

## Bone remodelling biomarkers after whole body cryotherapy (WBC) in elite rugby players

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### ABSTRACT

Whole body cryotherapy (WBC) consists of a brief exposure to extreme cold air ( $-110^{\circ}\text{C}$ ) in a controlled chamber and it is applied in sports medicine to improve recovery from musculoskeletal trauma. The aim of this study is to better define the beneficial effect of WBC on the musculoskeletal system of athletes, in particular on bone remodelling. Remodelling osteoimmunological biomarkers OPG, RANKL and RANK were measured after WBC treatment in 10 male rugby players randomly selected from the Italian National team. OPG levels were increased significantly, supporting the view that WBC induces an osteogenic effect. Further studies evaluating the effect of WBC on bone metabolism are desirable.

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### Introduction

The use of cryotherapy (the application of cold for the treatment of injury or disease) is widespread in sports medicine established method of treating acute soft tissue injury. Cold is commonly used to reduce the recovery time as part of the rehabilitation programme both after acute trauma and for the treatment of chronic injuries.<sup>1</sup> Its physiological and biological effects are due to the decline of temperature in the treated tissues and the associated neuromuscular action and relaxation of muscles produced by the application of cold leading to a decrease of the local inflammatory reaction. Whole body cryotherapy (WBC) consists in a brief exposure to extreme cold air ( $-110^{\circ}\text{C}$ ) in a controlled chamber.<sup>2</sup> It is applied to treat such conditions as rheumatoid arthritis, fibromyalgia and ankylosing spondylitis. In sports medicine, cryotherapy is acknowledged as a valid method to improve recovery from muscle trauma. The general effects of this method can also be beneficial for minimising the risk of injury after intense training and competition seasons. WBC may be beneficial for athletes since prompt recovery from muscle injury is a primary

concern for both athletes and sports physicians. Body cooling is also used in endurance exercise; in particular pre-cooling is applied to reduce body temperature before exercise, thereby increasing the margin of metabolic heat production and the time to reach the critical limiting temperature when giving exercise intensity can no longer be maintained. This strategy is particularly effective for enhancing exercise performance in endurance and resistance sports.<sup>3</sup> WBC has been shown not to be harmful as it improves the antioxidant capacity exposure to intense exercise.<sup>4</sup> In a previous study, we demonstrated that WBC has no negative effect on haematological values, as measured by haemoglobin concentration and count of leukocytes, erythrocytes, reticulocytes and platelets in peripheral blood in athletes.<sup>5</sup> Moreover, we recently described the anti-inflammatory effects of WBC on elite rugby players, as indicated by a reduction in proinflammatory cytokine and chemokines levels and an increase in anti-inflammatory cytokines after WBC treatment.<sup>5</sup> To date, no published data are available about the aftermath effect of WBC on the bone system. Bone metabolism and biomarkers, such as osteocalcin and type I collagen for bone formation and resorption, respectively, are widely applied in sport medicine for the evaluation of acute and long term exercise during and after a competitive season. Recently, a new family of molecules, the OPG–RANK–RANKL system,<sup>6,7</sup> have been considered as biomarkers of the bone system, in particular bone remodelling, thus defining the emerging concept of “osteimmunology” as a common pathway and a regulatory

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mechanism linking bone and the immune system.<sup>8–11</sup> The cellular receptor RANKL (receptor activator of nuclear factor NFκB ligand) and the decoy receptor OPG (osteoprotegerin) constitute a novel cytokine system.<sup>12</sup> RANKL is produced by osteoblasts, activates T lymphocytes and promotes osteoclast activation and survival, thus resulting in bone resorption. Osteoclast, as well as dendritic cells, produce RANKL specific receptor, RANK, which activates the RANKL mediated intracellular signalling cascade leading to bone resorption.<sup>12</sup> The effects of RANKL on RANK are counteracted by the decoy receptor OPG, acting as soluble neutralising receptor. Abnormalities of the OPG–RANK–RANKL system have been implicated in a variety of bone diseases, such as osteoporosis, osteopetrosis, rheumatoid arthritis, bone tumours and bone metastasis.<sup>13,14</sup> In the herein study, we aimed to better define the beneficial effect of WCB on the musculoskeletal system of athletes, in particular on bone remodelling by measuring the levels of bone remodelling biomarkers OPG, RANKL and RANK at baseline and after one week of WCB treatment in 10 male rugby players selected randomly from the Italian National Team.

