Bone and Energy Metabolism Parameters in Professional Cyclists during the Giro d’Italia 3-Weeks Stage Race

Giovanni Lombardi1*, Patrizia Lanteri1, Rosa Graziani2, Alessandra Colombini1, Giuseppe Banfi1,3, Roberto Corsetti4

1 IR.C.C.S. Istituto Ortopedico Galeazzi, Milano, Italia, 2 CEDAL Lab, Gallarate, Italia, 3 Chair of Clinical Biochemistry, School of Medicine, University of Milano, Milano, Italia, 4 Liquigas Cannondale pro-cycling team, Medical board, Sesto al Reghena, Italia

Abstract
Cycling is a not weight-bearing activity and is known to induce bone resorption. Stage races are really strenuous endurance performances affecting the energy homeostasis. The recently highlighted link, in the co-regulation of bone and energy metabolism, demonstrates a central role for the equilibrium between carboxylated and undercarboxylated forms of osteocalcin. Aim of this study was to understand the acute physiological responses to a cycling stage race in terms of bone turnover and energy metabolism and the possible co-regulative mechanisms underlying their relationship. We studied nine professional cyclists engaged in 2011 Giro d’Italia stage race. Pre-analytical and analytical phases tightly followed academic and anti-doping authority’s recommendations. Bone and energy metabolism markers (bone alkaline phosphatase, tartrate-resistant acid phosphatase 5b, total and undercarboxylated osteocalcin, leptin and adiponectin) and related hormones (cortisol and testosterone) were measured, by Sandwich Enzyme Immunoassays, at days -1 (pre-race), 12 and 22 during the race. The power output and the energy expenditure (mean and accumulated) were derived and correlated with the biochemical indexes. During the race, bone metabolism showed that an unbalance in behalf of resorption, which is enhanced, occurred along with a relative increase in the concentration of the undercarboxylated form of osteocalcin that was indirectly related to the enhanced energy expenditure, through adipokines modifications, with leptin decrease (high energy consumption) and adiponectin increase (optimization of energy expenditure). The exertion due to heavy effort induced a decrease of cortisol, while testosterone levels resulted unchanged. In conclusion, during a 3-weeks stage race, bone metabolism is pushed towards resorption. A possible relationship between the bone and the energy metabolisms is suggested by the relative correlations among absolute and relative concentrations trends of undercarboxylated OC, adipokines concentrations, BMI, fat mass (%), power output and the derived energy expenditure.

Citation: Lombardi G, Lanteri P, Graziani R, Colombini A, Banfi G, et al. (2012) Bone and Energy Metabolism Parameters in Professional Cyclists during the Giro d’Italia 3-Weeks Stage Race. PLoS ONE 7(7): e42077. doi:10.1371/journal.pone.0042077

Editor: Alejandro Lucia, Universidad Europea de Madrid, Spain
Received February 17, 2012; Accepted July 2, 2012; Published July 27, 2012
Copyright: © 2012 Lombardi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was funded by the Italian Ministry of Health. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Competing Interests: The authors have declared that no competing interests exist.
* E-mail: giovanni.lombardi@grupposandonato.it

Introduction

Professional cycling 3-weeks stage races such as Tour de France, Vuelta a España and Giro d’Italia, very popular sport events, could be included among the most strenuous athletic performances [1]. These races last 21–22 days with more than 3000 km covered and only one or two days of rest; athletes are submitted to very intense metabolic effort, combining aerobic and anaerobic metabolism, particularly during mountain stages and time trials [2,3,4]. Thus, studies concerning the metabolic changes occurring during these competitions are of particular interest for describing the biochemical pathways orchestrating the energy expenditure required to perform the mechanical work, as highlighted by previous studies on endurance athletes [5,6].

Although, papers concerning hormonal, haematological and biochemical changes during cycling races are available [7,8], the link between bone and energy in cycling has never been investigated.

The modification of the hormonal profile during the Vuelta a España was described in detail [2,7,9]. Morning serum concentrations of testosterone, FSH, LH and cortisol, in nine cyclists during the Vuelta 1999, measured before the race (at the end of the first, the second and the third week of competition) showed no variations in gonadotropins, and a decrease in cortisol and testosterone as a result of long duration and intense exercise [2]. Interestingly, thyroid hormones, which are known to be involved in adaptation of an organism to physical exercise [9], were modified during a Vuelta. Hormones were measured in 16 cyclists before the race and at the end of each of the three weeks of competition: free 3,5,3’-triiodothyronine, total and free-9-triiodothyronine remained unchanged. The increase in thyroid hormones might be due to haemoconcentration or to the release of thyroid hormone-transporting proteins. These effects could be also related to an impaired peripheral conversion of thyroxine to 3,5,3’-triiodothyronine [9], possibly brought by cortisol increase that is in turn induced by leptin decrease [10], as a result of an energy unbalance. This response is typical of the hardest part of the race, when mountain stages, requiring high energy expenditure, are common [2,11]. The intensity and the duration of the
exercise during 3-weeks stage races induce a rise of insulin growth factor I concentrations during the first week, and, after an adaptation, its stabilization after three weeks. This parameter is linked to nutritional status and to both insulin and growth hormone production and release [7].

