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The effect of the BDNF Val66Met polymorphism on BDNF expression and the counteract power of the exercise

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INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a protein robustly expressed by neurons and neural tissue, especially in brain areas that are involved in essential mechanisms of cognition and behavior. BDNF plays an important role in the development and maintenance of adult neurons and is a putative regulator of neurogenesis, neuroprotection, neurodegeneration and synaptic plasticity. In particular, BDNF is widely expressed in the hippocampus where it associates with long-term potentiation of hippocampal neurons and memory processes. More recently, an exercise dependent secretion of BDNF has been acknowledged as the main mechanism by which the exercise positively influences on neuroplasticity and cognition (de Assis and de Almondes 2017).

Importantly, it has been supposed that a genetic variant in BDNF gene (*BDNF*) may alter the regulated functionality of BDNF, and impact on the exercise benefits to its production. Current animal studies report that the Val66met polymorphism within the pro region of the *BDNF* is associated with lower-activity dependent secretion and such disruption might be critical for emotional and cognitive processes (Egan et al. 2003).

BACKGROUD RESEARCH

My research on BDNF began in the occasion of the "Project Water walking" regarding the obtention of a Master of Science degree. The project involved patients with advanced Parkinson disease engaged in a short period of training - 4 weeks - (see more at de Assis et al., 2018), and analysis of blood BDNF levels of the subjects submitted to the exercise revealed prospective results. The circulating levels of BDNF in individuals of the exercise group showed an acute increase post- training session, and their resting BDNF levels were higher than control's, at the end of the Project. Moreover, their levels of BDNF associated to their performances in executive tests. Although such data was not included for publication due to a relatively small sample size, later on, after running a systematic review it was possible to confirm that the exercise, specifically those predominantly aerobic, increases secretion of BDNF (de Assis et al. 2018) with a positive impact on cognitive capabilities and the course of neurodegeneration (de Assis and de Almondes 2017).

These findings led me to the decision to continue the investigation on the physiological mechanisms by which BDNF is modulated by the exercise, as well as the potential implications of the *BDNF* Val66met polymorphism, in a PhD study realized in AWFiS. Initially, we observed that the metabolic mechanism linking exercise and BDNF regards the demands of

oxygen-dependent energy, as a main source of adenosine triphosphate (ATP). As a consequence of high energy demands, a parallel increase in the activity of 5' adenosine monophosphate-activated protein kinase (AMPK) leads to the expression and release of the co-transcription activator proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a central up-regulator of several metabolic responsive genes, such as *FNDC5* (which encodes for Irisin) and *BDNF*. Further, regulation of cortisol levels by the exercise works as a counteraction to eventual glucocorticoid receptor resistance that, in turn, results in failure to down-regulate inflammatory response. Additionally, the effects of exercise on adipocytes secretion of Leptin may ultimately regulate central mechanisms food intake control thereby contributing to the body weight and mind health. Altogether, increases in levels of BDNF, which serve as both a material support for building up neuronal connections – synaptogenesis and neuroplasticity – and a backup support against natural neurodegeneration – cognitive reserve, gathered with hormonal (cortisol) and hormone peptide (Leptin) control confer to the exercise a protective character against disease and disorders related to neurodegeneration (**Papers 01 - 02**: de Assis, 2020; de Assis & Cieszczyk, 2020).

STUDY 01

Observing that circulating levels of BDNF might be altered by physical effort, we then decided to test the thesis that the secretion of BDNF is metabolic-responsive, and verify what type of exercise effort (aerobic or anaerobic) would trigger BDNF increase response (**Paper 03:** Murawska-Ciałowicz E., de Assis, et al., 2021). For this purpose, we have submitted thirty-five health active adults (27.80 ± 3.59 years; 25.55 ± 2.88 kg/m2 body mass index (BMI); 4.41 ± 1.14 kg/m2 fat mass index) to a 9-week training program composed of 4 different high-intensity training groups – with similar exposition to high intensity aerobic stimulus **(Table 1)**.

Table 1. Subjects and groups characteristics.

