Zhang et al., 2010].

Increased resistin levels caused by periodontal inflammation may mediate the relationship between periodontitis and cardiovascular disease [Furugen et al., 2008].

### ***Resistin and bone metabolism***

Resistin acts on skeletal muscle myocytes, hepatocytes, and adipocytes reducing their sensitivity to insulin, and, has therefore, been suggested to be a molecular link between obesity and type 2 diabetes [Steppan et al., 2001]. The underlying belief among those in support of this theory is that serum resistin levels will increase with increased adiposity. [Steppan et al., 2001]. Conversely, serum resistin levels have been found to decline with decreased adiposity following medical treatment [Valsamakis et al., 2004].

It is known that body fat is positively correlated with increased bone mineral density and decreased fracture risk [Reid, 2002]. Resistin may affect bone metabolism via several mechanisms, where the two main actions seem to be the enhancement of osteoclast differentiation and the recruitment of osteoblasts [Thommesen et al., 2006]. The highest expression of resistin amongst bone cells was found in the early differentiation stages of human peripheral blood mononuclear cells (PBMC) or monocytes into osteoclasts. This is in accordance with studies demonstrating that cells from the monocyte/macrophage lineage are the predominant source of circulating resistin in humans [Kaser et al., 2003]. The resistin receptor is not yet described, however resistin itself is expressed in murine and human osteoblast and osteoclasts, and increases differentiation and NFκB activity in osteoclasts, the observed increase in resistin during osteoclast differentiation could stimulate, in turn, the osteoblast recruitment, indicating all together a potential role for resistin in bone metabolism and remodelling [Thommesen et al., 2006].

## **Materials and methods**

### ***Cell cultures***

The human osteoblast (NHOst cell system) and mesenchymal stem cells (MSC) were obtained from Cambrex BioScience (Walkersville, MD, USA). NHO were grown in Osteoblast Basal Media (OBM, Cambrex BioScience), containing ascorbic acid, foetal calf serum and gentamycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cultured osteoblasts were exposed to hydrocortisone hemisuccinate (200 nM) and β-glycerophosphate (10 nM) in ambient medium to facilitate differentiation and mineralization.

Human peripheral mononuclear cells (PBMC) were isolated from peripheral blood from 3 healthy donors using a Percoll density gradient. Cell culture media from EMD treated cells were a gift from Sofia Almquist, Goteborg University, Sweden.

### ***Size Exclusion Chromatography***

Freeze dried Emdogain was provided by Straumann (Basel, Switzerland). Twenty milligrams of Emdogain were dissolved in 0.75 mL of 0.125 M formic acid and subjected to size exclusion chromatography using a 90 x 1.6 cm column of Bio Gel P10 (Bio Rad, Hemel Hempstead, UK). The column was eluted with 0.125 M formic acid at a flow rate of 0.3 mL/min. The column eluant was monitored at 280 nm and 5 mL fractions collected.

Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated based on their size. The particles of different sizes will elute (filter) through a stationary phase at different rates. This results in the separation EMD solution in thirteen fractions based on size.

### ***Mechanical load***

Loading was conducted using centrifugation (Allegra TM 21 with a S2096 microplate Rotor). G-values were calculated according to the following formula:  $RCF=1,12r(RPM/1000)^2$ . The radius (r) of the rotor S2096 was 110 mm. RCF corresponds to G-value and RPM is rotations pr minute.

The cells were centrifugated for 30 min at respective G force, followed by incubation at 37°C at 1G for 1 and 3 days before harvesting.



### ***Experimental design***

Briefly, cells were seeded in 24-well trays, 0.5 mL/well. Three parallel experiments were conducted. Cultured 90% confluent cells were treated with growth medium (untreated control), EMD and various fractions of EMD separated in size exclusion column (from Steven Brooks, Leeds). Cell culture media were harvested after 6 hours, 1, 2, and 3 days incubation. The cell well also treated with leptin (50 mg/ $\mu$ l) and recombinant human ameloblastin (AMB) (10 $\mu$ g/ml, 1-1447aa, a gift from Dan Deutsch, institute for dental sciences, Hebrew University, Jerusalem, Israel), factors also known to stimulate resistin secretion.

