Free Radical Biology and Medicine 246 (2026) 660–667

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Circulating Neuregulin-4 tracks acute hyperbaric and workload stress in human divers, preceding oxidative injury markers

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ARTICLE INFO

Keywords:
Neuregulin-4 (NRG4)
Hyperbaric stress
Oxidative stress
Adipokines
Diving physiology

ABSTRACT

Acute hyperbaric stress during diving combines increased ambient pressure, hyperoxia, hemodynamic shifts, and often muscular workload. Identifying real-time blood biomarkers sensitive to these individual and combined physiological loads remains a challenge. Neuregulin-4 (NRG4), an adipokine secreted by thermogenic and subcutaneous white fat, responds to adrenergic stimulation and modulates redox homeostasis. We investigated NRG4 dynamics alongside oxidative protein carbonyls in divers in warm (thermoneutral) water (~33.6 °C ambient water temperature) to avoid cold stress.

Two field campaigns were conducted: a first depth response campaign involved divers exposed to 20, 30, or 40 m on separate days, without exercise, with serial blood sampling; a second physical effort study involved 15 m dives with or without slow-pedalling exercise. Serum NRG4 was quantified by ELISA and expressed as $\log_2$ fold change relative to baseline. Protein carbonyls were measured as markers of oxidative damage. Statistical analysis employed single-sample tests and false-discovery rate control.

NRG4 exhibited a robust early increase at 30 m, significant after correction, and nominal elevations at 40 m, but remained unchanged at 20 m. Exercise at 15 m triggered a significant early NRG4 rise absent during passive dives at the same depth. Protein carbonyls remained stable in early post-emersion windows but increased significantly at later time points (180- and 240-min post-emersion) following dives to 40 m, indicating delayed oxidative burden.

Our findings position NRG4 as a fast, pressure- and workload-responsive biomarker of diving stress, temporally distinct from classical oxidative injury markers that manifest later. This temporal dissociation underscores the potential of NRG4 for real-time monitoring of acute physiological load during hyperbaric exposure, integrating pressure- and workload-related stressors.

1. Introduction

Environmental stress imprints molecular signatures well before clinical manifestations, transforming physiological landscapes in a manner not immediately visible yet detectable by precise blood analyses. Even a single dive in humans can acutely and transiently perturb networks across cardiovascular, endocrine, and oxidative domains—alterations that, while occult at the macroscopic level, emerge rapidly in the circulatory compartment [1–4]. This convergent model makes diving an exquisite natural laboratory for probing responses to acute environmental load, wherein pressure, hyperoxia, submersion-induced hydrostatic shifts, physical workload, and, at times, thermal stress, are orchestrated deliberately in a controlled but ecologically meaningful setting.

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https://doi.org/10.1016/j.freeradbiomed.2026.02.002
Received 20 November 2025; Received in revised form 28 January 2026; Accepted 1 February 2026
Available online 2 February 2026

Within this framework, neuregulin-4 (NRG4) has emerged as a compelling biomarker candidate, given its dual roles in stress physiology and metabolic regulation as a fat-derived endocrine ligand. NRG4 belongs to the epidermal growth factor family, showing preferential enrichment in thermogenic adipocytes and detectable presence in subcutaneous white adipose tissue. Mechanistically, it acts predominantly via ErbB4 receptors expressed in hepatic tissue to restrain lipogenesis and stabilize glucose–lipid equilibrium [5,6]. Pathophysiologically, circulating NRG4 is reduced in human metabolic disease. Lower levels are reported in obese adults with metabolic syndrome, in newly diagnosed type-2 diabetes, and in inflammatory metabolic states such as non-alcoholic fatty liver disease [7–10]. Conversely, structured exercise training—particularly high-intensity interval and circuit resistance protocols—reliably increases circulating NRG4 in men with obesity [11]. Recent experimental evidence reveals that cellular knock-down of NRG4 drives oxidative stress and induces mitochondrial dysfunction in adipocytes [12], suggesting that this adipokine not only signals exercise and metabolic physiological stress but participates actively in redox safeguarding. Together, these mechanistic insights position NRG4 as an integrative and rapidly responsive marker for exposures involving environmental challenge.

Diving is exceptionally suited to interrogate such candidates, as the acute rise in ambient pressure not only increases gas density and the work of breathing, but also precipitates dramatic shifts in cardiovascular load. In parallel, full-body submersion during diving elicits the classical diving response, characterized by reflex bradycardia, peripheral vasoconstriction, and central blood redistribution [13]; these stressors are known to trigger sympathetic nervous-system activation, oxidative stress and inflammatory responses [3,14–16]. While several classic hormones — including catecholamines, prolactin and cortisol — have been explored as markers in diving studies, their patterns are strikingly inconsistent and highly sensitive to variations in depth, water-temperature, exercise and individual status [2,17–19]. In contrast, molecular markers of oxidative imbalance show robust and reproducible perturbations — for example increases in lipid peroxidation (8-iso-$PGF_{2}\alpha$), oxidized nucleic acids (8-OHdG), protein carbonyls, and transient increases in pro-inflammatory mediators [16,20,21]. Yet most of these endpoints peak well after exposure — sometimes hours post-dive — creating a temporal gap in real-time environmental stress monitoring.