Bone metabolism, especially bone formation, is known to be enhanced by exercise. Physical activity is indeed recommended for assuring correct bone density and homeostasis, and to prevent bone mineral depauropation. Bone mineral density (BMD), assessed in athletes practicing different sports, showed general positive effect, but with wide differences between disciplines, especially when considering distinct bone districts, genders and weight-bearing exercise level. On the other hand, no univocal data have been obtained from bone metabolism markers, although it is evident that, at least long-lasting loads increase the circulating levels of formation markers [12, 13].

Cycling is characterized by a low level of skeletal load, with the training specifically based on the non-weight-bearing bike riding performed across the whole year. As a consequence, cyclists have lower BMD than runners [14] and age-matched controls [15]; furthermore, bone health status in adolescent cyclists is affected by the heavy training and the exercise in absence of load [16].

Bone markers were never studied in long-term cycling performances. Therefore, no data about the possible bone metabolism changes during a high-demanding 3-weeks stage race exist. Noteworthy, a modern approach to bone function is claiming its active role in regulating energy metabolism [17]. The regulation of osteoblasts and osteoclasts activity has recently been integrated into an endocrine perspective, which includes a consistent link with energy metabolism and sympathetic nervous system. The endocrine function of the bone is exerted through the release of a series of hormones and, among them, osteocalcin (OC), a molecule with a role in local bone physiology, but also able to regulate fat and glucose metabolism, insulin secretion and pancreatic β cells proliferation; moreover, in adipocytes, OC induces the release of adiponectin which, in turn, reduces insulin resistance [12, 17, 18]. OC is a 5.8 kDa, hydroxyapatite-binding, protein exclusively synthesized by osteoblasts, odontoblasts and hypertrophic chondrocytes. It possesses 3 vitamin-K dependent, γ-carboxyglutamic acid residues (Gla-OC), which are responsible for the calcium binding properties of the protein. There are some post-transcriptional forms of OC. In energy metabolism pathway, it is of particular interest the undercarboxylated form (Glu-OC) which could be directly released, by osteoblasts, in balance with the carboxylated form [17, 18], or released from the main OC molecule, when the bone environment is acidified by acid phosphatase activity, expression of activation of osteoclasts [19]. Circulating Glu-OC acts on insulin-sensitive tissues and pancreatic β cells [18].

The control of OC secretion is assured by sympathetic tone, which is in turn controlled by leptin, an adipocyte-derived hormone, which can regulate osteoblasts: its action is mediated through two different neural pathways. Leptin concentrations are not influenced by short exercise, but are decreased by long-term exercise or by exercise with high energy expenditure; adiponectin, instead, presents a delayed increase. In general, long-term exercise could decrease leptin and increase adiponectin. Adiponectin concentrations are negatively related with body fat, fasting plasma insulin and oral glucose tolerance and positively related with glucose disposal. No data concerning leptin and adiponectin concentrations at rest and after exercise in cyclists exist [20].

Leptin and adiponectin correlate positively and negatively with fat mass, respectively in normal and overweight subjects. Among professional athletes, cyclists appear to have the lowest body mass index (BMI), fat percentage and adipokines concentrations, especially during long-term training and competition. BMI in cyclists could significantly change during a 3-weeks stage race [11]; this finding should be taken into account for interpreting metabolism modifications.

The regulation of energy metabolism in cyclists during a high-demanding and long-lasting race is crucial for maintaining a sufficient level of performance for three weeks. This study is aimed to study bone metabolism markers, adipokines and hormones involved in the loop of energy metabolism regulation in professional cyclists who competed in the 2011 Giro d’Italia. The choice of cyclists involved in a 3-week stage race, in order to study the integrative physiology of bone and energy metabolism, was derived from the evidence of peculiar body composition and accelerated bone turnover of these athletes associated with the extreme energy demands necessary to carry out the race.

Materials and Methods

Ethics Statement

The study was approved by the Reference Ethical Committee (ASL Milano 1). The athletes involved in this study have given written consent for sample collection and data analysis. Clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Subjects

Nine professional cyclists belonging to the Líquigas-Cannondale team were recruited. They were involved in the Giro d’Italia 2011 and followed across the race, from May 8th to May 29th.

The route characteristics are reported in Table 1.

Mean athletes’ age was 26.7 ± 2.5 years. Weight and height were measured in the morning before the stage in fasting conditions and body mass index (BMI) was calculated as weight/height²; anthropometrical parameters are reported in Table 2.