Rats (n = 12) were divided in 2 groups receiving either saline (control) or 2 mg EMD intramuscular every 3th day for 14 days. Blood samples were collected after 1, 3, 6 and 14 days, and bone marrow from femur were isolated after 14 days.

Wound fluid from a patient before and after undergoing EMD treatment (Xelma) was provided by Mölnlycke (Gothenburg, Sweden).

### ***Measurement of protein in cell culture medium***

Colorimetric detection and quantization of total protein in the samples was performed by BCA Protein Assay kit (Pierce Biotechnology, USA) according to the producer's instructions.

ALP activity was quantified by measuring the hydrolysis of p-nitrophenyl phosphate (pNPP) (Sigma) at 405 nm. Standard curves, constructed using calf intestinal alkaline phosphatase (CIAP) (Promega, Madison, WI, USA), were run in parallel for quantification purposes. The total protein content in the culture medium was determined using Sigma Microprotein PR assay kit with a Protein Standard Solution Calibrator (Sigma Diagnostics St.Louis, USA). Analyses were performed using a Cobas Mira chemistry analyzer (Roche Diagnostics, Germany). Intra-assay and inter-assay variability were less than 2.4 % and 3.2 %, respectively. The assay detection range was 10-2000 mg/l. ALP activity was calculated in terms of nmol of pNPP/min/mg of total protein in each individual sample. The activity was finally expressed as a % of the controls at the different time points.

Multianalyte profiling of the level of cytokines in the cell culture medium of MSC, NHO and monocytes was performed on the Luminex-100 system (Luminex Corporation, Austin, TX). Calibration microspheres for classification and reporter readings as well as sheath fluid were

also purchased from Luminex Corporation. Acquired fluorescence data were analyzed by the STarStation software (Version 2.0; Applied Cytometry Systems, Sheffield, UK). Amount of Resistin and IL- 6 into culture media was measured by Human Adipocyte Lincoplex Kit (Linco, Missouri, US) according to the manufacturer's instructions.

***Rat resistin ELISA kit (UM-104101) B-Bridge***

The detection and quantification of resistin in rat serum was performed by using the B-Bridge Rat Resistin ELISA Kit.

Standards and samples are incubated in microtiter wells coated with a rabbit polyclonal anti-rat resistin antibody. The wells are washed and biotin-labeled rabbit polyclonal anti-rat resistin antibody is added and incubated with the captured resistin. After a thorough wash, streptavidin-horseradish peroxidase (HPR) conjugate is added. Following subsequent incubation, and washing, the bound conjugate is allowed to react with the substrate H<sub>2</sub>O<sub>2</sub>-tetramethylbenzidine (TMB). The reaction is quenched by addition of acidic solution and absorbance of the resulting product is measured at 450 nm. The absorbance correlates to the concentration of resistin standard concentrations and concentrations of unknown samples are determined by interpolation on this curve.

## Results

### *Effect of EMD in vitro*

Upon treatment with EMD, the secretion of resistin to the cell culture medium from MSC and NHO enhanced significantly compared to untreated cells as well as cells treated with another factor known to stimulate resistin secretion (leptin; 50 ng/ml) and another enamel protein (AMB, ameloblastin; 10 µg/ml) (Fig 1).

