Irisin – a myth rather than an exercise-inducible myokine

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The myokine irisin is supposed to be cleaved from a transmembrane precursor, FNDC5 (fibronectin type III domain containing 5), and to mediate beneficial effects of exercise on human metabolism. However, evidence for irisin circulating in blood is largely based on commercial ELISA kits which are based on polyclonal antibodies (pAbs) not previously tested for cross-reacting serum proteins. We have analyzed four commercial pAbs by Western blotting, which revealed prominent cross-reactivity with non-specific proteins in human and animal sera. Using recombinant glycosylated and non-glycosylated irisin as positive controls, we found no immune-reactive bands of the expected size in any biological samples. A FNDC5 signature was identified at ~20 kDa by mass spectrometry in human serum but was not detected by the commercial pAbs tested. Our results call into question all previous data obtained with commercial ELISA kits for irisin, and provide evidence against a physiological role for irisin in humans and other species.

In 2012, Boström et al.1 described irisin as a cleaved and secreted part of the transmembrane protein FNDC5 (fibronectin type III domain containing 5). They proposed irisin as an exercise-induced myokine triggering “browning” of white adipose tissue. These findings sparked a debate mainly turning on two issues: (I) the relevance of irisin in humans, (II) the specificity of commercially available enzyme-linked immunosorbent assays (ELISA), and more specifically the polyclonal antibodies on which they were based.

First, following the initial study1, it was realized that the start codon of the human FNDC5 gene is mutated from the normal ATG to ATA. There are examples of proteins being expressed from unusual start codons2, however, Raschke et al.3 found that FNDC5 transcripts derived from the AUA start codon were translated to protein with extremely low efficiency as compared to the normal AUG start codon. All other animal species have an ATG as start codon at this position. This suggests that the human species has an effective gene knockout of FNDC5 and, consequently of irisin. Furthermore, Timmons et al.4 expressed doubts about the response of FNDC5 mRNA in human muscle to exercise, based on their previous and larger data sets, which showed no such response. Nevertheless, a number of research groups around the world have examined the effects of exercise on irisin levels in human serum. These studies, mostly using commercial ELISA kits that are questioned here, have given contradictory results. Huh et al.5 observed a significant increase in serum irisin in response to acute exercise after 1 week of moderate training whereas no effect of acute exercise was found after 8 weeks of training intervention. Short term effects of exercise on irisin levels were also reported by some authors5-9. In contrast, no systematic effects of exercise on circulating irisin were found in several other studies10-17.

Second, the FNDC5 antibody used in the initial study1 was raised against the C-terminus of the protein (amino acids [aa] 149–178), which is not part of the cleaved irisin peptide (aa 32–143; GenPept accession number NP_715637). Thus, as initially noted by Erickson18, the 20 kDa band detected in western blots in that study should not be irisin, but is probably a non-specific cross-reacting protein. Further studies employed western blots with different antibodies against this epitope and found immune-reactive bands in the range of 20–26 kDa in serum or plasma of rats, mice and humans19-22. Again, all these antibodies were generated against the C-terminal FNDC5 segment, which is not part of circulating irisin. An antibody raised against partial irisin (aa 42–112), which should detect irisin, stained a band at 25 kDa as well as bands of higher molecular weight in western blots of the secretome of cultured rat muscle cells and adipocytes21. In previous studies, we used an antibody against full-
length irisin (aa 32–143) and observed an immune-reactive band at ~13 kDa, the theoretical size of non-glycosylated irisin, in murine serum but not in bovine plasma.23,24.

The therapeutic potential of irisin to fight obesity and diabetes has aroused extensive interest. Several commercial sources have marketed kits for ELISA, EIA, and RIA to detect and quantify irisin in different biological fluids, under different exercise interventions and/or in different diseases (reviewed by Sanchis-Gomar et al.25, Elsen et al.26). A striking feature of these investigations was the vast variation of reported irisin levels, differing by orders of magnitude even in healthy subjects. This was the case for results obtained with tests from different manufacturers but also with the same test from a single supplier used in different laboratories. More than 80 studies have been published with irisin levels ranging between 0.01 and more than 2,000 ng/mL in human serum or plasma. These commercial kits were usually tested for the ability to detect bacterially expressed irisin (recombinant protein produced in the bacterial cytoplasm, and therefore not glycosylated), and a wide range of sensitivity was reported. For example, a polyclonal antibody used in an ELISA kit (designated here pAb-A) was reported by the supplier to have a detection range of 0.38–205 ng/mL, and a linear range of 4–41 ng/mL. We believe this is an acceptable validation of the sensitivity of the ELISA kit for assaying purified recombinant irisin, in the absence of other proteins.