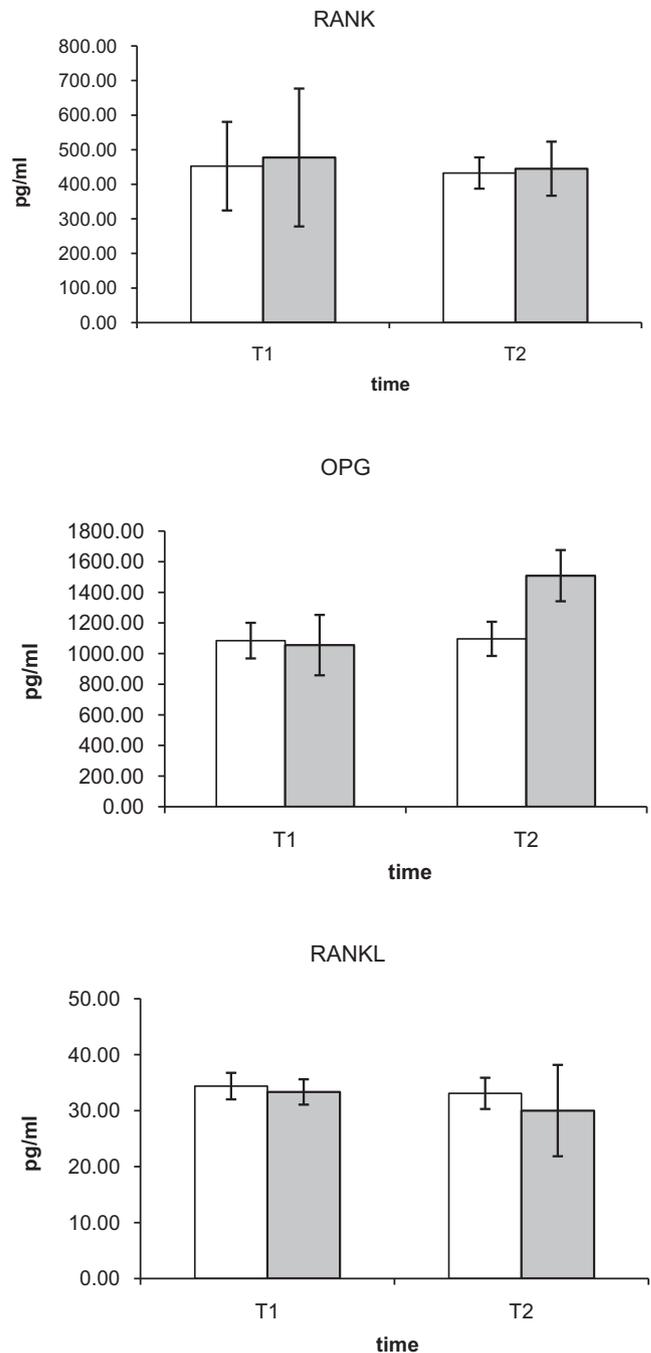
### Patients and methods

The 10 (males) subjects were chosen randomly from the National Team consisting of 30 athletes; all gave informed consent for the study protocol and blood drawing. The mean age was  $26 \pm 2.5$  years; the mean body-mass index (weight in kg divided by height in metres squared) was  $27.5 \pm 2.3$  kg/m<sup>2</sup>. The subjects underwent daily sessions of WCB for 5 days at the Center of Spała (Poland). Wearing minimal clothing, the subjects were exposed to very cold air ( $-60$  °C) for 30 s in a temperature-controlled room, then to extremely cold air ( $-110$  °C) for 2 min. During the study period, they continued with the regular training regimen; the workload was identical to that of the previous 6 weeks. Training consisted of 3 h of exercises every day: maximal training for the first morning hour; sub maximal effort for the second hour; sub maximal training and conditioning for the third hour in the afternoon. Blood samples were collected by means of Vacutainer tubes at 8 a.m. on the Monday morning prior to administration of WCB and on the following Monday after the end of training. The same protocol of blood drawing was applied to a control group of rugby players undergoing the same training session, but without WCB treatment. The mean age was  $26 \pm 1.9$  years; the mean body-mass index (weight in kg divided by height in metres squared) was  $28.5 \pm 1.3$  kg/m<sup>2</sup>. Blood drawings were performed by using evacuated 7-mL plain tubes (Becton Dickinson, Rutherford, USA). Sera were separated from whole blood after complete blood coagulation by centrifugation at 3000 rpm for 10 min using (what equipment, Make city?). Sera were stored at  $-70$  °C until analyses performed in a single batch. The time period since the last heavy training session was the same for both blood drawings. Sera