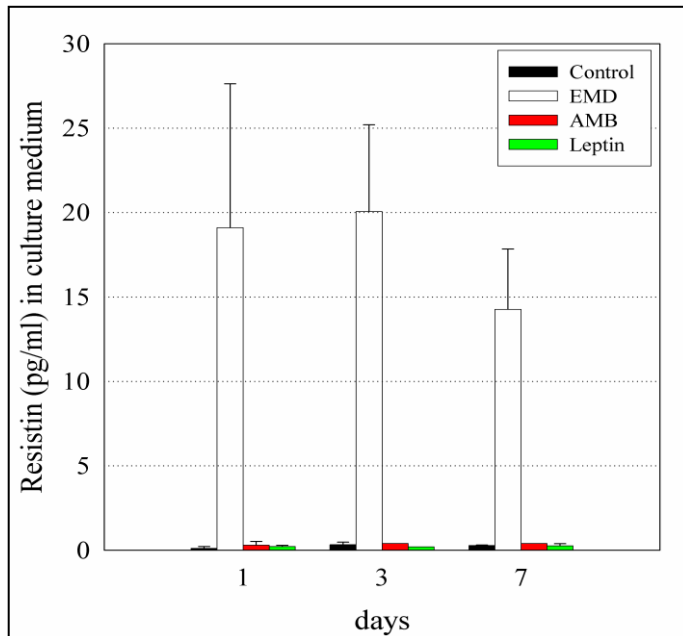


Fig. 1

The concentration of resistin in the cell culture medium of MSC treated with EMD, ameloblastin (AMB) and leptin after 1, 3 and 7 days of incubation. Control is untreated cells at the same time points.

Data is presented as mean ( $\pm$ SD) of 3 different donors.

The secretion of resistin from NHO was also enhanced upon EMD stimulation (data not shown).

It has been shown that amelogenin participates in the maturation and growth of dental pulp cells during tooth formation [Oida et al., 2002]. The major (>95%) component of EMD is amelogenins. Upon treatment with EMD both PULP- and PDL cells enhanced the secretion of resistin to the cell culture medium (Fig. 2).

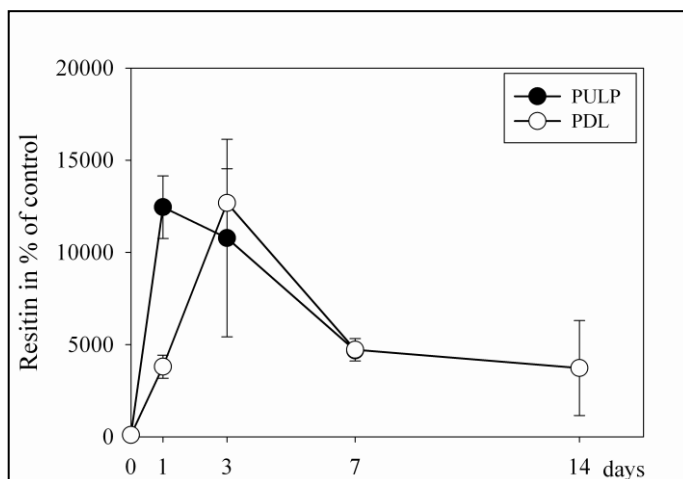
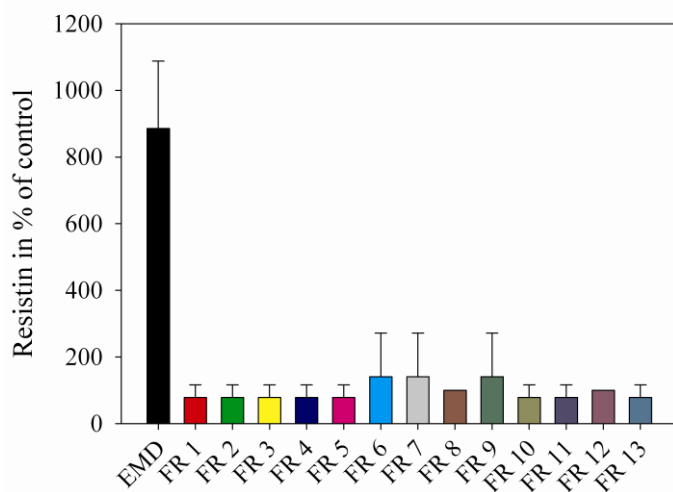


Figure 2

Resistin in cell culture medium from human pulp and periodontal ligament cells (PDL) treated with EMD (50 µg/ml). Resistin levels at each time point were calculated in percentage of the level from untreated cells (control) at the same time points. Results are presented as mean ( $\pm$ SD) for 3 individual experiments for each cell type.