Given this landscape, we sought to define whether NRG4 could act as an early, load-sensitive biomarker in divers, capable of discriminating responses to both depth and muscular activity within minutes post-exposure and temporally dissociated from slower oxidative markers. We deliberately employed dives in warm water to remove thermal artifacts, isolating pressure and exercise as principal drivers of physiological changes. By aligning NRG4 responses to concurrent protein carbonyl measurements, our design directly tests whether endocrine and redox biomarkers provide complementary temporal diagnoses of the dive-induced stress continuum.

2. Materials and methods

2.1. Study design and setting

Two complementary, real-world field studies were conducted in Montegrotto (Italy) according to the diving protocol described by Aquilano et al. (2021) [22]. The depth response study examined depth-dependent changes following recreational-style dives at 20, 30, or 40 m, performed on separate days, with repeated early post-exposure blood sampling (0-, 90-, 180- or 240-min post-emersion) to capture short-term responses.

The physical effort campaign investigated the contribution of muscular workload at 15 m, comparing dives performed with exercise (slow pedalling on an underwater cycle ergometer for 30 min at depth) versus passive diving without exercise. Exercise consisted of continuous, low-intensity pedalling performed at a steady, self-selected pace and was not quantitatively measured, as the aim was to introduce a predefined muscular workload rather than to assess exercise performance or maximal capacity. All dives were performed in warm water (~33.6 °C) to minimize thermal stress. The design prioritized early post-dive windows, where endocrine and oxidative responses were expected to be most pronounced, in line with established hyperbaric physiology principles [23], although later response peaks cannot be excluded.

2.2. Participants and exposure protocols

Participants were healthy adult volunteer divers. Dives followed standard safety procedures and recreational-style durations. Briefly, divers used identical neoprene suits, compressed air as breathing gas, individual dive computers for depth monitoring, and buoyancy compensator devices, following the ascent profile defined in the referenced protocol [22]. Target depths were 20, 30, and 40 m in the depth response study and 15 m in the physical effort campaign. In the exercise condition at 15 m, participants performed a predefined bout of pedalling on an underwater bike during the entire bottom phase of the dive; in the no-exercise condition, only routine manoeuvring occurred. Bottom times and workload characteristics were kept as consistent as feasible within each condition under field constraints. Ambient water temperature was maintained at ~33.6 °C throughout.

2.3. Sampling schedule

Venous blood was obtained at baseline (pre-dive) and at four post-emersion timepoints (0, 90, 180 and 240 min after emersion) in the depth exposure trial. Venipuncture was performed at each timepoint with single-use sterile devices. No indwelling venous catheter was maintained; each blood draw was obtained via a separate peripheral venipuncture. Standard antiseptic procedures were applied before each sampling to minimize infection risk. During the physical workload study, samples were collected at baseline (pre-dive) and at two post-emersion timepoints (0 and 180 min after emersion) under both exercise and no-exercise conditions. Samplings were scheduled within comparable daytime ranges to minimize diurnal variance.

2.4. Blood processing and storage

Blood was collected into serum separator tubes (SST) according to manufacturer instructions, allowed to clot, and centrifuged to obtain serum. Aliquots were transferred into cryovials, kept cold during transport, and stored long-term under frozen conditions. Freeze–thaw cycles were minimized by aliquoting. All samples from a given subject and session were processed in parallel to limit pre-analytical variability.

2.5. Quantification of circulating NRG4

Serum NRG4 was quantified using a human NRG4 ELISA (RayBiotech, ELH-NRG4-1). Each plate included calibrators and internal controls per kit specifications. Samples were assayed in quadruplicate, and technical replicates were averaged. Potential plate effects were mitigated by randomizing sample order and batching samples from the same condition within a run.

2.6. Quantification of protein carbonyls

Serum protein carbonyls were measured as a marker of oxidative protein modification using a commercial ELISA-based kit (Cayman Chemical, Item No. 10005020) following manufacturer instructions. Optical density values were converted to concentrations and expressed as fold change relative to each participant's pre-dive baseline. The same serum aliquots used for NRG4 assays were employed to ensure comparability of sampling windows.

2.7. Data structuring and normalization

Within-subject change was expressed as fold change relative to each subject's baseline (pre-dive set to 1.0). For both NRG4 and protein carbonyls, values were $\log_2$-transformed before inference so that 0 denoted no change, +1 a doubling, and −1 a halving. In the depth exposure experiment, post-emersion values were normalized within each depth series; in the physical workload study, values at emersion and 180 min post-emersion were normalized within condition (exercise vs no-exercise).

2.8. Inclusion, missingness, and outliers

All available observations were included, and no imputation was performed. No data points were excluded as outliers; visual inspection confirmed that $\log_2$ transformation improved symmetry of distributions and residuals.

2.9. Statistical analysis

The primary question was whether circulating NRG4 or protein carbonyls deviated from baseline at each post-exposure timepoint.

One-sample tests. On $\log_2$ fold changes values, we ran two-sided one-sample t-tests versus 0 at each timepoint within depth (depth-dependent study) or condition (exercise exposure campaign). Wilcoxon signed-rank tests were used as sensitivity checks when normality was doubtful (directionally concordant results).