were separated within 3 h of blood collection and stored at  $-20$  °C until assayed. Levels of soluble OPG, RANKL and RANK in serum specimens were determined by commercially available assays according to the manufacturers' instructions (Duo Set Development System, human OPG and RANK, R&D Systems, Minneapolis, MN, USA; sRANKL ELISA, Biomedica GmbH, Vienna, Austria). The sRANKL test kit is an enzyme immunoassay designed to determine soluble, uncomplexed human RANKL directly in biological fluids. In a first step, sample and the biotinylated anti-sRANKL detection antibody are pipetted into the wells (100 μL). Human sRANKL, if present in the sample, binds to the precoated recombinant osteoprotegerin (OPG) and forms a sandwich with the detection antibody. After a washing step (five times), which removes all non-specific bound material, streptavidin–HRP conjugate is added to the wells (200 μL). After removal of unbound conjugate by washing, 100 μL of AMPLIFIER A and 100 μL AMPLIFIER B are added to the wells. The colour development is stopped with 50 μL of STOP SOLUTION and the

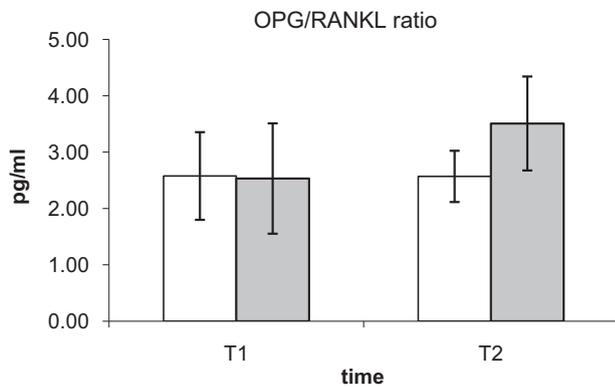
intensity of the colour is measured using a micro plate reader set to 490 nm.

### Statistical analysis

We used Kolmogorov Smirnov for evaluating normal distribution of the values and Student *t* test for paired values for comparing data by means of the programme MedCalc (Maria-werke, Belgium). The normality was accepted for RANK values before WCB ( $p = 0.19$ ), after WCB ( $p = 0.90$ ) and for control ones



**Fig. 1.** Serum concentration (pg/mL) of bone remodelling markers, RANK (panel A), OPG (panel B) and RANKL (panel C), before (T1) and after (T2) whole body cryotherapy (WCB) in 10 top-level rugby players (grey bars) and in 10 not WCB treated rugby players undergoing the same training session (white bars). Results are shown as mean  $\pm$  SD.



