Measurement of Autoantibodies Against Osteoprotegerin in Adult Human Serum: Development of a Novel ELISA Assay

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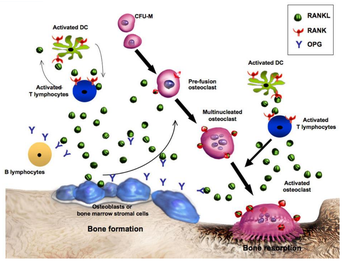
Introduction

The RANK/RANKL/OPG signalling pathway is essential for osteoclastogenesis.

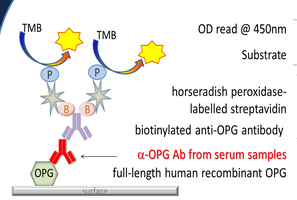
Osteoprotegerin (OPG) is a decoy receptor for RANKL. By binding to RANKL, OPG blocks RANKL-RANK interaction, inhibiting the differentiation of the osteoclast precursor into a mature osteoclast and thereby protecting the skeleton from excessive bone resorption.



RANK/RANKL/OPG signalling pathway.

- Auto-antibodies against Osteoprotegerin (α-OPGAb), by capturing OPG, enable sustained interaction of RANKL with RANK and over-activation of osteoclasts.
- Such antibodies have been identified in 2009, in a man with coeliac disease associated with severe osteoporosis[1] and later in 2013, in patients presenting with rheumatoid arthritis, systemic lupus erythematosus, spondyloarthritis and osteoporosis[2].
- These findings suggest a role for α-OPGAb as primary cause of high bone turnover.
- We aimed to develop an enzyme linked immunosorbent assay (ELISA) for detection and quantification of α-OPGAb in patient serum samples.

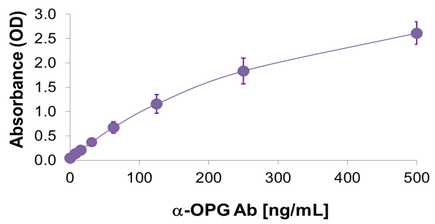
Development of a sandwich-like assay:



Reagent	Provider
Maxisorp™ F8	ThermoFisher Scientific, Loughborough, UK
Coating Buffer	AbD Serotec, Kindlington, UK
Blocking Buffer	AbD Serotec, Kindlington, UK
rhTNFRSF11B	Novoprotein, Summit, USA
STD: α-OPGAb (rabbit)	Abnova, Taipei, Taiwan
CONJ: Donkey α-rabbit IgG, Biotin	ThermoFisher Scientific, Loughborough, UK
SA-HRP	Jackson ImmunoResearch Lab., West Grove, USA
TMB	Sigma Aldrich, Dorset, UK

Schematic of α-OPGAb assay principle and table of reagents.

- **Typical standard curve** antibody against human α-OPGAb. Circulating antibody concentration is calculated against a 4-Parameter Logistic equation (Typical obtained using a polyclonal r^2=0.9916).



- **Finalised protocol:** using a sample/standard and control volume of 50µL which are incubated for 3hrs at RT, the assay has been developed to be performed within a day (6.5hrs)
- The format of the assay (volumes, temperatures and times) are convenient to transpose it onto an automated platform.

References:

1-Riches et al. (2009) N Engl J Med:361 pp1459-65. **2**-Hauser et al. (2013) Bone Abstracts: 1 pp383. **3**-Nielen et al. (2004) Arthritis and rheumatism:50 pp380-386. **4**-Jorgensen et al.(2008) Annals of the rheumatic diseases:67 pp860-866.**5**- Dahlqvist (2003) ACP journal club 139 pp50. **6**-Whittingham et al. (1997) Diabetic medicine : a journal of the British Diabetic Association:14 pp678-685.**7**-Eriksson et al. (2011) Arthritis research & therapy:13 pp30. **8**-Arbuckle et al. (2003) N Engl J Med:349 pp1526-1533. **9**-Jonsson et al. (2013) JAMA:310 pp1854-1855. **10**-Conrad & Bachmann (2005) Pre-symptomatic Tumor Diagnostics Vol.1 pp22-77.

Assay Characteristics

- Imprecision:

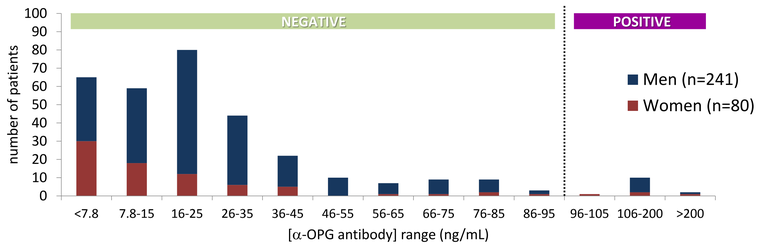
Intra-assay imprecision: 3.1% at 98.6 ± 3.1 and 7.3% at 274.4 ± 20.1 ng/mL.

Inter-assay imprecision: 5.9% at 80.9 ± 4.5 and 10.1% at 254.2 ± 25.6 ng/mL.

- Linear range was 0-500ng/mL.
- Lower and upper limit of quantification were 3.9 and 500 ng/mL.
- Cross-reactivity was assessed against human sera (10 anonymised NNUH patients samples) containing raised thyroid antibody (TPO>150kU/L; ref range 0-34kU/L). No samples showed elevated α-OPGAb concentration (green box-plot).