Multiple testing. For the depth profile investigation, we controlled the false discovery rate (FDR) across four windows (0–240 min post-emersion) within each depth. For the exercise trial, we controlled FDR across 0- and 180-min post-emersion within each condition. Adjusted q-values are reported alongside nominal p.

Estimation. Group estimates are presented as geometric mean (GM) fold change with 95% confidence intervals (CIs), obtained by computing mean ± CI on the $\log_2$ scale and back-transforming.

Assumptions. Analyses assume independence across subjects and stable pre-analytical handling. Results were robust to analysis on raw FC values, but the $\log_2$ scale provided more symmetric intervals and improved residual behaviour.

2.10. Visualization

Figures were generated in GraphPad Prism. Scatter plots display individual values overlaid with mean ± 95% CI at each timepoint. Reference lines denote 1.0 (fold) or 0 ($\log_2$). For the depth variation trial, panels display post-emersion values stratified by depth; for the exercise exposure experiment, panels display the values monitored at emersion and 180 min post-emersion stratified by exercise vs no-exercise. Asterisks mark nominal $p < 0.05$, with FDR-significant results starred in the main panels; figure notes indicate nominal-only findings where relevant.

2.11. Group size considerations

No formal a priori power calculation was performed, as the field design was constrained by logistical and safety considerations inherent to controlled human hyperbaric exposures. Sample size was therefore determined by feasibility and is comparable to prior exploratory and pilot field studies in human diving physiology.

The cohorts were exploratory and convenience-sampled, with group sizes of n = 4–5 per cell in the depth-response series and n = 4 per condition in the exercise versus no-exercise experiment, assessed at emersion and 180 min post-emersion. The study design prioritized detection of within-subject changes over between-group contrasts. Accordingly, statistical inference focused on one-sample departures from baseline, with effect size estimates and confidence intervals reported, and within-family false discovery rate (FDR) control applied to balance type I error against limited sample size.

2.12. Ethics, consent, and reporting

The Ethics Committee of the "LAZIO 2" ASL Roma 2 approved this study (approval no. 0207553/2022). All procedures adhered to the Declaration of Helsinki and to the International Code of Ethics for Occupational Health Professionals (International Committee of Occupational Health, 2014), as adopted by the Istituto Nazionale Assicurazione contro gli Infortuni sul Lavoro (INAIL). Written informed consent was obtained from all participants prior to sampling. The study is reported in line with STROBE recommendations for observational studies where applicable. No identifiable information is presented.

2.13. Software and reproducibility

Statistical analyses were performed in GraphPad Prism and cross-checked in a scripting environment (Python) for summary statistics. The analysis pipeline (baseline normalization → $\log_2$ transform → one-sample tests with within-family FDR) and figure specifications are described here to enable reproduction. De-identified raw data tables (subject × timepoint × condition) and analysis code are available upon reasonable request.

3. Results

3.1. Participant characteristics

The study group comprised six experienced, non-smoking professional divers (3 males and 3 females). Mean age was 42 ± 5 years, height 169 ± 14 cm, and body weight 67 ± 15 kg (mean ± SD). Anthropometric and physiological characteristics of the participants included a body mass index (BMI) of 25.4 ± 3.0 kg/m^2 and a fat mass (FM) of 22.2 ± 6.3%.

3.2. Depth-dependent NRG4 responses in the depth dynamics field study

To rigorously assess whether circulating NRG4 is modulated by ambient pressure independently of confounding variables, we conducted a controlled dive protocol where divers were exposed to 20, 30, or 40 m in warm water, deliberately excluding exercise as an additional stressor. For each participant, individual NRG4 values were computed as $\log_2$ fold changes ($\log_2$FC) relative to their pre-dive baseline. Post-emersion values were statistically compared against zero across designated sampling windows (0-, 90-, 180- and 240-min post-emersion), providing a direct measure of acute endocrine shifts (Fig. 1, Table I).

At 20 m, group-level means oscillated minimally around baseline, with confidence intervals very consistently crossing zero in all post-emersion periods—evidence against any reproducible pressure-induced NRG4 drift. In marked contrast, a dive to 30 m provoked an unequivocal, early rise in circulating NRG4 directly post-emersion, achieving statistical significance that withstood robust within-depth FDR correction. This early elevation was transient, regressing toward baseline during subsequent timepoints, highlighting NRG4's role as a rapid and transient stress signal.

Dive to 40 m yielded a qualitatively similar yet less robust pattern: during the early post-emersion phase (up to 90 min post-emersion) NRG4 levels showed an upward trend from baseline, nominally significant, yet non-significant after multiple-testing correction. Variability across subjects at this depth may reflect differences in adaptive capacity or sub-threshold physiological responses. Notably, late windows (180- and 240-min post-emersion) at all depths were indistinguishable from baseline, reinforcing the concept of a temporally constrained NRG4 response.