No drugs or supplements influencing the iron metabolism were taken by athletes; only non-steroidal anti-inflammatory drugs and antibiotics were administered where needed. The Giro 2011 was a “no-needle” race: therapies and drugs were allowed only for evident diseases. Diet was strictly controlled by team physicians and was composed by a 45% of carbohydrates, 36% of proteins and 19% of lipids. The caloric intake was balanced on the base of the energy expenditure; it was set at 6000 kcal/day and kept for the whole study.

Blood drawings were performed on the day before the start (day -1), and on days 12 and 22 of the race (Table 1).

At day -1 athletes performed 3 h of light training at 55% VO₂max including a short bout (30 min) of medium-high levels sub-threshold commitment at 60% VO₂max. The diet on day -1 had the same composition of that carried out during the race.

Power Output and Net Energy Expenditure Measurements

The individual power output was measured for each stage, through the power sensor PowerMeter™ (SRM GmbH, Julich, Germany) integrated within the bike pedal (sensitivity ±2%), as previously described [21]. Briefly, the PowerMeter™ utilizes 8 strain gauges on 16 grids, within a Wheatstone’s bridge: when the force is applied on the pedals the resistance to the transmission impresses a pressure on the measuring bridge. The pressure intensity proportionally modifies the strain gauge length that is translated in a change of the electrical resistance and thus in a proportional variation of the output frequency. The instanta-
neous torque ($\tau$), calculated as the output frequency subtracted of the basal frequency, and the instantaneous angular velocity ($\omega$) are related to the power output ($P$) as follow:

$$P(W) = \tau(Nm) \times \omega(rad/s)$$

The PowerMeter$^{TM}$ automatically derived the instantaneous energy expenditure (kcal) from the instantaneous power output by applying the following unit conversion equivalence:

$$W = 2.39 \times 10^{-4} \text{kcal/s}^{-1}$$

The total energy expenditure is given by the sum of the instantaneous expenditures.

Blood Drawings

Pre-analytical warnings were strictly followed [22] to avoid any possible factor affecting the analytical phase [23]. Particularly, International Cyclist Union (UCI) and World Anti-Doping Agency (WADA) guidelines for collection and transport of specimens were followed [24]. Blood was drawn between 0800 and 1000 h in fasting subjects resting in bed ten minutes after their awaking. Evacuated tubes (BD Vacutainer Systems, Becton–Dickinson, Franklin Lakes, NJ, USA) were used for analyzes measurement (BD K2EDTA 3.5 mL and 7.0 mL plain tubes, BD SSTII Advance). Immediately after drawing, tubes were inverted ten times and stored in a sealed box at 4°C. Controlled temperature was assured during transportation: a specific tag (Libero Ti1, Elpro, Buchs, Switzerland) was used for temperature measurement and recording. Samples were transported by car or by both train and car: the time elapsed from drawing to laboratory was 1 h 30 min at day -1, 7 h 50 min at day 12, and 1 h 30 min at day 22. The differences in delay were due to the sampling performed in places at different distances from the laboratory. Such delays do not affect the analytical output for the measured parameters [25].

The K2EDTA-anticoagulated blood was homogenized for 15 min prior to be analyzed, as recommended by UCI and WADA [24]. Official anti-doping controls were performed, by antidoping agencies, prior the start of the race (day -3) and during the final phases (day 20); moreover, one of the recruited subjects was submitted to an additional check at day 5 (Table 2).

### Table 1. Characteristics of the route.

| Day | Stage | Stage Length (km) | Level Difference (m) | Kind of Stage | Blood Drawing |
|-----|-------|------------------|---------------------|---------------|---------------|
| -3  | /     | /                | /                   | /             | Official Antidoping |
| -2  | /     | /                | /                   | /             |               |
| -1  | /     | /                | /                   | /             |               |
| 1   | 1     | 19.3             | 96                  | Flat          | Time Trial   |
| 2   | 2     | 244.0            | 780                 | Flat          |               |
| 3   | 3     | 173.0            | 2340                | Flat          |               |
| 4   | 4     | 216.0            | 2779                | Medium Mountain |               |
| 5   | 5     | 191.0            | 3637                | Medium Mountain | Official Antidoping |
| 6   | 6     | 216.0            | 3200                | Flat          |               |
| 7   | 7     | 110.0            | 2796                | Medium Mountain |               |
| 8   | 8     | 217.0            | 912                 | Flat          |               |
| 9   | 9     | 169.0            | 5412                | Mountain      |               |
| 10  | Rest  | /                | /                   | /             |               |
| 11  | 10    | 159.0            | 882                 | Flat          |               |
| 12  | 11    | 144.0            | 3097                | Medium Mountain | Study |
| 13  | 12    | 184.0            | 463                 | Flat          |               |
| 14  | 13    | 167.0            | 4664                | Mountain      |               |
| 15  | 14    | 210.0            | 6643                | Mountain      |               |
| 16  | 15    | 229.0            | 9490                | Mountain      |               |
| 17  | Rest  | /                | /                   | /             |               |
| 18  | 16    | 12.7             | 756                 | Time Trial    |               |
| 19  | 17    | 230.0            | 5336                | Medium Mountain |               |
| 20  | 18    | 151.0            | 1949                | Flat          | Official Antidoping |
| 21  | 19    | 209.0            | 3569                | Mountain      |               |
| 22  | 20    | 242.0            | 3464                | Mountain      | Study         |
| 23  | 21    | 31.5             | 67                  | Time Trial    |               |