However, in addition to recognizing the specific antigen, polyclonal antibodies often cross-react with non-specific proteins. When an ELISA kit, based on a polyclonal antibody against irisin, is used to assay a complex protein sample such as plasma or serum, the signal will include that from any irisin but also that from cross-reacting proteins. Some of these may be present at high levels and can dominate the signal. To control for this, it is necessary to validate the antibody by western blot in the target tissue or fluid. Western blots were shown in the original study4, but these showed multiple strong cross-reacting proteins and were based on an inappropriate polyclonal antibody18. Only a few of the subsequent studies have presented full-size western blots that reveal cross-reacting proteins in plasma or serum.21,23,24,27. Many studies using only ELISA have apparently ignored the possibility of cross-reacting proteins contributing to the signal.

In our present study, we examined the specificity and sensitivity of currently available irisin antibodies by comparative western blot analysis. For standards we produced recombinant non-glycosylated irisin (rNG-irisin), which runs on SDS-PAGE as a sharp band at 13 kDa, and recombinant, glycosylated irisin (rG-irisin), expressed in mammalian HEK293 cells and running as a smeared band at 18–25 kDa, with a peak at about 20 kDa. By diluting rNG-irisin into plasma and running western blots we achieved two calibrations: determination of the sensitivity of the assay for irisin, and detection of cross-reacting proteins of various molecular weights. We then extended the assay to survey a range of animal species for the presence of irisin in serum or plasma. In addition, we analyzed samples from a previously published training intervention study13 with an additional ELISA and western blots to compare irisin levels obtained with different methods. Mass spectrometry was used to search for FNDC5 or irisin signatures in human serum at different sizes after SDS-PAGE. Finally, RNA sequencing was employed to gain insight about the abundance of different transcripts of FNDC5 in human muscle.

Results
Detection of rNG-irisin with pAb-A. Dilution series of rNG-irisin into either phosphate buffered saline (PBS) or bovine plasma were analyzed with anti-irisin pAb-A, raised against full length NG-irisin (Fig. 1a). Bovine plasma was used for the initial test because our previous study had shown no detectable circulating irisin4. Two murine sera with unknown irisin levels, human serum samples with irisin levels previously measured with a corresponding ELISA kit (based on pAb-A), and a murine muscle sample were analyzed on the same blot. The antibody reacted with a single band at ~13 kDa in PBS and bovine plasma containing the higher concentrations of added rNG-irisin (Fig. 1a). This band could be completely quenched by preincubation of the primary antibody with 5-fold the amount of rNG-irisin (Fig. 1b). Densitometric analysis of the irisin dilution in bovine plasma revealed linearity in the range from 4 to 0.125 ng irisin/lane (lanes 10–15 in Fig. 1a; $R^2 = 0.9947$, Fig. 1c). Recovery

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**Figure 1 | Western blot of dilution series of rNG-irisin, using pAb-A.**

(a) rNG-irisin was diluted into PBS (lanes 1–8; absolute amounts 20, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 ng irisin/lane) or diluted in bovine plasma (lanes 9–16; identical amounts). Two samples of mouse serum (lanes 17, 18) and samples of human serum (lanes 19–24) are included. Mouse muscle protein extract is in lane 25. (b) pAb-A was blocked with 5-fold amount rNG-irisin prior to staining the blot. Images were taken after 2 (a) and 4 (b) min exposure to adjust for different background chemiluminescence, and were equally enhanced in contrast. (c) Band volumes of recombinant NG-irisin (lanes 9–15 in (a)) were densitometrically analyzed and plotted against irisin amount (ng) in bovine serum. The highest (20 ng) and lowest (0.0625 ng) concentrations were omitted from this analysis.
rates for the spiked irisin ranged from 75% to 96% in the lanes used for determination of linearity. Because the sample contained 1 μL of plasma we were able to detect irisin at a concentration of 125 ng/mL with this western blot.