Taken together, the depth-response data delineate a pronounced,

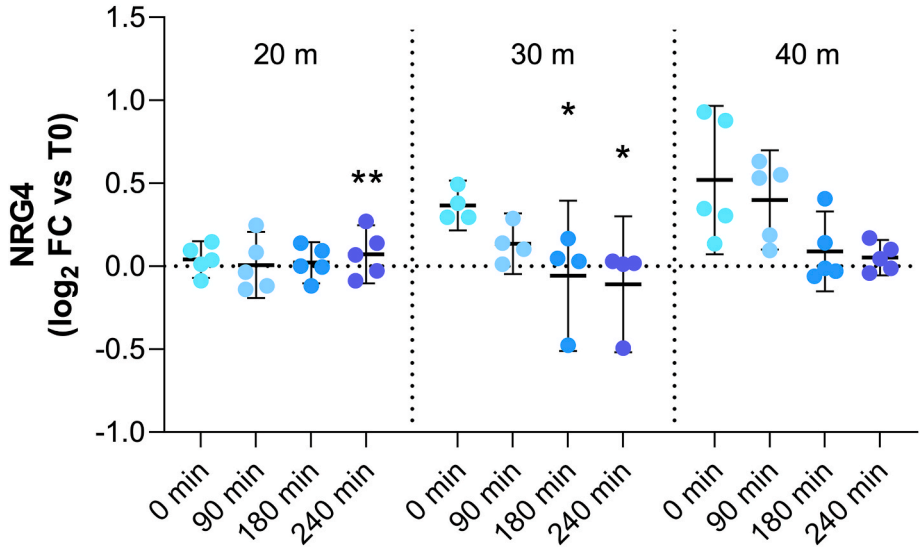

Fig. 1. Depth-dependent NRG4 responses in the field. Serum NRG4 was measured in divers exposed at 20, 30, or 40 m in thermoneutral water without exercise. Values are expressed as $\log_2$ fold change ($\log_2$FC) relative to each subject's pre-dive baseline. Points represent individual participants; bars denote mean ± 95% CI. Dashed lines indicate unity (fold = 1). Asterisks mark nominal $p < 0.05$ (two-sided one-sample *t*-test vs 0 within depth); FDR-adjusted q values across 0–240 min post-emersion within depth are reported in Table I. A reproducible early rise emerged at 30 m immediately after emersion, which remained significant after within-depth FDR correction, while effects at 40 m and 90–180 min post-emersion were nominal but not robust to correction.

Table 1
Depth-dependent NRG4 responses after hyperbaric exposure. Serum NRG4 is expressed as $\log_2$ fold change ($\log_2$FC) vs each subject's pre-dive baseline; summary values are geometric mean (GM) fold with 95% CI obtained by back-transforming the mean ± CI on the $\log_2$ scale. p: two-sided one-sample t-tests vs 0 within depth. **q**: BH–FDR within depth across 0–240 min post-emersion.

Depth	Time	n	GM fold (95% CI)	$\log_2$FC mean (95% CI)	*p*	*q* (FDR within depth)
20 m	0 min	5	1.0288 (0.9531–1.1104)	0.0409 (−0.0693–0.1511)	0.3610	0.7220
	90 min	5	1.0052 (0.8760–1.1535)	0.0075 (−0.1909–0.2060)	0.9213	0.9213
	180 min	5	1.0152 (0.9318–1.1060)	0.0217 (−0.1020–0.1454)	0.6514	0.8686
	240 min	5	1.0512 (0.9314–1.1863)	0.0720 (−0.1025–0.2465)	0.3160	0.7220
30 m	0 min	4	**1.2885 (1.1613–1.4296)**	0.3657 (0.2158–0.5156)	**0.0044**	**0.0178**
	90 min	4	1.0985 (0.9681–1.2464)	0.1355 (−0.0467–0.3177)	0.0989	0.1977
	180 min	4	0.9608 (0.7015–1.3159)	−0.0577 (−0.5114–0.3961)	0.7129	0.7129
	240 min	4	0.9278 (0.6986–1.2322)	−0.1081 (−0.5175–0.3013)	0.4624	0.6165
40 m	0 min	5	**1.4334 (1.0512–1.9546)**	0.5194 (0.0720–0.9669)	**0.0322**	0.0644
	90 min	5	**1.3193 (1.0720–1.6236)**	0.3997 (0.1003–0.6992)	**0.0207**	0.0644
	180 min	5	1.0636 (0.9004–1.2565)	0.0890 (−0.1514–0.3294)	0.3621	0.3621
	240 min	5	1.0370 (0.9633–1.1164)	0.0524 (−0.0540–0.1588)	0.2431	0.3242

pressure-linked increase in NRG4 that peaks sharply at 30 m—particularly at the first post-dive interval—where significance is most compelling, while a similar directional pattern emerges at 40 m under stricter statistical scrutiny. This constellation of findings situates NRG4 as a sensitive, rapid biomarker, acutely responsive to ambient pressure shifts within thermoneutral diving exposures.

3.3. Depth-dependent oxidative balance in depth-profile study

To determine whether biochemical indices of oxidative stress reflect or temporally lag behind the endocrine response, we analysed serum protein carbonyl concentrations from the same depth-response study. Protein carbonyl values were normalized as $\log_2$ fold changes against pre-dive baseline, then assessed at defined post-emersion points (0-, 90-, 180- and 240-min post-emersion) and stratified by depth to preserve the explicit gradation in pressure exposure. For each depth, we used two-sided one-sample tests against zero for primary inferential analysis, reporting nominal *p* values to inform figure annotations and controlling for multiplicity by within-depth FDR adjustment (Fig. 2, Table II).