In the Table are reported the correspondence between day, length (km), level difference (m) of each stage along with the kind of stage and the blood sampling performed for this study and the blood sampling for official anti-doping testing.

doi:10.1371/journal.pone.0042077.t001
Renal Function

Renal function was monitored by using creatinine and cystatin C. Estimated glomerular filtration rate (eGFR) was calculated by different equations based on these two parameters [26].

Analytes Measurement

Total Osteocalcin (Gla-OC) and undercarboxylated Osteocalcin (Glu-OC) were measured, in plasma, by Sandwich Enzyme Immunoassays (EIA - Takara Bio Inc., Otsu, Shiga, Japan) using specific monoclonal antibodies directed against the different forms of OC, following the manufacturer specifications. The specificity of each analyte was evaluated in duplicate. The higher the zero standard curve, all samples and standards were controlled by both internal and external quality control schemes; moreover, all analytes were assayed in a single batch and by the same technician to limit analytical variability.

Bone-specific alkaline phosphatase (BAP) and Tartrate-resistant Acid Phosphatase 5b (TRAP5b) activities were measured in serum by immunoassay methods (Quidel Corporation, San Diego, CA, USA) based on the conversion of the uncolored p-nitrophenylphosphate into its yellowish-colored dephosphorylated form, the p-nitrophenol in alkaline or acidic environment, respectively. The sensitivity of BAP assay kit was 0.7 U/L and CVs were 5.8% and 6.0% for TRAP5b. The reaction was stopped with 1N H2SO4. All steps were performed at RT. The absorbance was read at λ = 450 nm (VICTOR X3, Perkin Elmer, Waltham, MA, USA). The analytes concentrations in samples were calculated against their respective standard curves. All samples and standards were evaluated in duplicate.

Serum cortisol and testosterone concentrations were tested on a Bayer Elecsys 2010 (Bayer AG, Leverkusen, Germany). Sensitivities were 0.018 µg/dl for cortisol and 0.025 ng/mL for testosterone, while CVs were 2.8% and 3.4%, respectively.

During the study the analyzers were regularly calibrated and controlled by both internal and external quality control schemes; moreover, all analytes were assayed in a single batch and by the same technician to limit analytical variability.

Statistical Analysis

Statistical analysis was performed by GraphPad Prism v5.0 software (GraphPad Software Inc., LaJolla, CA, USA). All values in the descriptive analysis are expressed as the mean ± SD. Normal distribution of values was assayed by Kolmogorov-Smirnov normality test, while one-way Analysis of Variance (ANOVA) for repeated measures, with the Bonferroni’s correction, was used to compare data over time. Paired comparisons were performed by two-tailed t test. In the case of not normally distributed values, repeated measures were compared with the Kruskal-Wallis test with the Dunns’ correction. Correlation analysis was performed by the two-tailed Pearson correlation test (Spearman’s test for not normally distributed values); the same test was conducted to evaluate the correlation between the trends of these parameters across the time-points. The significance level was set at 0.05.

Results

Anthropometric and Power Measurements

Body weight significantly decreased in the second phase of the race, with the 22nd day significantly differed from day -1 and day 12 (Table 2). Consequently, the BMI at day 22 (20.4±1.06) was significantly lower than those calculated for day -1 (20.9±1.27, p<0.001) and day 12 (20.8±1.14, p<0.001).

Table 2. Anthropometric and power-related measurements across the stage race.

| Parameters                        | day -1   | day 12  | day 22  | P-value |
|-----------------------------------|----------|---------|---------|---------|
| Height (m)                        | 1.82±0.05| /       | /       | /       |
| Weight (kg)                       | 69.1±5.24| 68.9±4.94| 67.4±5.06| <0.05   |
| Mean power output (W)             | /        | 214.3±14.96| 256.2±14.63| <0.001  |
| Mean power output/Weight (W/kg)   | /        | 3.1±0.17 | 3.7±0.16 | <0.001  |
| Accumulated power output (W)      | /        | 1929.0±134.60| 2305.5±131.69| <0.001  |
| Accumulated power output/Weight (W/kg) | /        | 27.9±1.56 | 33.4±1.43 | <0.001  |
| Mean net energy expenditure (kcal) | 2213.1±156.01| 3401.5±249.41| 3755.9±239.96| <0.001  |
| Accumulated net energy expenditure (kcal) | 2213.1±156.01| 30613.4±2244.66| 33803.0±2159.62| <0.001  |