The western blot showed several cross-reacting proteins staining much more intensely than even the higher concentrations of added NG-irisin. The band at ~45 kDa is approximately the size for IgG. However, cross-reaction of IgG with the secondary detection system can be excluded because the TrueBlot® system used here is designed to avoid reaction with denatured IgG, and control blots omitting the primary antibody showed no unspecific staining. The band at ~30 kDa is too large to be irisin. The smear from 50–70 kDa and the higher molecular weight bands include albumin and unknown proteins. However, it is clear that these cross-reacting proteins would dominate the signal if this pAb-A is used to assay bovine plasma or human serum.

Notably, irisin was not detected by pAb-A in human serum although levels between 324 and 864 ng/mL were previously reported using the corresponding ELISA kit13. This is equivalent to absolute amounts between 0.324 and 0.864 ng/lane on the blot (Fig. 1a), clearly in the linear range of detection by our western blot. A weak band slightly smaller than rNG-irisin was visible in murine serum, but as discussed below is apparently not irisin.

The experiment was repeated with antibodies pAb-B, C, and D (Supplementary Fig. 1). All antibodies proved capable of detecting rNG-irisin added to PBS or bovine plasma in a similar linear range. However, none of them stained a band of the expected size for glycosylated irisin in murine or human samples. A strong immune-reactive band at ~25 kDa was observed only with pAb-C in human but not in murine samples which is the upper size limit for glycosylated irisin. However, this band was not detected by any other irisin antibody, thus it is probably a cross-reaction specific for pAb-C. Analysis of serum samples with pAb-E (raised against aa 149–178 of FNDC5) revealed no specific band in serum but in murine muscle extract it stained a sharp, single band with the size of full length FNDC5 (~24 kDa; Fig. 2a). The strong doublet band at about 25 kDa stained uniquely by pAb-C (Fig. 2b) was tested for deglycosylation. In contrast to rG-irisin, whose size was significantly reduced by PNGase, the ~25 kDa doublet was unaffected (Fig. 2b,c). This band was analyzed by mass spectrometry, described later.

Besides the variability in band patterns observed between the tested antibodies, a high lot-to-lot variability was observed for antibodies pAb-A and C (Supplementary Fig. 2). Different lots of antibodies pAb-B and D were not compared.

**Western blot analysis of irisin in serum and plasma of mammalian species.** Sera were analyzed from 3 horses undergoing extreme physical exercise (160 km endurance race) and from 1 horse with established metabolic syndrome. For comparison, samples from cattle, domestic pig, wild boar, donkey, goat, rabbit and mouse were included (Fig. 3). Human samples with irisin levels of 76 ng/mL and 864 ng/mL, previously measured with ELISA (pAb-A), were from 1 healthy and 1 pre-diabetic individual prior to or 2 hours after a single bout of acute exercise13. All samples were analyzed by western blot with antibodies pAb-A and pAb-C (Fig. 3a,b). Recombinant NG- and rG-irisin were used as positive controls. No antibody detected proteins at the size of the positive controls, indicating that neither non-glycosylated nor glycosylated irisin circulated in the serum/plasma of any species. However, immune-reactive bands were observed at ~16 kDa in a human sample and at ~25 kDa in both human and goat samples with pAb-C. These bands were consequently further analyzed by HPLC/mass spectrometry.

Separate analysis of 11 samples from 6 horses with pAb-C showed a similar band pattern to that in lanes 3–6 (Fig. 3b). Band volumes were not related to physical activity or disease and were not correlated to results obtained with pAb-B on the same samples (Supplementary Fig. 3).