**Fig. 2.** OPG/RANKL ratio, before (T1) and after (T2) whole body cryotherapy (WBC) in 10 top-level rugby players (grey bars) and in 10 not WBC treated rugby players undergoing the same training session (white bars).

( $p = 0.36$ ). The normality was accepted for correspondent OPG values at  $p$  values of 0.70, 0.66 and 0.53.

## Results

Blood values of OPG, RANK and RANKL before (T1) and after (T2) WBC are summarised in Fig. 1.

RANK peripheral blood values, as shown in Fig. 1 (panel A), were not dramatically altered by WBC. Pre-WBC RANK peripheral blood mean values ( $477.47 \pm 199.44$ ) were not homogeneous, with a quite high SD indicating a quite large value distribution. WBC did not induced a significant change ( $p = 0.33$ ). Post WBC values ( $445.13 \pm 78.17$  pg/mL) displayed a lower standard deviation, indicating a greater uniformity of the data, but they were still very similar to pre-WBC values. RANK serum levels were measured at the same time points also in a control group of rugby players undergoing the same training session, but without WBC treatment. RANK levels were comparable pretreatment values and in WBC treated players and between the two time points ( $452.47 \pm 128.13$  and  $432.63 \pm 45.21$  for T1 and T2, respectively).

Panel B (Fig. 1) shows OPG peripheral blood values before ( $1054.94 \pm 197.11$ ) and after WBC ( $1508.16 \pm 167.12$ ), indicating a very strong and statistically significant difference ( $p < 0.001$ ) induced by this treatment on OPG values. OPG serum levels were measured at the same time points also in a control group of rugby players undergoing the same training session, but without WBC treatment. RANK levels were comparable with pretreatment values and in WBC treated players and between the two time points ( $1804.11 \pm 116.26$  and  $1095.66 \pm 111.51$  for T1 and T2, respectively).

Panel C (Fig. 1) shows RANKL peripheral blood values before ( $33.33 \pm 2.26$ ) and after WBC ( $30.01 \pm 8.16$ ), indicating a weak but not statistically significant decrease induced by WBC treatment ( $p = 0.099$ ). RANKL serum levels were measured at the same time points also in a control group of rugby players undergoing the same training session, but without WBC treatment. RANK levels were comparable with pretreatment values in WBC treated players and between the two time points ( $34.38 \pm 2.37$  and  $33.08 \pm 2.79$  for T1 and T2, respectively).

In order to define the net effect induced by WBC on bone resorption or formation, we compare the bone resorption and formation markers, OPG and RANKL, respectively, as OPG/RANKL ratio. Fig. 2 shows that WBC induced a statistically significant increase in the OPG/RANKL ratio ( $2.53 \pm 0.98$  and  $3.51 \pm 0.83$  before and after WBC, respectively,  $p < 0.05$ ). The same ratio was measured in the control group and no difference was observed between the two time points ( $2.58 \pm 0.78$  and  $2.57 \pm 0.45$  for T1 and T2, respectively).