In the Table are reported the results of the measurements at the same time-points of blood sampling, with the exception of the height that is reported only for day -1. Mean power output and accumulated power output, as both raw data and corrected for body weight, refer to the mean or the sum, respectively, of the consecutive values. The same applies to mean net energy expenditure and accumulated net energy expenditure for whom the rest values (day -1) correspond to the basal metabolic rate. Measurements are expressed as mean±SD and values bearing different letters in apex are significantly different (p<0.05).

doi:10.1371/journal.pone.0042077.t002
To characterize the metabolic effort in which the athletes are involved, the power output and the net energetic expenditure were derived. Values are reported in Table 2. The mean of all the measurements relative to the first half of the race was significantly lower than those of the second phase (p < 0.001) as well as the sum of all the measurements (accumulated power output) until day 11 compared with those accumulated until day 21 (p < 0.001). Even for what concern the net caloric expenditure, an increase was evident throughout the race with significant differences among the basal value, the mean and the accumulated expenditures until the 12th and the 22nd day.

Both the mean and the accumulated power outputs strongly correlated with the accumulated and the mean net energy expenditures (r = 0.95, p < 0.001), and when they are corrected for body weight, this relationship is approximate to perfect fit (r = 0.99, p < 0.001).

Renal Function
No significant differences were reported on the serum parameters and also in glomerular filtration rate estimated using the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) creatinine equation, as previously reported [26].

Bone Metabolism Markers

The trend of the bone metabolic markers is illustrated in Figure 1. BAP was unchanged (Figure 1A), whilst TRAP5b increased in the final part of the race respect to day -1 (p < 0.01) (Figure 1B).

To give a numerical evaluation of the difference in metabolic activity of osteoblast and osteoclast, the ratio between the main metabolic indexes of these cells (BAP/TRAP5b) was calculated. The BAP/TRAP5b ratio significantly decreased from 8.3 ± 2.51 at day -1 to 7.4 ± 2.33 at day 12 (p < 0.05) and to 6.5 ± 2.10 at day 22 (p < 0.001).

A fair correlation was found between BAP and TRAP5b trends (r = 0.44, p < 0.05), while no correlation was found with the indexes of energetic expenditure and power output.

Osteocalcin

The data of total OC and Glu-OC are illustrated in Figure 2. Total OC decreased along the race (p < 0.01 between day -1 and day 12; p < 0.05 between day -1 and day 22) (Figure 2A). Absolute Glu-OC concentrations, at the contrary, were unchanged during the race, but they showed a higher inter-individual variability (Figure 2B).

The relative concentration of Glu-OC, i.e. the percentage of total OC represented by Glu-OC, increased in the first part of race (p < 0.01) (Figure 2C).

Moderate to good correlations were found between TRAP5b and total OC (r = 0.50, p < 0.05), total OC and Glu-OC (r = 0.51, p < 0.05), Glu-OC and Glu-OC percentage (r = 0.80, p < 0.001); total OC fairly correlated with BAP/TRAP5b ratio (r = 0.37, p < 0.05). Total OC correlated with mean and accumulated power outputs in a fair indirect manner (r = -0.44, p < 0.05 for raw data, and r = -0.46, p < 0.05 for weight corrected power outputs) and inversely with accumulated and mean net energy expenditures (r = -0.44, p < 0.05).

Energy Metabolism Markers

Adiponectin behavior during the race is shown in Figure 3. While adiponectin constantly rose (p < 0.001 between day -1 and day 22, p < 0.01 between day 12 and day 22) (Figure 3A), leptin steadily decreased (p < 0.05 between day -1 and day 12; p < 0.01 between day -1 and day 22) (Figure 3B). Adiponectin and leptin were inversely fairly related (r = -0.41, p < 0.05) and they fairly correlated with the BAP/TRAP5b index (r = 0.30, p < 0.05; r = 0.32, p < 0.05, respectively), while only adiponectin correlated with total OC in a fair indirect manner (r = 0.37, p < 0.05). Adiponectin, but not leptin, and fairly correlated with Glu-OC (r = 0.30, p < 0.05). Of note, the GluOC (%) directly fairly correlated with BMI (r = 0.41, p < 0.05) and the fat mass percentage (r = 0.48, p < 0.05), while absolute concentrations of GluOC fairly correlated with fat mass percentage (r = 0.45, p < 0.05). Parallel, adiponectin, but not leptin, showed a direct moderate correlation with BMI (r = 0.51, p < 0.001).