**Comparison of ELISA data with semi-quantitative western blot analyses.** A total of 156 serum samples from 26 individuals from a previously published exercise study13 were re-analyzed with an additional irisin ELISA based on pAb-C and by semi-quantitative western blot analysis with antibody pAb-C. Irisin levels were previously measured with ELISA based on pAb-A and the data were used for correlation analyses with the results of the present study. Western blot analyses revealed 3 prominent bands at ~16 kDa (larger than rNG-irisin), ~25 kDa, and ~30 kDa in all samples (Fig. 4a). The 16 kDa band was not observed in previous tests with pAb-A (Fig. 1a). Regions of interest were selected and individual band volumes were measured densitometrically (Fig. 4b). There was no significant correlation between normalized volumes of any of the 3 bands or their combined volumes and irisin concentrations measured with the corresponding ELISA kit (pAb-C; r = −0.109, p = 0.46; Fig. 4c). Furthermore, irisin levels measured with the 2 different ELISA kits differed by a factor 18 on average (overall means 2,961 ng/mL vs. 164.5 ng/mL) and were not correlated at all (r = 0.034, p = 0.67; Fig. 4d). In contrast, the volume of a non-specific band observed at ~50 kDa with pAb-A (Fig. 4e) was significantly correlated to irisin levels measured with the corresponding ELISA (pAb-A) in an arbitrarily selected subset of samples (n = 24, r = 0.730, p < 0.001; Fig. 4f). Thus, using the ELISA based on pAb-C (Supplementary Fig. 4) we were unable to repeat the previous finding of an acute effect of exercise on irisin levels in serum, which used an ELISA based on pAb-A13.

**Immuo-precipitation and protein identification by mass spectrometry.** The immuno-reactive bands at ~25 kDa stained by pAb-C in human (Supplementary Fig. 1b, Fig. 3b) and goat serum (Fig. 3b) were purified by immuo-precipitation with pAb-C cross-linked to magnetic beads (Fig. 5a,b). An additional band at ~16 kDa observed earlier in human serum (Fig. 3b) was also included in subsequent mass spectrometric analysis. No peptide corresponding to FNDC5 or its irisin-part was identified in any of the precipitated samples in
Samples in lanes 20–25 differ between (a) and (b). (a) and 20 min (b) exposure and equally enhanced in contrast. Boxes indicate bands of rNG-irisin (rNGI), rG-irisin (rGI), and synthetic (SI) irisin.

Expression of FNDC5 mRNA in human skeletal muscle. Re-analysis of samples of m. vastus lateralis from control and pre-diabetic subjects in a training intervention study by RNA-sequencing confirmed a small but statistically significant increase of total FNDC5 mRNA levels after 12 weeks of exercise compared to baseline levels (fold change = 1.185, p < 0.001). Effects of the health status were not observed in accordance to the previous analysis.

The current NCBI RefSeq model of the human FNDC5 gene predicts 3 transcripts (T1: NM_001171941.2, T2: NM_153756.2, T3: NM_001171940.1). Transcript 1 is proposed to be produced by alternative splicing from an upstream exon 1a, whereas the two other transcripts share exon 1b (Fig. 6a). Transcript 1 would produce a truncated FNDC5 protein from an in-frame AUG codon in exon 3, and the proposed irisin peptide would miss the first 44 amino acids.

However, the Kozak sequence around this AUG is very weak and there are 3 other upstream out-of-frame AUG codons that would make translation of this truncated protein unlikely. Thus, analysis of 154 muscle biopsies in the training intervention study revealed no expression of exon 1a specific for this transcript. In contrast, transcripts 2 and 3 were expressed at high levels as indicated by the data of their shared exon 1b (Fig. 6b). A dissection of the expression to specific transcripts 2 or 3 is not possible due to the structure of the locus (Fig. 6a).

Discussion
Almost 3 years after the discovery of irisin the debate over its potential role in human metabolism has not been settled. Only recently, Elenet al. noted that the role of irisin in humans appears still questionable despite the numerous studies. Spiegelman & Wrann commented in reply that there are more than 45 articles reporting human irisin levels measured with a variety of immunoassays with independently derived antibodies and thus they considered the existence of human irisin in blood a closed issue. However, not one of those studies has addressed the possibility that the immunoassays might be reporting cross-reacting proteins in serum or plasma, and not irisin itself.

Our present study examined 4 antibodies of which 3 were used in corresponding ELISAs. More than 80 studies have been published based on these assays. We showed that all antibodies had prominent cross-reactions with non-irisin proteins in serum or plasma of different species. For one antibody we found a strong correlation between the volume of a prominently stained ~50 kDa band in human serum, which is clearly not irisin, and irisin levels measured with a variety of immunoassays with independently derived antibodies and thus they considered the existence of human irisin in blood a closed issue. However, not one of those studies has addressed the possibility that the immunoassays might be reporting cross-reacting proteins in serum or plasma, and not irisin itself.