At 20 m, distributions were tightly centred at zero throughout all post-dive windows, with overlapping confidence intervals and absence of any sustained or reproducible deviation. This pattern designates the 20 m depth as a robust comparator, supporting the integrity of the biomarker under low-stress conditions. At 30 m, measurements during early post-emersion phase (0 and 90 min) showed only sporadic changes without a consistent trend. By the later post-emersion window (180 min), values exhibited a nominal rightward skew, suggesting a potential late-onset oxidative effect; however, this did not survive FDR correction and levels returned promptly back toward baseline by 240 min post-emersion.

In marked contrast, dives to 40 m provoked a delayed but pronounced oxidative response: protein carbonyls were indistinguishable from baseline at both 0- and 90-min post-emersion yet rose markedly at 180 min post-emersion and peaked further at 240 min post-emersion, with both time points retaining statistical significance following FDR adjustment. These data define a pressure threshold for manifest oxidative injury, in which biochemical evidence of molecular damage consolidates only following sustained, maximal hyperbaric exposure and with a clear lag behind the acute phase.

When considered alongside the NRG4 analyses, these results reveal a

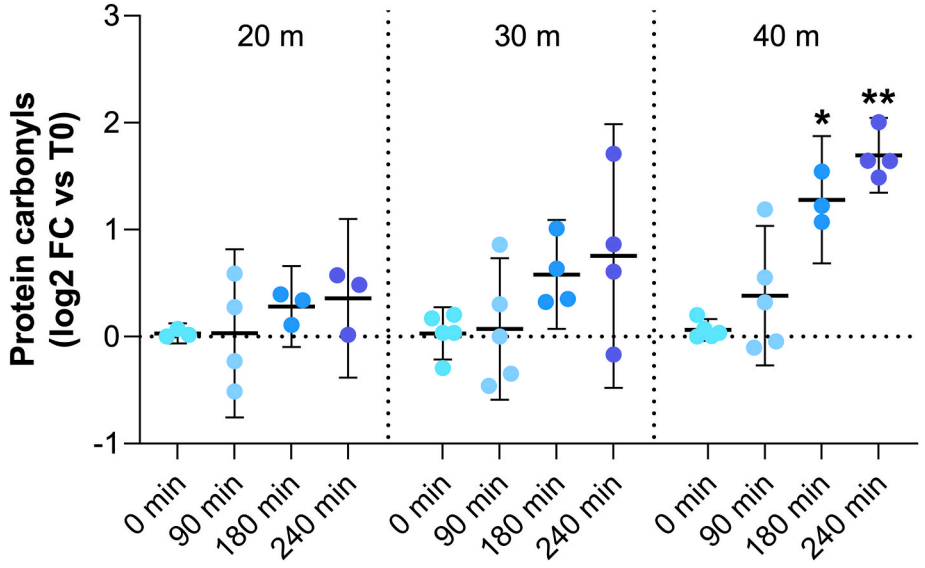

Fig. 2. Depth-dependent oxidative balance in the field. Serum protein carbonyls were quantified in the sera of the divers belonging to the depth exposure study. Values are expressed as log_2FC relative to pre-dive baseline. Symbols show individual observations; bars are mean ± 95% CI. Tests were one-sample (two-sided) against zero within each depth; dashed lines mark baseline. Nominal p values are annotated on the plot, and FDR-adjusted q values across 0–240 min post-emersion within depth are summarized in Table II. At 20 m, values remained near baseline. At 30 m, a nominally significant shift appeared late at 180 min post-emersion but did not survive FDR. At 40 m, delayed increases consolidated at 180 and 240 min post-emersion, both surviving within-depth FDR correction, delineating a late oxidative signature at the highest pressure.

Table 2
Serum protein carbonyls after hyperbaric exposure. Serum protein carbonyls are expressed as log_2 fold change (log_2FC) relative to each subject's pre-dive baseline; summary values are reported as geometric mean (GM) fold with 95% confidence intervals (CI), obtained by back-transforming the mean ± CI on the log_2 scale. *p* values derive from two-sided one-sample t-tests vs baseline (within depth). *q* values are adjusted by Benjamini–Hochberg false discovery rate (FDR) within each depth across 0–240 min post-emersion.