Groups	Age (years)	Weight (kg)	Height (cm)	BMI (kg/m^2)	FMI (kg/m^2)	WHR
HIFT	26.9 ± 4.20	83.12 ± 7.30	1.78 ± 0.04	26.19 ± 2.48	4.58 ± 0.93	0.87 ± 0.04
HIPT	28.9 ± 3.70	79.93 ± 6.78	1.81 ± 0.07	24.48 ± 2.03	4.96 ± 1.46	0.88 ± 0.03
HIIT	26.5 ± 3.30	83.99 ± 6.50	1.81 ± 0.06	25.59 ± 4.38	3.81 ± 0.65	0.85 ± 0.06
HIET	28.9 ± 3.10	81.42 ± 6.50	1.77 ± 0.04	26.07 ± 2.04	4.31 ± 1.03	0.91 ± 0.02

Blood samples for BDNF analyses were taken pre- and post- a VO_2max test (BRUCE protocol on treadmill) and the standard anaerobic capability test (Wingate on cycloergometer). Samples procedures were performed both at baseline and after the 9-week training sessions **(Table 2)**.

Table 2. Overview on training sessions. TC = technique correction; EL = exercise learning.

Summary of TRAINING SESSION								
Groups	**Warm-up**	**Work**	**Total work**	**Rest**	**Total rest**	**TC /EL**	**Cool-down**	**Σ time/session**
HIPT	10 min	8 rep × 30 s	4 min	8 rep × 150 s	20 min	20 min	5 min	59 min
HIIT	10 min	6 rep × 90 s	9 min	6 rep × 90 s	9 min	20 min	5 min	53 min
HIET	10 min	12 min	12 min	–	–	20 min	5 min	47 min
HIFT	10 min	8 rep × 30 s	4 min	8 rep × 150 s	20 min	20 min	5 min	59 min
Summary of MICROCYCLE								
Groups	**Warm-up (Σ)**	**Work**	**Total work**	**Rest**	**Total rest**	**TC /EL**	**Cool-down (Σ)**	**Σ time /week**
HIPT	3 × 10 min (30 min)	3 × 4 min	12 min	3 × 20 min	60 min	3 × 20 min (60 min)	3 × 5 min (15 min)	177 min
HIIT	3 × 10 min (30 min)	3 × 9 min	27 min	3 × 9 min	27 min	3 × 20 min (60 min)	3 × 5 min (15 min)	159 min
HIET	3 × 10 min (30 min)	3 × 12 min	36 min	–	–	3 × 20 min (60 min)	3 × 5 min (15 min)	141 min
HIFT	3 × 10 min (30 min)	4 + 9 + 12 min	25 min	20 + 9 min	29 min	3 × 20 min (60 min)	3 × 5 min (15 min)	159 min
Summary of the 9 week MESOCYCLE								
Groups	**Warm-up (Σ)**	**Work**	**Total work**	**Rest**	**Total rest**	**TC /EL**	**Cool-down (Σ)**	**Σ time/9 weeks**
HIPT	9 × 30 min (270 min)	9 × 12 min	108 min	9 × 60 min	540 min	9 × 60 min (540 min)	9 × 15 min (135 min)	1593 min (26 h 33 min)
HIIT	9 × 30 min (270 min)	9 × 27 min	243 min	9 × 27 min	243 min	9 × 60 min (540 min)	9 × 15 min (135 min)	1431 min (23 h 51 min)
HIET	9 × 30 min (270 min)	9 × 36 min	324 min	–	–	9 × 60 min (540 min)	9 × 15 min (135 min)	1269 min (21 h 09 min)
HIFT	9 × 30 min (270 min)	9 × 25 min	225 min	9 × 29 min	261 min	9 s × 60 min (540 min)	9 × 15 min (135 min)	1431 min (23 h 51 min)

Our results confirmed that in face of an aerobic stimulus – especially of high intensity – the secretion/release of BDNF is increased and higher levels of BDNF can be found in the blood stream. And as a result of physiological adaption, the effect of the aerobic stimulus on BDNF secretion is diminished as the individuals improve in fitness condition **(Fig 1 – 2)**.

Interestingly, our results helped to identify that, as opposed to aerobic stimulus, anaerobiosis appeared to suppress BDNF secretion. The evaluation of anaerobic performance imposed by the Wingate test resulted in a diminishment in levels of blood BDNF. Notwithstanding, the physiological adaptations yielded by the training program showed a positive impact on the individuals' anaerobic capability. It was possible to perceive that declines in BDNF secretion were milder in those individuals who were submitted to higher loads of the high-intensity exercise, featuring the impact of physiological adaptations to both aerobic and anaerobic energetic systems **(Fig 3 – 4)**.