For what concern the mean and the accumulated power outputs they both equally correlated with the energy markers adiponectin (r = 0.44, p < 0.05) and, inversely, but more strongly, with leptin (r = -0.72, p < 0.001). These correlations were kept when the power outputs were adjusted on body mass. The energy expenditures indexes showed a good correlation, directly, with adiponectin (r = 0.51, p < 0.01) and inversely with leptin (r = -0.69, p < 0.001).
Hormones

Testosterone concentration was unchanged during the race (Figure 4A), whereas cortisol concentration at day 22 were decreased in respect to day -1 (p<0.01) (Figure 4B).

No differences in the ratio cortisol/testosterone were observed throughout the study; the values attested at 51.5±16.57 at day -1, 44.6±14.86 at day 12 and 48.3±15.25 at day 22.

Cortisol was directly fairly related with GlaOC (r = 0.41, p<0.05) and moderately with leptin (r = 0.55, p<0.01) and body mass (r = 0.56, p<0.01). Testosterone was directly moderately correlated with body weight (r = 0.56, p<0.01). Moreover, cortisol, but neither testosterone nor cortisol/testosterone, was inversely related with accumulated and mean power outputs, both as raw data (r = -0.45, p<0.05) and corrected for weight (r = -0.47, p<0.05). Finally accumulated and mean net energy expenditures were fairly inversely related to cortisol levels (r = -0.44, p<0.05).

Discussion

Three-weeks cycling stage races represent unique and paradigmatic situations of long and strenuous daily competition [27,28] and they represent ideal models for investigating the homeostatic responses to strenuous and continuous workload. Cycling is one of the hardest sport discipline, owing to high demanding training and competition, long-lasting season, high level of competition and high selection within athletes that results in only about 800 professionals in the world. The 3-weeks stage races represent the highest level of fatigue [28], possibly affecting the athletes’ health.

A recent knowledge in endocrinology suggests a fundamental role of bone in energy metabolism. Bone is not a passive tissue but it is able to secrete hormones, and, among them OC, which act on pancreatic cells to regulate insulin and, consequently, glucose.

**Figure 2.** Modification of plasma osteocalcin levels and relative concentration of Glu-OC over the race. The Figure shows the trends of plasma total OC (panel A), plasma Glu-OC (panel B) and relative percentage concentration of Glu-OC (panel C) over the stage race. ** and * indicate significant variations (p<0.01 and p<0.05, respectively).

**Figure 3.** Modification of adipokines levels over the race. The Figure shows the trends of serum adiponectin (panel A) and serum leptin (panel B) over the stage race. ***, ** and * indicate significant variations (p<0.001, p<0.01 and p<0.05, respectively).

doi:10.1371/journal.pone.0042077.g002
doi:10.1371/journal.pone.0042077.g003
For what concern the bone metabolism, BAP activity remained stable (Figure 1A), testifying that osteoblast metabolism, and thus bone formation, are preserved during such a strenuous exercise. BAP activity decreased the day after an ultramarathon of 245 km and then recovered pre-race values after 3 and 5 days [33]. Throughout the training year BAP activity was reported to decrease in professional triathletes [34] and to increase in alpine skiers [13]. Thus, BAP activity is modified in long-term periods in athletes: a stage race could be too short for evidencing modifications or even, cyclists could have specific stability of bone formation parameters, as deducted from the narrow inter-individual variability in these athletes; however, data on this specific topic are lacking.

The bone resorption appear to be stimulated during the race as witnessed by the increase in TRAP5b activity more evident in the second phase of the race when, however, a higher inter-individual variability is present (Figure 1B). The resorption seems to be strictly connected to this specific continuous and stressful exercise, since it is not reported, after long periods of training, in top-level athletes of other disciplines [12,34,35] However, when TRAP5b is evaluated, its increase is evidenced, in top-level athletes, during the most intense part of the season [13]. The balance between the metabolic activities, operating within the bone remodeling unit, could be represented by the ratio between BAP and TRAP5b. During the race this ratio decreases, better demonstrating the imbalance towards the resorption. This trend, due to osteoclasts activation specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity exercise specifically marked by TRAP5b, which could enhance OC decarboxylation, may represent a common trait of high-intensity...
Bone and Energy Metabolism in Competing Cyclists

through only 5 days. Heavy increase in OC is likely due to the stressful initial mechanical stimulation exerted on the bone; finally, no information about the form of OC recognized by the assay were reported [11]. The energy metabolism is potentely enhanced during the 3-weeks stage race; despite of the very high-caloric diet, the tremendous need of energy, testified by the increase in the net energy expenditure and the decrease in weight and, thus, in BMI (Table 2), stimulates adipokines and related bone metabolites. Moreover, adipokines trends were clearly related to the power output and the energy expenditures.