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Importantly, our Western blots failed to detect irisin or glycosylated irisin in plasma or serum of human and several animal species, even after extreme exercise in the case of horses. A 13 kDa band in mouse serum, identified previously as potential irisin 23, was stained by pAb-A, but not by -B, -C or -D. This strongly suggests that it is also a cross-reacting protein specific to pAb-A. The detection limit of the most sensitive western blots was about 100 ng/mL being a potential limitation of this study. However, this corresponds to approximately 10 nM which is below the concentration of recombinant FNDC5 or irisin (20 nM) used in cell culture assays 13. Because hormones are typically present in circulation at pM to nM concentrations, it cannot be ruled out that circulating irisin at less than 10 nM could have activity in vivo.

Our data on FNDC5 expression in skeletal muscle demonstrate that the human gene is exclusively translated from transcripts with a mutant start codon. Considering the translation efficiency of such transcripts – 1% compared to transcripts with a canonical start codon 1 – very low amounts of FNDC5 could be expected in skeletal muscle. Hence, it is unlikely that irisin can be cleaved and secreted into circulation at amounts measured with ELISAs previously. This is further supported by the observation that acute exercise did not cause a significant up-regulation of FNDC5 and that the mRNA abundance increased only slightly after 12 weeks of training intervention. In contrast to most studies, which have neglected the possibility of cross-reaction, a recent study 22 employed western blots of human serum as the first step. These blots showed several strong cross-

Figure 4 | Semi-quantitative analysis of western blots with pAb-C and comparison with ELISA data (based on pAb-A and pAb-C). (a) Western blot (pAb-C) of serum samples of 4 individuals (lanes 1–4) at 6 time points (lanes a–f). RNG-irisin was included as positive control (lane 5). The image was taken after 10 min exposure. (b) Quantification of chemiluminescence of targeted bands (crosses) in marked lanes. Note that contrast enhancement in the region of measurement is only for visualization and does not influence the results. This measurement was done in all 156 samples from 26 individuals of a previous study 13. (c) Plot of normalized, combined band volumes against irisin levels measured with ELISA based on pAb-C. (d) Plot of irisin levels measured with ELISA based on pAb-A against ELISA based on pAb-C. (e) Quantification of a band (crosses) at 50 kDa detected by western blot of human serum samples with pAB-A. Samples were arbitrarily chosen from the previous study 13. (f) Volume of the non-specific 50 kDa band was plotted against irisin concentration measured with ELISA based on pAb-A.
reacting bands of higher molecular weight, but the study focused on bands of 32 and 24 kDa, interpreting them as glycosylated and deglycosylated irisin. As shown in our present report the molecular weights of purified rG- and rNG-irisin are actually 20 and 13 kDa, so the bands identified in that study are too large. Furthermore, they used the same antibody as Boström et al.\textsuperscript{1}, designated pAb-E here, which was raised against a C-terminal sequence that is not included in irisin. Finally, they analyzed the 32 and 24 kDa bands by mass spectrometry and reported a single tryptic peptide corresponding to the irisin sequence. We did not find any FNDC5 or irisin signatures in our immune-precipitated bands identified by western blots of human serum. However, we were able to detect irisin spiked into human serum at the predicted size for glycosylated and non-glycosylated irisin.

Mass spectrometry analysis of the ~20 kDa zone cut from SDS-PAGE of human serum detected a peptide corresponding to FNDC5 or irisin. This is the same peptide identified by Lee et al.\textsuperscript{22}, although they found it in ~32 and ~24 kDa bands stained with a pAb against C-terminal FNDC5. Consequently, our result is the first mass spectrometry identification of an irisin peptide at the correct size, and might be considered as supporting the existence of irisin in human serum. However, none of the tested irisin antibodies stained a band at this size in serum. This underlines that glycosylated irisin has not been detected by western blot before and that none of the ELISA kits used in previous studies have measured irisin levels in serum or plasma of any species.