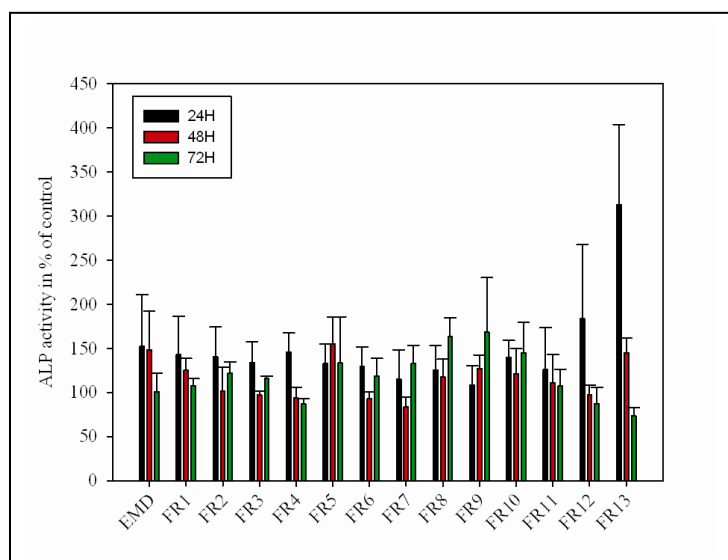
To examine whether EMD fraction could modulate induction of resistin, we compare the effect of EMD (10  $\mu\text{g/ml}$ ), with thirteen fractions of EMD separated on a size exclusion column (Fig. 3). The concentration of each fraction was equivalent to the content in 10  $\mu\text{g/ml}$  EMD.



*Figure 3*  
The effect of EMD and fractions (FR1-13) of EMD on the secretion of resistin from NHO-cells after 72h incubation. Data calculated in % of untreated cells (control = c), and presented as mean ( $\pm$ SEM) of 3 individual experiments.

Although the secretion of resistin was enhanced in MSC, NHO, pulp and PDL cells after exposure to EMD, we were not able to identify one or more active EMD fractions giving the same effect as whole EMD.

ALP, are osteoblast phenotype-related genes associated with extracellular matrix maturation [Lian and Stein, 1992]. Earlier studies show that the effect of EMD on bone formation and enhanced osteoblast maturation was verified by a more than twofold increase in ALP activity into medium [Reseland, 2006].

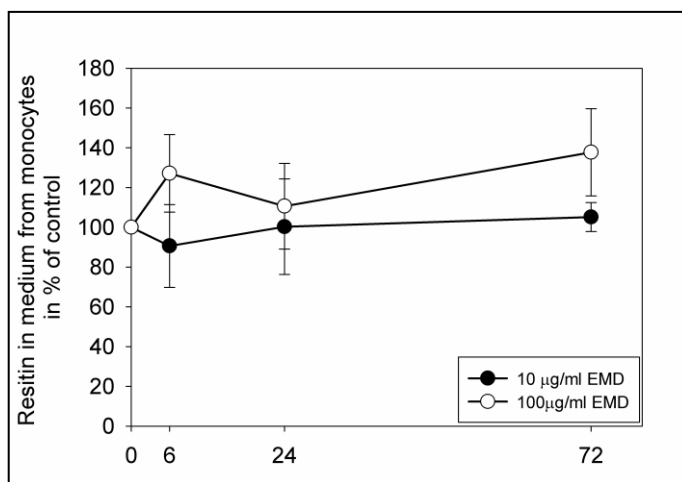


*Figure 8*  
The effect of EMD and fractions (FR1-13) of EMD on the secretion of ALP from NHO-3 cells after 1, 2 and 3 days of incubation. Data calculated in % of untreated cells (control = c), and presented as mean ( $\pm$ SEM) of 3 individual experiments.

NHO-3 cells treated with EMD shows an increase in ALP after 24 and 48h in our study. We found no evidence for the involvement of the fractions (Fr1-FR13) in increasing of ALP, more or less similar picture with fractions as with EMD.

The main source of circulating resistin in the human body is monocytes/macrophages [Furugen et al., 2008]. Monocyte is a type of white blood cell, part of the human body's immune system. Recently, several groups have reported a close relationship between resistin and inflammation [Son et al., 2010]. Therefore, we wanted to examine whether EMD induced an increase in the resistin level in monocytes. Two concentrations of EMD were tested (10 and 100  $\mu\text{g/ml}$ ) for 6, 24 and 72 hours, however we found no significant changes in the secretion of resistin to the cell culture medium (Fig. 4) compared to untreated cells.