Even if the only correlation among adipokines and bone OC forms was found between adiponectin and Glu-OC, evident correlations were found among Glu-OC, Glu-OC (%) and fat mass (%) and BMI as well as between the adipokines and these anthropometrical features. These findings suggest the presence of a relation between undercarboxylated OC and the body composition in line with previous reports. However, we speculate the existence of another factor involved in their molecular link and further studies must be addressed to uncover this marker. This link is stressed by the relationship, even if fair, between the bony metabolic index BAP/TRAP5b and levels of adiponectin and leptin.

The decrease of cortisol (Figure 4A) was similar to that one previously described during Vuelta [11]. Cortisol trend should be linked to an exhausting mechanism, as suggested by the relationship the indexesthe of metabolic effort. At the same time, a decrease in endurance efforts and in adrenal sensitivity to adrenocorticotropic hormone stimulation, and a decreased hypothalamic-pituitary axis sensitivity to cortisol negative feedback, may occur [42]. Catabolic status, which is expected during a stage race, is usually marked by an increase of cortisol. In our study, despite the cortisol decrease, the catabolic status is, however, manifest and it is demonstrated, at the adipose tissue level, by the leptin decrease and, at the bony level, by the decrease in total OC (Figure 2) and BAP/TRAP5b. Remarkably, positive relationships were found among cortisol and leptin and among cortisol and total OC. A possible explanation of this phenomenon could be hide in the activation of the sympathetic nervous system, which is known to affect the physiological homeostasis.

We did not observe significant decrease in testosterone concentrations (Figure 4B), differently from a previous report [11] where testosterone constantly decreased through the three weeks of race of Vuelta, in16 cyclists belonging to two different professional teams. Testosterone was significantly different between the two teams, but the decreasing trend was observed for both [11]. This decrease was explained by body mass loss accompanied by the reduction in fat mass; similarly, in our study, we observed correlation between testosterone values and weight loss, despite the decrease of testosterone levels was not significant. Of note, our basal levels were lower than those reported before Vuelta, while similar during the competition. A previous study showed that testosterone values in professional cyclists, similar to ours, were no different from those in controls, who had a higher bone density [37]. We did not observe any correlation between cortisol, testosterone, cortisol/testosterone and bone markers, suggesting that the control of bone metabolism in cyclists is uncoupled from these hormones.

The variations of testosterone and cortisol in cyclists, even testifying a catabolic state, did not automatically lead to a decrease in performance or to a state of overtraining, as demonstrated in other sport disciplines [43]. Thus, further specific studies in cycling, during stage races and also during a whole season, should be necessary to better define hormonal modifications.

We finally want to point out that, to our knowledge, this is the first study in which the pre-analytical management of the samples has been accurately checked and, thus, we highlight a warning about the previous researches concerning the pre-analytical phase, mostly undescribed.

In conclusion, during a 3-weeks stage race, bone metabolism parameters showed an unbalance towards resorption. A possible relationship between bone and energy metabolisms is suggested by the relative correlations among absolute and relative concentrations trends of undercarboxylated OC, adipokines concentrations, BMI, fat mass (%), power output and the derived energy expenditure. The presence of this association, although a direct link cannot be demonstrated, supports the evidence of a strict involvement of bone in the regulation of the energy metabolism.

Acknowledgments

We are grateful with the Liquigas-Cannondale pro-cycling team for the availability and the logistics of the study. The authors would also thank Dr. Antonino Coco who performed the blood drawings at day 12.

Author Contributions

Conceived and designed the experiments: GB PL GL RC. Performed the experiments: PL GL. Analyzed the data: PL GL. Contributed reagents/materials/analysis tools: AG RG. Wrote the paper: GB PL GL.