The birth of the new, promising myokine irisin\textsuperscript{1} has been complicated from the beginning by multiple contradictions and obscurities in the interpretation of western blot data\textsuperscript{18}. Numerous subsequent studies relied on ELISAs with commercial antibodies that were not sufficiently validated in biological fluids. This resulted in highly contradictory data concerning the existence of irisin and its role in humans and other species. Our study targeted the basic question behind the controversy – the physical existence of the proposed cleavage product of FNDC5 in circulation. The key to this was to use western blots to visualize and avoid the false signal from cross-reacting proteins of the polyclonal antibodies, and to use recombinant, non-glycosylated and glycosylated irisin as positive controls. In addition to calling into question all previous studies using commercial ELISAs, we found no evidence for circulating irisin in human or several animal species when examined by western blot with 4 different antibodies and a sensitive detection system. Although we report the first peptide signature size-matched to rG-irisin by HPLC/mass spectrometry in human serum, the apparently low concentration – below the detection limit of the tested antibodies – makes a physiological role for irisin very unlikely. Atherton & Philips\textsuperscript{32} raised the question whether irisin is a “Greek goddess or a Greek myth”. Our results provide experimental evidence for irisin being a myth.

**Methods**

**Ethical approval.** The human exercise study named MyoGlu adhered to the Declaration of Helsinki and was approved by the National Regional Committee for Medical and Health Research Ethics North, Tromsø, Oslo, Norway. The study was registered with the US National Library of Medicine Clinical Trials registry (NCT01803568). Written informed consent was obtained from all participants prior to any study-related procedure. Equine blood samples were taken in accordance with the local animal ethics committee (Etat de Vaud, Service Vétérinaire, Switzerland).
Strength and endurance training intervention. Healthy, sedentary men (40–65 years) were recruited in 2 groups; controls with normal weight (23.5 ± 2.0 kg/m²) and normal fasting and 2 h serum oral glucose tolerance test (OGTT) levels (n = 13) or overweight (29.0 ± 2.4 kg/m²) with abnormal glucose metabolism (pre-diabetes group, n = 13). Abnormal glucose metabolism was defined as fasting glucose ≥ 5.6 mmol/L and/or impaired glucose tolerance (2 hours serum OGTT ≥ 7.8 mmol/L). The participants underwent 2 endurance bicycle sessions (60 min) and 2 whole-body strength-training sessions (60 min) per week for 12 weeks. A 45 min bicycle session at 70% of VO₂max was performed before and after the 12 week training period as an acute work challenge 13.

Blood and tissue sampling. Blood and muscle samples were taken before, directly after, and 2 h after the 70% of VO₂max bicycle test, before as well as after 12 weeks of training. Blood samples were taken by standard antecubital venous puncture. Biopsies from m. vastus lateralis were immediately rinsed in phosphate buffered saline (PBS), dissected free of blood, before they were transferred to RNA-later (Qiagen, Limburg, Netherlands; overnight at 4°C) and finally transferred to −80°C. Serum was stored at −80°C until analysis.

Animal serum and plasma samples. Samples for species comparison were obtained from commercial suppliers (donkey, goat, rabbit), from previous studies in swine and wild boar 33, and from our own investigations in cattle 24 and mice 23. These samples were not further characterized and represented the respective species only.

Serum of a horse that underwent a moderate physical exercise program (45 min with intervals of walking, trotting and 3 times 1500 m at a speed of 8.5, 8.4, and 10.3 m/s) was sampled in the Swiss National Stud in Avenches (Switzerland) immediately and 30 min post exercise. Further samples were taken from 5 horses immediately or 60 min after participation in a 160 km endurance race (Rambouillet, France).