Figure 4



Effect of EMD (10,100  $\mu\text{g/ml}$ ) on monocytes after 6, 24 and 72h. Resistin levels were calculated in percentage of control (untreated cells) at each time point. Results are presented as mean ( $\pm$  SEM) from 3 different donors.

Osteoblasts (NHO), pulp and PDL cells are exposed to mechanical forces like load. To investigate whether load in combination with EMD affected the secretion of resistin, PDL cells, with and without EMD (50  $\mu\text{g/ml}$ ) were exposed to different loads (1, 6 and 30G) for 30 min by centrifugation and incubated at 1G for 24 and 71 hours (Fig. 5). EMD enhanced the secretion of resistin significantly after 72 hours in PDL cells; however the EMD induced increase was reduced with increasing load compared to normal gravity (1G).

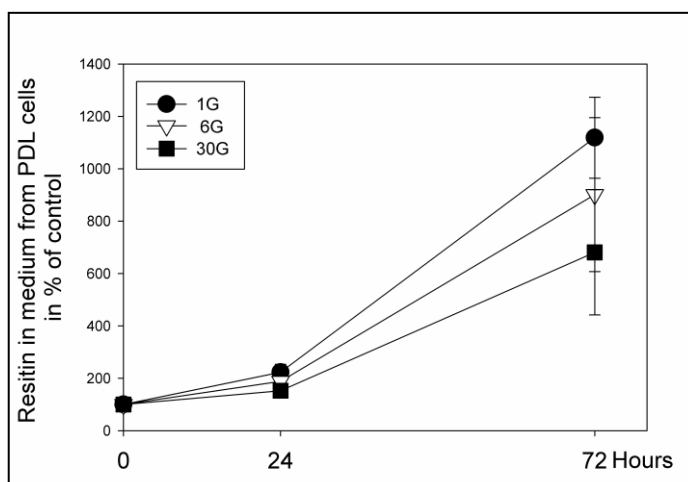


Figure 5  
The effect of EMD and load (1, 6 and 30 G) resistin secretion from PDL cells. Resistin levels are calculated in % of untreated cells at each time point, and presented as mean ( $\pm$  SEM).

### Effect of EMD on circulating resistin levels in vivo

To assess the role of EMD in vivo, 2mg EMD was injected intramuscular (gluteus maximum) of rats every 3th day for 14 days.

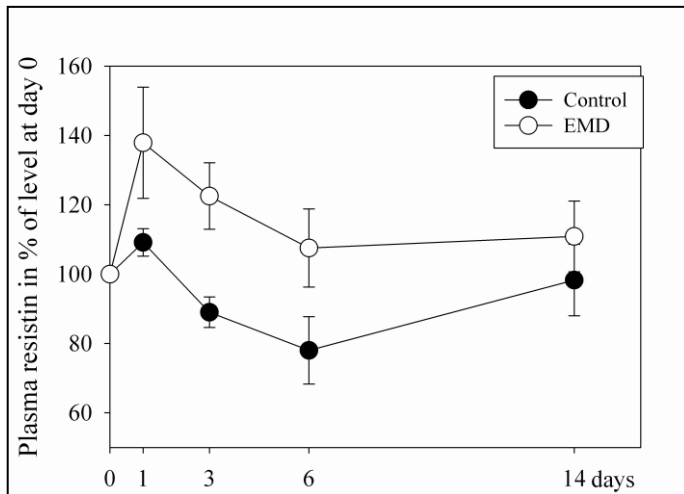


Figure 6

In vivo effect of EMD shows the level of resistin in blood samples measured after 1, 3, 6 and 14 days from rats. Resistin levels at each time point were calculated in percentage of the level at time 0 for each individual animal. Results are presented as average (SD) for treatment (EMD) and control group (n=6).

Results from in vivo effect of EMD shows that rats with injected EMD have a significant increased level of circulating resistin levels compare to controls (injected with saline).