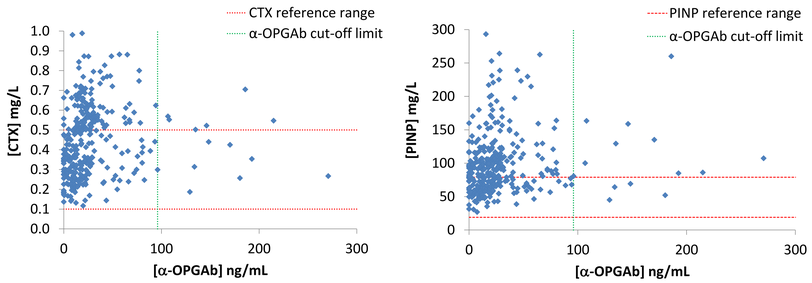
Determination of α-OPGAb reference intervals in healthy individuals

- Serum samples were collected from healthy volunteers following and in accordance with the Ministry of Defence Research Ethics Committee (MODREC-165). 321 serum samples (241 men and 80 women, aged 17-32 years) were used to assess the distribution of α-OPG Ab.



- Skewed distribution of α-OPGAb in healthy population
- Adult population would be considered positive with a titer above the cut-off limit (95%) of 96ng/mL calculated using the geometric mean of log10 dataset
- 4.3% of the population presented elevated α-OPGAb. There was no significant effect of gender on the distribution (♀:7.6%; ♂:5.3%).

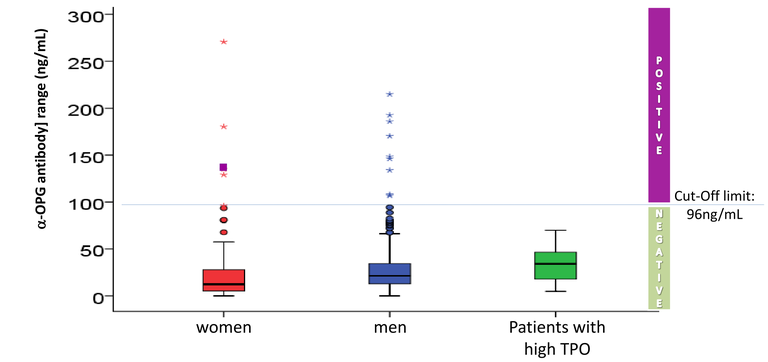
Association of α-OPGAb with bone turn-over markers in young and healthy population



Of αOPGAb positive samples 42.9% had CTX (bone-resorption marker) above the reference range and 71.4% had PINP (bone-formation marker) above the reference range.
No clear correlation was observed.

Case study

History- Mrs JY, 49, presented with multiple bone aches involving mainly the axial skeleton and severe joint pain (hips, knees, wrists and small joints of the hands). Biochemical investigations detected an adjusted calcium of 3.56mmol/L (ref range 2.15-2.60mmol/L), markedly elevated bone turnover CTX 3.95µg/L (0.1-0.5µg/L), P1NP 1200µg/L (19-89µg/L), Bone ALP 224IU/L (14-50IU/L) but all other investigations including autoantibody screen were normal and joint aspiration was negative. Following zoledronate therapy, adjusted calcium normalised at 2.34mmol/L and bone turnover decreased, CTX 0.97µg/L, P1NP 175µg/L, bone ALP 64IU/L.



Box-Plot showing the distribution of α-OPGAb in healthy women and men and in patients with high TPO (green). Mrs JY (▪) showed elevated levels of serum α-OPGAb.

Conclusions

- We developed a novel plate-based ELISA assay for the detection and measurement of α-OPGAb in human serum. The assay showed good assay characteristics, suitable for use in research and clinical hospital laboratories. However we are limited in using a standard antibody of animal origin (rabbit) allowing us to only perform a relative quantification of α-OPGAb in human serum.
- Autoantibodies have been shown to be present in the blood of patients with autoimmune diseases such as rheumatoid arthritis[3-5], diabetes mellitus[6], lupus[7,8] and retrospectively in patients with primary Sjögren syndrome[9] prior to development of any symptoms but also before development of tumors[10].

  Prevalence of α-OPGAb in healthy individuals suggests a possible autoimmune cause of metabolic bone diseases with presentation of pre-symptomatic antibodies. α-OPGAb in young (<50yrs) and healthy individuals tended to be present in individuals with high bone formation markers (PINP) rather than bone degradation markers (CTX). The subjects being young army volunteers subjected to heavy physical training a high bone turnover is not surprising. However, these results suggest that autoantibodies against osteoprotegerin in healthy individuals may be present in a non functional or dysfunctional state at this stage and may become functional later due to changes in the antibodies themselves or dysregulation in the OPG/RANK/RANKL/α-OPGAb system. Further study is needed to answer this question.
- The novel ELISA for detection and measurement of α-OPGAb is a potential diagnostic tool for patients with high bone turnover and for clinicians to identify appropriate treatment options. When α-OPGAb are present, suppressing the regulatory effect of OPG on the activation of RANK by RANKL, an appropriate treatment may be Denosumab (licenced osteoporosis treatment under the trade name Prolia and Xgeva) which is an antibody to RANKL.