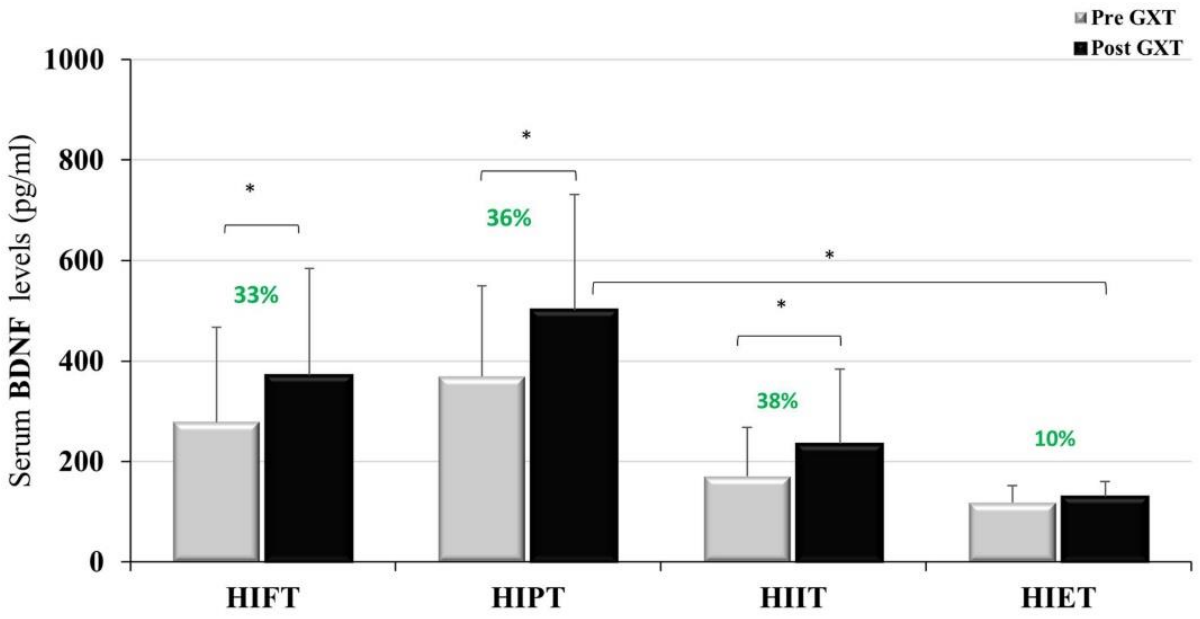


Figure 1. BDNF levels pre- post VO_2max test (GXT), at baseline. *p<0.05

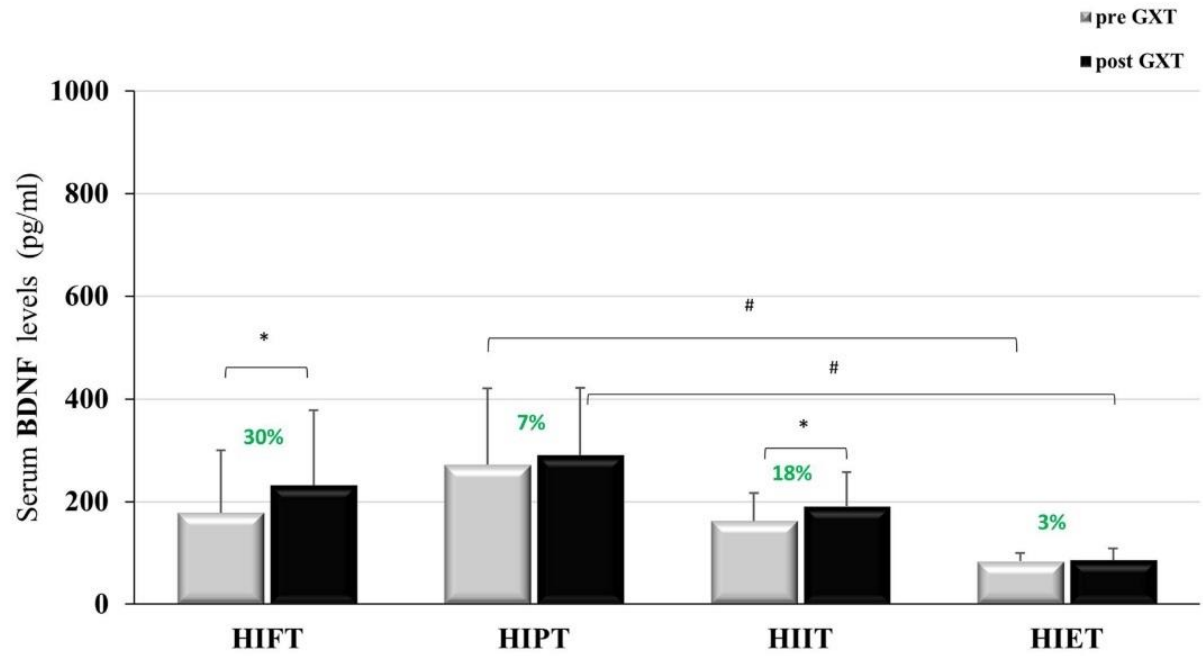


Figure 2. BDNF levels pre-post