EMD treatment on wound shows a remarkable increase in the content of resistin in the wound fluid (Fig. 7). We had only used one sample, so the experiment needs to be repeated before further evaluation. An interesting observation is that IL-6 is not changed upon EMD treatment.

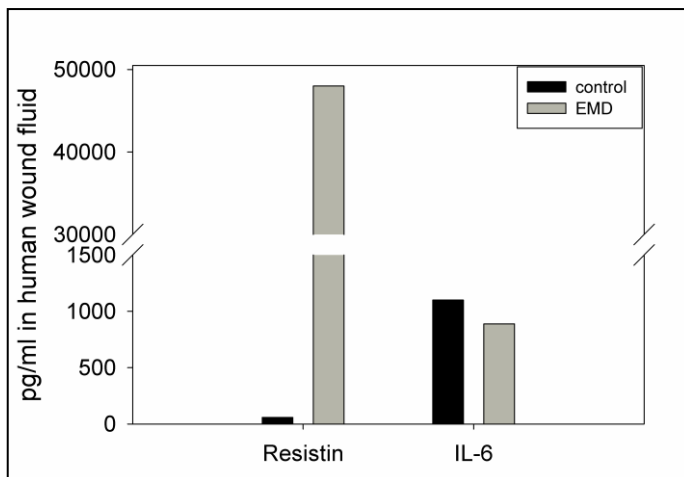


Figure 7

In vivo effect of EMD on wound. (n=1).

## Discussion

We observe an acute and dramatic increase in the secretion of resistin after incubation with EMD in primary human mesenchymal stem cells, osteoblasts, pulp and PDL cells, but not in monocytes. An enhancement of circulating resistin was observed after intramuscular injections of EMD in rats, and in wound fluid after EMD treatment.

We were not able to reproduce the effect by any of the fractions of EMD, indicating that the whole enamel matrix derivative is more active than isolated protein components.

Resistin was originally described as an adipokine promoting resistance against the insulin response and adipogenesis [Way et al., 2001] in rodents. Previous studies show that human resistin is mainly secreted from human immune cells such as macrophage and monocytes and organs such as the spleen and bone marrow [Son et al., 2010]. More recently, important roles for resistin in the human immune response and inflammation, including induction of pro-inflammatory cytokines have been reported [Son et al., 2010].

The lack of effect on resistin secretion from monocytes indicates that EMD-induced increase in resistin is not a general signal but, might only be in cells of mesenchymal origin.

It has been demonstrated radiographically, histological, and in re-entry procedures that new bone is regenerated in EMD-treated periodontal defects [Heijl, 2002]. We know that resistin has a potential role for in bone metabolism and remodelling, there it increases differentiation in osteoblasts and osteoclasts, in addition to increase the NF $\kappa$ B activity (pivotal factor for bone resorption) in osteoclasts. The EMD-induced increase in resistin from MSC and NHO might be one of the mechanisms inducing enhanced bone remodelling.

The expression of resistin has never been demonstrated in pulp or PDL cells previously, however we do find it to be expressed and the secretion to be regulated by EMD in these cells. Taken together, this data shows that EMD regulates resistin in MSC, osteoblasts, pulp, and PDL cells, all of mesenchymal origin. Intramuscular injection of EMD in rats resulted in enhanced circulating resistin levels compared to controls, indicating that our data can be reproduced in vivo as well. This is a strong evidence for resistin being one of the downstream factors executing the observed effects of EMD both in vivo and in vitro. The observed enhanced resistin levels in wound fluid after EMD (Xelma) treatment are supporting this, although narrow sample size limits the interpretations here.

Nevertheless, more effort is still needed in order to fully elucidate and understand the interplay between EMD and resistin.

### **Acknowledgments**

Resistin results from pulp and PDL cells exposed to EMD were obtained from the master project named “The role of adipokines in cranial development” by Hanne Tuvrønningen and Gry Andreassen.

Human peripheral mononuclear cells (PBMC) were isolated from peripheral blood from 3 healthy donors using a Percoll density gradient, a gift from Sofia Almquist, Goteborg University.



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