Depth	Time	n	GM fold (95% CI)	log_2FC mean (95% CI)	*p*	*q* (FDR within depth)
20 m	0 min	3	1.0209 (0.9569–1.0893)	0.0299 (−0.0636–0.1234)	0.3024	0.6049
	90 min	4	1.0216 (0.5922–1.7625)	0.0308 (−0.7560–0.8176)	0.9086	0.9086
	180 min	3	1.2148 (0.9347–1.5788)	0.2807 (−0.0975–0.6588)	0.0856	0.3426
	240 min	3	1.2826 (0.7672–2.1441)	0.3591 (−0.3823–1.1004)	0.1725	0.5176
30 m	0 min	5	1.0212 (0.8618–1.2100)	0.0302 (−0.2145–0.2750)	0.7489	1.0000
	90 min	5	1.0515 (0.6645–1.6639)	0.0724 (−0.5898–0.7345)	0.7766	1.0000
	180 min	4	**1.4954 (1.0508–2.1280)**	**0.5805 (0.0715–1.0895)**	**0.0360**	0.1440
	240 min	4	1.6865 (0.7184–3.9592)	0.7541 (−0.4771–1.9852)	0.1464	0.4392
40 m	0 min	5	1.0447 (0.9727–1.1220)	0.0631 (−0.0400–0.1661)	0.1644	0.3288
	90 min	5	1.3037 (0.8301–2.0475)	0.3826 (−0.2687–1.0339)	0.1782	0.3288
	180 min	3	**2.4275 (1.6075–3.6657)**	**1.2795 (0.6848–1.8741)**	**0.0115**	**0.0344**
	240 min	4	**3.2363 (2.5409–4.1222)**	**1.6944 (1.3453–2.0434)**	**0.0006**	**0.0024**

temporal dissociation intrinsic to the dive response: while NRG4 increases rapidly (0- and 90-min post-emersion) as an early endocrine response, protein carbonyls accumulate later (180- and 240-min post-emersion), capturing the metabolic cost of prolonged hyperbaric challenge. This staggered sequence underscores the complementary nature of stress axis versus redox axis readouts—together, they delineate a continuum from acute, immediate response to delayed molecular burden in the physiology of human diving.

3.4. NRG4 at shallow depth with and without exercise

To elucidate whether mechanical or energetic load can recapitulate the acute endocrine response in the absence of pronounced ambient pressure, we conducted a second protocol involving dives to 15 m. In this framework, subjects underwent either continuous exercise via fin-pedalling or passive dives, serving as distinct physiological arms for comparative analysis. NRG4 concentrations were quantified as log_2 fold changes from pre-dive baseline and statistically tested against zero at two strategic post-emersion intervals: immediately after emersion (0 min, early) and 180 min (later) post-emersion. Analysis employed two-sided, one-sample tests within each condition; nominal *p* values annotated the relevant figures, with Benjamini-Hochberg–FDR correction applied across timepoints per group. Direct comparisons (exercise versus rest) at corresponding timepoints were interpreted as exploratory given the limited sample size (Fig. 3, Table III).

In the exercise group, NRG4 exhibited a consistent and pronounced early post-emersion elevation, statistically significant ($p = 0.0058$) and robust to within-condition FDR correction ($q = 0.0114$). By the later interval (180 min post-emersion), mean values trended upward but no longer attained statistical distinction from baseline, suggesting a transient and rapidly resolving endocrine signal coupled to exertion. In contrast, passive dives at 15 m produced distributions that hovered close to zero at both timepoints, marked by broad scatter and absence of any systematic deviation, indicating that inactivity is insufficient to activate this adipose-derived biomarker under the given conditions.

Exploratory between-arm analysis revealed wider individual

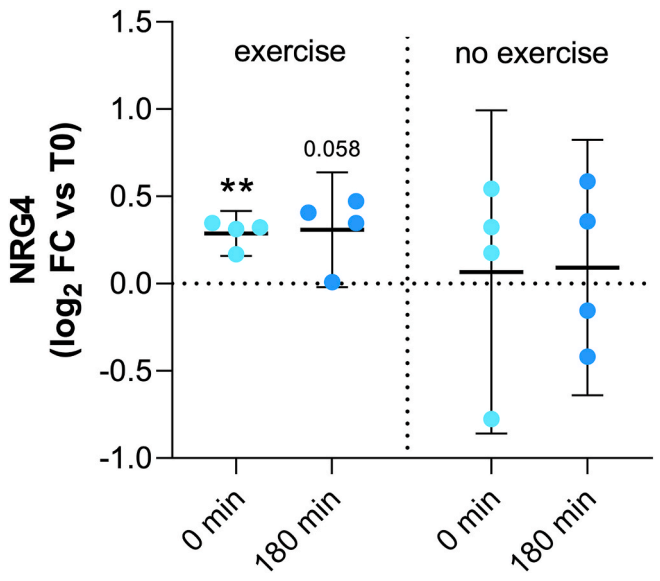

Fig. 3. NRG4 at shallow depth with and without exercise. A separate cohort dived at 15 m with or without exercise (fin-pedaling vs passive dives). NRG4 was measured as log_2FC vs pre-dive baseline immediately after emersion (0 min, early) and 180 min (later) post-emersion. Symbols represent individuals; bars are mean ± 95% CI. Dashed lines denote baseline. Within each condition, p values are from two-sided one-sample t-tests vs 0; FDR was controlled across the two windows per condition (see Table III). With exercise, NRG4 rose significantly immediately after emersion, remaining significant after within-condition FDR, while values returned toward baseline by 180 min post-emersion. Without exercise, NRG4 hovered around baseline at both windows. Exploratory between-condition contrasts were directionally consistent but underpowered.

variation and greater deviation from baseline under exercise at emersion, although none of these achieved statistical reliability within the constraints imposed by small n. These findings suggest but do not definitively establish heightened sensitivity of NRG4 to workload under these exposure regimes.