VO_2max test (GXT), after program. #p < 0.01; *p < 0.05.

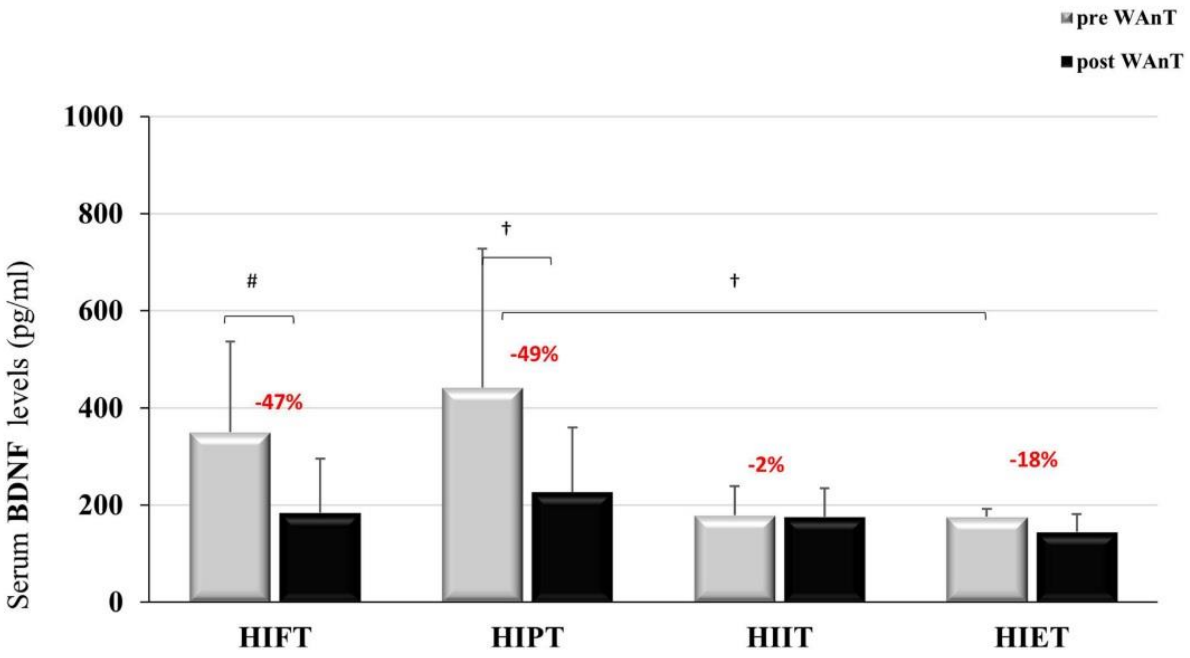


Figure 3. BDNF levels pre- post Wingate test, at baseline. *p < 0.05; #p < 0.01; †p < 0.001.