References

1. Foster C, Hoyos J, Earnest C, Lucia A (2005) Regulation of energy expenditure during prolonged athletic competition. Med Sci Sports Exerc 37: 670–675.
2. Lucia A, Diaz B, Hoyos J, Fernandez C, Villa G, et al. (2003) Hormone levels of world class cyclists during the Tour of Spain stage race. Br J Sports Med 35: 424–430.
3. Saris WH, van Erp-Baart MA, Brooks F, Westerterp KR, ten Hoor F (1998) Study on food intake and energy expenditure during extreme sustained exercise: the Tour of France. Int J Sports Med 10 Suppl 1: S26–31.
4. Padilla S, Mujika I, Nunthakul J, Impellizzeri FM, Goriena JJ (2008) Exercise intensity and load during uphill cycling in professional 3-week races. Eur J Appl Physiol 102: 431–436.
5. Hale R, Loucks AB (2004) Dose-response relationships between energy availability and bone turnover in young exercising women. JBone Miner Res 19: 1231–1240.
6. Zanker CL, Swaine II (2000) Responses of bone turnover markers to repeated endurance running in humans under conditions of energy balance or energy restriction. Eur J Appl Physiol 85: 434–440.
7. Chicharro JL, Lopez-Calderon A, Hoyos J, Martin-Velasco AI, Villa G, et al. (2001) Effects of an endurance cycling competition on resting serum insulin-like growth factor I (IGF-I) and its binding proteins IGFBP-1 and IGFBP-3. Br J Sports Med 35: 303–307.
8. Corretti R, Lombardi G, Lanteri P, Colombini A, Graziani R, et al. (2012) Haematological and iron metabolism parameters in professional cyclists during the Giro d’Italia 3-weeks stage race. Clin Chem Lab Med 50: 949–956.
9. Chicharro JL, Hoyos J, Bandres F, Terrados N, Fernandez B, et al. (2001) Thyroid hormone levels during a 3-week professional road cycling competition. Horm Res 56: 159–164.
10. McMurray RG, Hackney AC (2005) Interactions of metabolic hormones, adipose tissue and exercise. Sports Med 35: 393–412.
11. Fernandez-Garcia B, Lucia A, Hoyos J, Chicharro JL, Rodriguez-Alomo M, et al. (2002) The response of sexual and stress hormones of male pro-cyclists during continuous intense competition. Int J Sports Med 23: 555–560.
12. Bandi G, Lombardi G, Colombini A, Lippi G (2010) Bone metabolism markers in sports medicine. Sports Med. 697–714.
13. Lombardi G, Colombini A, Freschi M, Tavana R, Bandi G (2011) Seasonal variation of bone turnover markers in top-level female skiers. Eur J Appl Physiol 111: 433–440.
14. Rector RS, Rogers R, Ruebel M, Hinton PS (2008) Participation in road cycling vs running is associated with lower bone mineral density in men. Metabolism. 226–230.
15. Nichols JF, Palmer JE, Levy SS (2003) Low bone mineral density in highly trained male master cyclists. Osteoporos Int 14: 644–649.
16. Olmedillas H, Gonzalez-Aguero A, Moreno LA, Casajus JA, Vicente-Rodriguez G (2011) Bone related health status in adolescent cyclists. PLoS One 6: e24841.
17. Confavreux CB, Levine RL, Karsenty G (2009) A paradigm of integrative physiology, the crosstalk between bone and energy metabolisms. Mol Cell Endocrinol 310: 21–29.

18. Confavreux CB (2011) Bone: from a reservoir of minerals to a regulator of energy metabolism. Kidney Int Suppl: S14–19.

19. Ducy P (2011) The role of osteocalcin in the endocrine cross-talk between bone remodelling and energy metabolism. Diabetologia 54: 1291–1297.

20. Bousaada A, Chamari K, Zouali M, Feki Y, Zbidi A, et al. (2010) Review on leptin and adiponectin responses and adaptations to acute and chronic exercise. Br J Sports Med 44: 629–630.

21. Vogt S, Heinrich I, Schumacher YO, Blum A, Roecker K, et al. (2006) Power output during stage racing in professional road cycling. Med Sci Sports Exerc 38: 147–151.

22. Banfi G, Dolci A (2003) Preanalytical phase of sport biochemistry and haematology. J Sports Med Phys Fitness 43: 223–230.

23. Lippi G, Banfi G, Maffulli N (2010) Preanalytical variability: the dark side of the moon in blood doping screening. Eur J Appl Physiol 109: 1003–1005.

24. Meier C, Nguyen TV, Handelsman DJ, Schindler G, Kushir MM, et al. (2008) Endogenous sex hormones and incident fracture risk in older men: the Dubbo Osteoporosis Epidemiology Study. Arch Intern Med 168: 47–54.

25. Campion P, Nevill AM, Karlsson MK, Lounana J, Shabani M, et al. (2010) Bone status in professional cyclists. Int J Sports Med 31: 511–515.

26. Smathers AM, Bemben MG, Bemben DA (2009) Bone density comparisons in male competitive road cyclists and untrained controls. Med Sci Sports Exerc 41: 290–296.

27. Smathers AM, Bemben MG, Bemben DA (2009) Diet, body composition, and bone mass in well-trained cyclists. J Clin Densitom 13: 43–50.

28. Penteado VS, Castro CH, Pinheiro Mde M, Santana M, Bertolino S, et al. (2010) Diet, body composition, and bone mass in well-trained cyclists. J Clin Densitom 13: 43–50.

29. Hinton PS, Rolleston A, Rehner NJ, Hellemans IJ, Miller BF (2010) Bone formation is increased to a greater extent than bone resorption during a cycling stage race. Appl Physiol Nutr Metab 35: 344–349.

30. Duclos M, Corcuff JB, Rashidi M, Fougeres V, Manier G (1996) Does functional alteration of the gonadotropic axis occur in endurance trained athletes during and after exercise? A preliminary study. Eur J Appl Physiol Occup Physiol 73: 427–433.

31. Hoogeven AR, Zonderland ML (1996) Relationships between testosterone, cortisol and performance in professional cyclists. Int J Sports Med 17: 425–426.