## Discussion

The appropriate balance between training/competition stress and recovery is important for maximising the performance of athletes. A wide range of recovery modalities are used as integral part of training programmes of elite athletes,<sup>15,16</sup> in order to reduce the severity and duration of exercise induced musculoskeletal injury. In rugby, in particular, the use of cold water associated with active recovery and the use of immersion in hot and cold water are applied to recovery programme after intense training. We have previously described the effects of a particular kind of cryotherapy, WBC (whole body cryotherapy), applied to rugby elite players, as beneficial on cardiovascular markers,<sup>17</sup> immunological parameter and serum muscle enzymes.<sup>5</sup> Since specific studies on the effects of WBC on bone are lacking, here we focused on the effects of WBC on bone remodelling biomarkers in 10 top level rugby players of the Italian National Team, before and after a 1-week of daily session of WBC. There is a substantial body of evidence that physical exercise and sport affect bone metabolism, growth, remodelling and turnover.<sup>18</sup> Bone mass can be considered the net result of two counteracting metabolic process, namely bone formation and resorption<sup>19</sup> governing bone remodelling. Several blood and urinary molecules have been identified as markers of bone metabolic activity, providing estimations of bone formation and resorption.<sup>20,21</sup> These processes are under the control of several endogenous factors, such as hormones, growth factors and cytokines, and exogenous factors, such as mechanical loading.<sup>22</sup> Physical activity, in particular weight bearing exercise, is thought to provide the mechanical stimuli or loading, which are important for the maintenance and improvement of bone health.<sup>23</sup> Indeed, several studies on top level athletes indicates that they have a higher bone turnover than sedentary subjects.<sup>24,25</sup> In young adults, highest values of BMD (bone mass density) have been found in strength and power-trained athletes.<sup>26–28</sup> The effect of training on bone metabolism serum markers can be dependent on aerobic or anaerobic training, inducing bone formation and turnover, respectively.<sup>29</sup> These effects have been studied in a variety of sports.<sup>27,28,30–32</sup> In particular, bone metabolism varies depending on the type, frequency and intensity of sport.<sup>18,30</sup> The beneficial effects of physical activity on bone mineral mass depend on the type of activity undertaken and are due to the sensitivity of the osteogenic process to the type, rate, frequency, direction and magnitude of the strain.<sup>33</sup> Unloaded exercises, such as swimming, have no impact on bone mass, while walking has limited positive effects.<sup>18</sup> The highest correlation with bone metabolic activity is found in weight-bearing, impact, jumping or resistance sports with high magnitude strains applied to the skeleton, such as soccer, fighting sport (box) or rugby.<sup>34</sup> Rugby is a weight-bearing, intermittent high intensity sport that includes a great number of impacts. In addition, it involves rapid directional changes, starts, stops, jumps, rotation forces and strains resulting in a large ground reaction force at the skeleton. For this reason, it can be considered as a weight-bearing and high impact sport and, therefore, very osteogenic.<sup>35–37</sup> Moreover, rugby is a contact sport with very high musculoskeletal injury risks, due to physical contact, primary in the tackle.<sup>38–42</sup> Therefore, the musculoskeletal recovery and in particular its beneficial effect on bone is extremely relevant in this sport. So far, limited evidence is available describing the correlation between long-term rugby practice and bone system, comparing general bone parameters such as bone mass density (BMD) and bone mass content (BMC) in rugby players and controls.<sup>35</sup> According to the same authors, Elloumi et al., the evaluation of more specific and sensitive biomarkers may increase the understanding of the mechanism responsible of bone mass changes in rugby players.<sup>35</sup> Here we investigated the beneficial effect of WBC on rugby players, focusing in particular on specific

bone remodelling biomarkers belonging to the emerging field of the “osteimmunology”, the common pathway linking bone and immune system, namely the OPG–RANK–RANKL system.<sup>8–11</sup> These molecules have been previously reported to be a useful tool to monitor bone turnover rate<sup>43,44</sup> and have recently been applied to the evaluation of bone remodelling in athletes,<sup>45,46</sup> but no data are available up to now about their response to WBC. In our study, WBC did not increase the level of RANK, which is considered a marker of bone resorption because it is the receptor transducing the RANKL induced intracellular signal, leading to osteoclast activation. The same trend was observed for the osteoclastogenic RANKL, a strong marker of bone resorption. The same molecules display no differences in a control (not WBC treated) group of players undergoing the same training session. This result indicates that WBC has not bone resorptive effects. Like others bone metabolism markers, RANKL production is controlled by different cytokines. In particular, RANKL production is associated to the inflammatory chemokine IL-8,<sup>47</sup> and a previous study of this group indicated that WBC was able to decrease IL-8 serum level in rugby players. Therefore, the absence of an increase in RANKL serum level is in agreement with our previous evidences. The steady level of RANKL we observed after WBC is in accordance with other studies on different sports context indicating that bone resorption markers are less sensitive and responsive than bone formation markers, at least in the short time period.<sup>29</sup> On the other hand, the third parameter we evaluated, OPG, is a bone formation marker and our results clearly indicated a statistical significant increase after WBC, suggesting an osteogenic effect of WBC, while no difference were observed in the not WBC treated players (Fig. 1, panel B). Moreover, RANK and OPG levels were evaluated in the same athletes in a different moment before WBC exposure (mean value RANK  $458 \pm 129.2$  SD, mean value OPG  $1117.7 \pm 157.1$  SD), and their values were comparable with the ones measured immediately before WBC (*t* student value  $p = 0.54$  and  $p = 0.95$ , for RANK and OPG, respectively), thus suggesting that there is no spontaneous fluctuation of OPG and RANK levels, but this variation is due to WBC effect. For this reason we focus on a short time interval (1 week) to monitor the effect of an acute treatment of WBC.