Synthesizing data from both protocols, it becomes evident that the addition of muscular effort at 15 m depth is sufficient to elicit an early NRG4 response that is otherwise observed only at higher ambient pressures, a signal absent during passive dives despite identical environmental exposure. These findings demonstrate that circulating NRG4 does not respond to a single isolated stimulus, but rather integrates the overall acute physiological load imposed by hyperbaric exposure and muscular work. When integrated with the delayed increase in protein carbonyls observed in the depth-dependent study, the results demonstrate a clear temporal dissociation between early endocrine signaling and subsequent oxidative damage, reinforcing and extending the concept of NRG4 as an early, load-sensitive biomarker, operating upstream of classical oxidative injury markers.

4. Discussion

4.1. Dive stressors and the rationale for NRG4 as a rapid marker

Diving challenges the human organism with a distinctive suite of acute physiological stressors. Increased ambient pressure, hyperoxic shifts in the partial pressures of inspired gases, immersion-induced central fluid redistribution, and respiratory and cardiac workload act in concert beneath the surface. In parallel, diving elicits a well-described autonomic "diving response", characterized by vagally mediated bradycardia, peripheral vasoconstriction with redistribution of blood flow toward central organs, and a relative reduction in peripheral metabolic demand, contributing to oxygen conservation under hyperbaric conditions. Together, these forces evoke hormonal and molecular stress responses, notably increasing reactive oxygen species (ROS) formation, stimulating sympathetic outflow, and blood shift, as documented by heart-rate and cardiovascular monitoring studies in thermoneutral diving conditions [13]. This composite stress consistently triggers oxidative and endocrine perturbations: multiple studies have demonstrated robust associations between diving and both molecular oxidation biomarkers and stress hormone fluctuations [3,14,15,24].

Within this physiological landscape, NRG4 is gaining traction as a candidate biomarker integrating stress and metabolic axes. NRG4 is an adipokine expressed in brown/beige as well as white adipose depots, sensitive to both cold and β-adrenergic stimulation. Its pleiotropic functions include regulation of hepatic lipid metabolism, prevention of steatosis, and maintenance of systemic energy homeostasis [6,25,26]. Notably, NRG4 is upregulated in some contexts of exercise and metabolic challenge, suggesting a broader non-thermal stress responsivity [11,27]. Our work directly explored this possibility, using a thermoneutral field model that isolated pressure and muscular effort as primary, acute triggers.

4.2. Thermoneutral depth series: pressure triggers early NRG4 release

By maintaining water temperature around 33.6 °C for all exposures, we minimized cold stress and thermogenic activation, which are known to stimulate NRG4 release [5], thereby allowing a clearer dissection of the contribution of hyperbaric exposure to the observed endocrine response. Core body temperature was not directly measured; however, the use of thermoneutral water conditions was specifically intended to limit cold-induced confounding effects. Under these controlled conditions, incremental depth provided a defined hyperbaric "dose," enabling attribution of early changes in circulating NRG4 primarily to pressure-related physiological strain rather than to thermal stress. While ambient pressure was the primary variable experimentally isolated in this thermoneutral setting, it should be emphasized that pressure acts *in vivo* in conjunction with other submersion-related stressors, including increased work of breathing, hyperoxic exposure, central blood shift, and autonomic activation.

It should be noted that real-life dives are frequently associated with thermal stress, which contributes to oxidative and inflammatory responses, as documented by field studies reporting increased oxidative biomarkers under non-thermoneutral conditions [28,29]. In contrast, the present thermoneutral design was deliberately chosen to reduce such

Table 3
NRG4 at 15 m with and without exercise at emersion and late post-emersion. Values are expressed as log_2 fold change (log_2 FC) relative to pre-dive baseline, summarized as geometric mean (GM) fold (95% CI) (back-transform of log_2 means). p: two-sided one-sample t-tests vs 0 within condition; q: BH–FDR within condition across 0 min and 180 min post-emersion.

Condition (15 m)	Time	n	GM fold (95% CI)	log_2FC mean (95% CI)	p	q (FDR within condition)
exercise	0 min	4	1.2207 (1.1167–1.3344)	0.2878 (0.1593–0.4162)	0.0058	0.0114
	180 min	4	1.2384 (0.9859–1.5556)	0.3085 (−0.0204–0.6375)	0.0584	0.0584
no exercise	0 min	4	1.0472 (0.5512–1.9897)	0.0666 (−0.8594–0.9925)	0.8337	0.8337
	180 min	4	1.0656 (0.6416–1.7699)	0.0917 (−0.6403–0.8237)	0.7169	0.8337

confounders and to isolate hyperbaric load.

In the depth-response experiment, increased depth—from 20 to 30 m—was mirrored by an early increase in NRG4 immediately after emersion (0 min post-emersion), which was reproducibly significant at 30 m and directionally present though more variable at 40 m. After FDR adjustment, only the 30 m early-post-emersion window reached significance; however, the rapid onset and transient nature of the response delineate a canonical "fast-responder" pattern. The absence of significant NRG4 elevation at 20 m supports a true dose–response, with stronger ambient pressure required to elicit this molecular signal.