Figure 6 | FNDC5 transcripts and deduced peptides. (a) Transcript structure of human FNDC5 (T1: NM_001171941.2, T2: NM_153756.2, T3: NM_001171940.1) and deduced peptide structure (P1–P3). Numbers refer to nucleotides (T1-3) or amino acids (P1–3). Black bars represent exons. Exon numbers are given in circles. Start and stop codons are indicated above the bars. The irisin peptide is marked by a bold box. The open box marks the truncated irisin peptide in P1 theoretically resulting from transcript T1. The size of irisin and FNDC5 protein variants is given. The peptide signature identified by mass spectrometry is marked in red. SP: signal peptide. (b) Example for detection of FNDC5 transcripts by RNA-sequencing of skeletal muscle biopsies. Exon 1a is specific for transcript T1 whereas exon 1b is part of transcripts T2 and T3. The panels represent results for one individual before (A1–A3) and after 12 weeks of training intervention (B1–B3). A1 and B1 were measured before, A2 and B2 immediately after, and A3 and B3 2 hours after acute exercise 13.
Expression and purification of recombinant, non-glycosylated and glycosylated irisin. For convenient his-tag removal, the bacterial irisin expression construct\(^\text{24}\) was modified by inserting the 3C protease cleavage sequence (LEVLFQPG) in the Nde I site. This modification drastically reduced protein solubility when it was expressed in E. coli BL21 (DE3) at 37 °C, as the his-3C construct was expressed in E. coli C41 (DE3)\(^\text{35}\) at room temperature. Expressed proteins were purified with a cobalt column (TALON; Clontech, Mountain View, USA) using standard protocols. Purified proteins eluted with imidazole from the column were dialyzed against phosphate buffered saline (PBS, pH 7.2) in the presence of his-tagged 3C protease at column (TALON; Clontech, Mountain View, USA) using standard protocols.

### Table 1 | Polyclonal irisin antibodies used in this and related ELISA kits

| Designation (this study) | Manufacturer/ Vendor (Cat. #) | Antigen (epitope)\(^1\) | Manufacturer (ELISA #) | Range of ELISA\(^2\) | Remarks |
|--------------------------|---------------------------------|------------------------|------------------------|---------------------|---------|
| pAb-A                    | Aviscera/Phoenix (G-067-52)     | recombinant irisin (aa 33–142) | Phoenix (EK-067-52)     | 0.3–205 ng/mL       | antibody discontinued; ELISA replaced by EK-067-29 |
| pAb-B                    | Cayman (14625)                  | synthetic irisin (aa 33–142) | -                       | -                   | no ELISA available |
| pAb-C                    | Adipogen (A-258-0027)           | recombinant irisin (aa 33–142) | Adipogen (AG-45A-0046EK) | 1.0–5,000 ng/mL     | antibody is epitope |
| pAb-D                    | Phoenix (G-067-16)              | synthetic irisin (aa 33–142) | Phoenix (EK-067-16)     | 0.1–1,000 ng/mL     | affinity purified |
| pAb-E                    | BioVision/ BioCat (AP87468-ab)  | C-terminus of FNDC5 (aa 149–178) | -                       | -                   | no ELISA available antibody is epitope affinity purified |

\(^{1}\)refers to GenPept accession number NP_715637.2.  
\(^{2}\)Data of manufacturer/vendor.

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Trans-Blot Turbo transfer pack, Bio-Rad, Munich, Germany) using a semi-dry blotter (Trans-Blot, Bio-Rad). Equal loading of the gels and proper transfer of the proteins to the membranes were verified by Coomassie Blue staining according to standard procedures. Membranes were blocked for 1 h in either 1% Roti-Block (Boehringer Mannheim, Germany) for detection of irisin (pAb-A, B, C and D), or in 5% non-fat dry milk in Tris-buffered saline (TBS) for detection of FNDC5 (pAb-E). Membranes were incubated with primary antibodies (pAb-A: diluted 1 : 3,000, pAb-B: 1 : 500, pAb-C: 1 : 2,000, and pAb-E: 1 : 1,000) overnight at 4 °C.

After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (rabbit IgG TrueBlot, 1 : 25,000; 18–8,816, ebioscience, Frankfurt, Germany). Antibody label was detected with chemiluminescence substrate (Super Signal West Femto, Thermo Fisher Scientific, Bonn, Germany) and a Chemocam HR-16 imager (INTAS, Göttingen, Germany). LabImage 1D software (Kapelan Bio-Imaging, Leipzig, Germany) was used to quantify the volumes of specific bands.