Since OPG and RANKL serum levels might vary independently after a stimulus, the evaluation of RANKL and OPG are not only considered as single parameters, but in relation with OPG/RANKL ratio (Fig. 2), which provides a more clear indication of the bone formation or resorptive effect. Our results indicated that, after WBC, OPG/RANKL ratio increased with a very statistical significance, providing a more robust indication of the osteogenic effect of WBC, while no difference were observed in the control (not WBC treated) group of rugby players. The evaluation of the OPG–RANKL–RANK system in sports medicine is very recent and only few evidences are present so far, but they are in agreement with our results, indicating an increase of serum levels of OPG and a parallel decrease of RANKL after endurance physical activity,<sup>45</sup> thus suggesting that an increase of the OPG/RANKL ratio in athletes might protect bone health.<sup>46</sup> The increase of OPG/RANKL ratio after WBC indicated that this procedure is able to improve bone formation and bone recovery from exercise-induced musculoskeletal injury and/or damage associated with intense physical training in rugby players. Therefore, this result indicates that WBC, as observed in other sport disciplines, can be applied to rugby players to provide beneficial effect on musculoskeletal recovery. In particular in our study, WBC induced an osteogenic and osteoprotective effect on rugby players. Previous studies indicate that WBC improved the antioxidant capacity of organism exposed to intense exercise, increasing anti-oxidant agent levels, such as superoxide dismutase and glutathione peroxidase activity.<sup>4</sup> Moreover, cold inhibits inflammatory mediators, such as ICAM-1 (intracellular adhesion molecule-1)<sup>48</sup> and induced the expression

of the anti-inflammatory cytokine IL-10.<sup>49</sup> In this way hypothermia attenuates the inflammatory response during WBC, thus contributing to its beneficial role in tissue protection. Due to its osteogenic and its anti-inflammatory properties, WBC may have a role in the clinical setting for the recovery of post fracture patients. Future clinical trials are desirable to throw some light into this potential clinical application. Previous studies of this group indicated that WBC is not deleterious for cardiac function and it does not alter blood chemistry values,<sup>5,17</sup> suggesting that it cannot be considered illegal or doping procedure and might also be applied to training sessions in order to increase exercise performance. Limitations of this study include the lack, in this case, of case control subjects and additional time points to evaluate the follow up of the long term effect of the WBC. Additional studies and further investigations on physically active subjects, including case–control protocol, are desirable to provide more robust evidence about our findings.

## Conclusions

In this study to our best knowledge we described for the first time, beneficial effects of WBC in rugby players by means of new bone remodelling osteoimmunological biomarkers OPG, RANK and RANKL. The use of these new osteoimmunological markers in athletes, in particular in those sports undergoing long-term and heavy physical effort, training programmes, like rugby, can be a useful tool to monitor bone metabolism during the whole competitive season.

## Conflict of interest statement

All the authors declare no conflicts of interest.

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