Importantly, multiple physiological mechanisms are likely to contribute to the NRG4 response, including increased respiratory resistance, hydrostatic central shift, elevated cardiac preload and afterload, transient oxidative stress, and adrenergic signaling linked to both mechanical and metabolic strain [13,30]. The rapid NRG4 rise immediately after emersion, followed by a return toward baseline within 90 min, supports its role as an acute signaling marker rather than a delayed consequence of oxidative injury. Taken together, these findings indicate that hyperbaric exposure, under thermoneutral conditions, is sufficient to evoke a transient NRG4 pulse.

4.3. Exercise exposure: workload as a complementary trigger

The physical-effort campaign demonstrated that under modest pressure, muscular and energetic demand becomes crucial for eliciting the NRG4 response. Here, dives with active slow pedalling on an underwater bike at 15 m, designed to mimic typical fin-propulsion, reproduced the early NRG4 elevation otherwise seen only at higher ambient pressure. The specificity was striking: passive dives under the same conditions produced no comparable change, indicating that NRG4 secretion is not a direct effect of submersion but rather reflects the integrated physiological stress. Although no direct physiological measures of workload (e.g., heart rate or power output) were recorded, the experimental contrast between active and passive dives was intentionally designed to introduce a defined muscular load rather than to quantify exercise intensity. This workload-response experiment thus positions NRG4 as a composite biosensor, responding rapidly to either elevated pressure or increased muscular activity.

4.4. Temporal coupling of endocrine and oxidative responses

Simultaneous measurement of protein carbonyls within the same sera enabled a direct comparison of redox and endocrine timing. Protein carbonyls—classical indicators of oxidant-driven protein modification [31]—remained near baseline (pre-dive) at 20 and 30 m across all early post-emersion windows (0–90 min), diverging only during the later recovery phase (180–240 min after emersion), where significance survived FDR correction. Thus, while biochemical injury is eventually reflected in protein oxidation, its appearance lags far behind the rapid NRG4 rise, supporting a model of temporally stratified stress markers.

A plausible mechanism emerges in which NRG4 release, although classically linked to brown adipose activation, is here rapidly orchestrated via adrenergic pathways—possibly involving sympathetically innervated white adipose depots responsive to workload and barometric stress. The independence from cold exposure in this setting highlights pressure and mechanical strain as sufficient triggers for NRG4 secretion. The coordinated changes in sympathetic tone, breathing work, blood redistribution, and oxygen partial pressure likely converge to exceed an endocrine threshold for acute NRG4 release. In contrast, the subsequent accumulation of protein carbonyls likely represents a secondary wave of molecular stress, signaling cumulative oxidative burden rather than immediate alarm.

4.5. Integrating endocrine and redox markers across time and load

Classical stress and adaptation markers in diving—such as catecholamines, prolactin, and cortisol—show considerable variability in magnitude, timing, and inter-individual patterning [30,32,33]. Oxidative stress biomarkers, including lipid-peroxidation products (e.g., 8-iso-$PGF_2\alpha$) and antioxidant-enzyme responses, may increase after dives but often peak well after emersion or exhibit inconsistency due to sampling time, assay variability, and confounding factors such as depth, dive count, and water temperature [3,16,33]. In this landscape, the performance of NRG4—rapid, reproducible, and scaling with exposure load—fills an important gap, offering time-resolved quantitation of physiological stress unattainable with canonical endocrine or redox measures.

This combined depth-profile and exercise-exposure approach uniquely leveraged thermoneutral diving, dual biomarker analysis, and both pressure and workload gradients to disentangle stressor contributions over time. Early and late sampling provided insight into immediate and follow-up phases of recovery. Nonetheless, sample size limitations call for replication to validate weaker or transient effects. Incorporating more granular endpoints (sympathetic mediators, cytokines, comprehensive oxidative panels) could enrich mechanistic resolution. Improved workload quantification, gas-mix recording, and stratification by diver phenotype (sex, fitness, adiposity) would further strengthen interpretation and generalizability. Methodological rigor in timing and sample handling remains crucial for reproducibility.

5. Conclusions

NRG4 emerges as a sensitive, rapid, and load-graded endocrine marker of diving stress, responsive to both elevated pressure and increased physical effort. Oxidative injury, manifested as protein carbonyl accumulation, follows with delayed kinetics and consolidates primarily under high barometric load. Together, these findings support a temporal model in which acute and cumulative stress can be mapped through an early-peaking endocrine signal (NRG4) and slower-developing oxidative markers. Such stratification may enhance personalized monitoring and risk assessment in divers and could have broader applications in other environmental or occupational stress contexts.

CRediT authorship contribution statement

Claudia Di Biagio: Writing – original draft, Methodology, Investigation. **Paola Giglio:** Methodology, Investigation. **Matteo Bordi:** Investigation. **Giovanni Larotondo:** Investigation. **Riccardo Turchi:** Investigation. **Luigi Fattorini:** Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Enrico Marchetti:** Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Daniele Lettieri-Barbato:** Writing – review & editing, Supervision. **Costanza Montagna:** Methodology, Investigation. **Giuseppe Filomeni:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation. **Katia Aquilano:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation.

Funding

This work was carried out with the financial support of Istituto Nazionale Assicurazione contro gli Infortuni sul Lavoro (INAIL, Italy) BRIC Grants 2016 ID 25, 2019 ID 31, 2022 ID 38.

Declaration of interest statement

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available upon request.

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