### Analysis of serum irisin.

Concentrations of irisin were measured in duplicates in human serum using enzyme-linked immuno-sorbent assays (ELISA [based on pAb-C]; Table 1) according to the manufacturer’s protocol. Optical density was determined using a microplate reader (Thermo Scientific Multiscan EX, Vantaa, Finland) set to 450 nm. Data from a previous measurement of irisin in plasma samples of the same individuals\(^1\) with a different ELISA (based on pAb-A; Table 1) was used for comparison. Letters assigned to the ELISAs correspond to the letters designated to the antibodies.

### Immuno precipitation and SDS-PAGE of serum samples with added irisin.

MagnaBind magnetic beads (Pierce, Thermo Fisher Scientific, Bonn, Germany), pre-coated with anti-rabbit IgG antibody, were incubated with anti-irisin antibody (Adipogen) for 60 min under permanent agitation (Dynabeads MX Mixer, Life Technologies, Darmstadt, Germany). Separation steps were performed using InvivMag (STRATEC, Berlin, Germany) magnetic separator. Binding of antibodies to the beads was made permanently according to standard protocols by using cross-linking buffer (25 mM DMAP) at room temperature for 45 min. To stop the reaction, blocking buffer (0.1 M Ethanolamine in PBS, pH 8.2) was used. Unbound antibody was eluted from the beads with elution buffer (1 M glycine-HCL, pH 3). Serum (500 µl) was denatured for 5 min at 95 °C and added to the antibody-coated beads. Incubation was performed overnight at 4 °C under permanent agitation. After several washes, proteins were eluted from beads with elution buffer (1 M glycine-HCL, pH 3). To optimize the protocol for subsequent mass spectrometry, the elution buffer was changed (8 M urea, 20 mM Tris pH 7.5, 100 mM NaCl) according to standard protocols (abcam, Cambridge, U.K.) in a repeated trial.

Eluted proteins were separated on a 15% polyacrylamide gel and stained with QuickCoomassie (Serva, Heidelberg, Germany) according to manufacturer’s instructions. Bands of interest were cut out of the gel and submitted to analysis by mass spectrometry. Furthermore, human serum samples were albumin-depleted with Aurum Affi-Gel Blue mini columns (Bio-Rad) as described elsewhere\(^\text{30}\). One hundred or 500 ng of either NG- or rG-irisin were then added to the samples. The samples (20 µg total protein) were separated by SDS-PAGE (15%) and stained with QuickCoomassie (Serva). Serum samples without addition of NG-/rG-irisin were also analyzed. Recombinant NG-/rG-irisin (500 ng) diluted in PBS were used for size comparison. Regions of interest were cut out of the gel and analyzed by mass spectrometry.

### Protein identification by mass spectrometry.

The Coomassie stained gel bands were in-gel digested using trypsin. The generated peptides were purified using p-C18 ZipTips (Millipore, Oslo, Norway), dried in a SpeedVac, dissolved in 10 µL 1% formic acid, 2.5% acetonitrile in water. Half of the volume was injected into a nano-UHPLC system (Ultimate 3000 RSLC, Dionex, Sunnyvale, CA, USA) coupled to an ESI-ion trap/Orbitrap (LTQ Orbitrap XL, Thermo Scientific, Bremen, Germany) mass spectrometry.
spectrometer. For peptide separation, an Acclaim PepMap 100 column (50 cm × 75 μm) packed with 100 Å C18 3 μm particles ( Dionex; Neflen Meszoly, Oslo, Norway) was used with a flow rate of 300 nL/min and a solvent gradient of 3% B to 35% B for 45 min. Solvent A was 0.1% formic acid and solvent B was 0.1% formic acid/90% acetonitrile. Survey full scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with the resolution R acid/90% acetonitrile. Survey full scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with the resolution R acid/90% acetonitrile. Survey full scan MS spectra (from m/z 300 to 2000),...
Author contributions
E.A., F.N., B.T., T.O., T.H., I.S., S.L., S.T. and J.B. performed the experiments, provided samples, materials and data, analyzed the data, and drafted parts of the manuscript. S.M., H.P.E. and C.A.D. conceived the study. S.M. coordinated the experiments and S.M. and H.P.E. wrote the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Albrecht, E. et al. Irisin – a myth rather than an exercise-inducible myokine. Sci. Rep. 5, 8889; DOI:10.1038/srep08889 (2015).