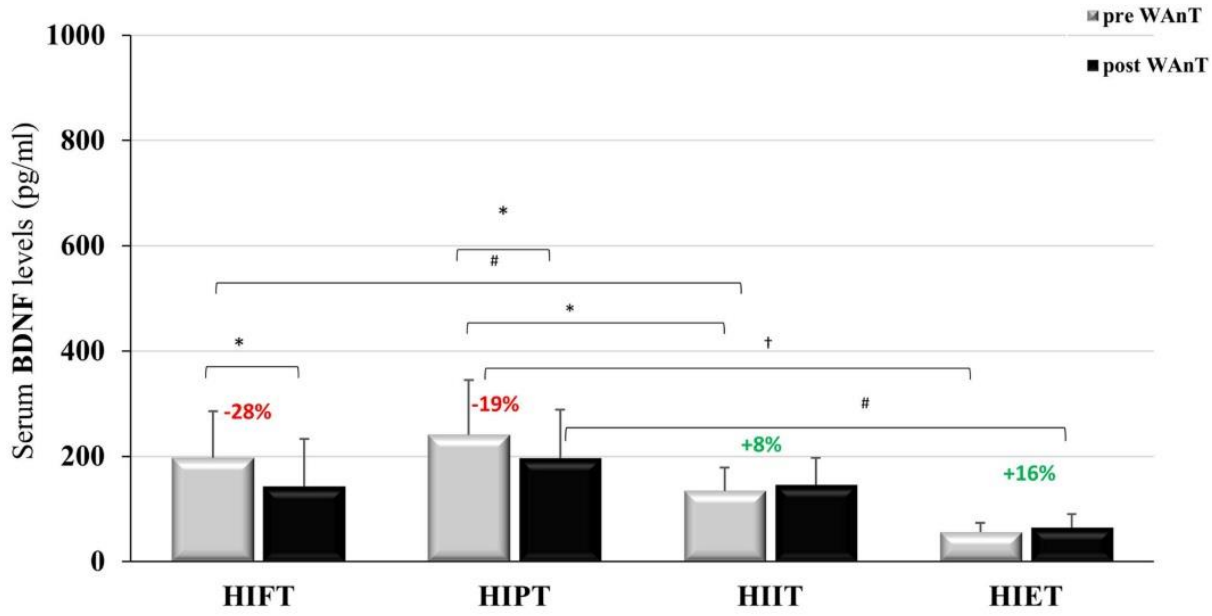


Figure 4. BDNF levels pre- post Wingate, after program. *p < 0,05; #p < 0,01; †p < 0,001.

Altogether, we confirmed that the secretion/release of BDNF is responsive to aerobic metabolism in a sense that a well-trained individuals display of higher capability of releasing BDNF and lower BDNF suppression upon anaerobic conditions.

STUDY 02

The *BDNF* Val66Met polymorphism, previously investigated only in animal experiments, is associated to multiple forms of neuropsychiatric illness and might have an effect on the expression/production of BDNF. Therefore, we have tested the hypothesis that the Val66Met polymorphism specific to human *BDNF* gene has an effect on BDNF production. Descriptive studies have pointed the Val66met polymorphism as a potential factor for the development of severe conditions in both cognitive and psychiatric disorders. The incidence of this polymorphism is higher amongst individuals sharing severe forms of bipolar disorder, cognitive impairment, depression and schizophrenia (Bath and Lee 2006; Chen et al. 2006; Froud et al. 2019; Jiang et al. 2017; Peters et al. 2020; Pezawas et al. 2004; Rybakowski et al. 2006; Shimizu, Hashimoto, and Iyo 2004; Ward et al. 2015). Such thesis becomes stronger when clinical studies report that there might be an impact of the *BDNF* Val66met polymorphism on the effect of exercise on BDNF and cognition. When comparing results of individuals positive vs negative for the *BDNF* Val66met polymorphism, the polymorphism presence appears to modulate both the cognitive and physiological responses to exercise (Abraham et al. 2019; Keyan and Bryant 2017; Ward et al. 2015).

Nevertheless, to understand better the potential implications of the *BDNF* Val66met polymorphism for stress related mental illness, we performed a systematic review that revealed that the polymorphism presence should not be considered a determinant factor for the development or the severity of psychiatric disorders. On the other hand, we observed that the acute stress and cortisol response, that is implicated in the etiology of mainly all mental disorders, consistently shows to be related to the *BDNF* Val66met polymorphism (de Assis and Gasanov 2019).

In order to take one step ahead of the current knowledge on *BDNF* Val66met polymorphism, we developed a 'clinical experimental' protocol, and tested the expression of *BDNF* in human muscle samples, which has been already demonstrated by Brunelli et al., (2012) and Marosi & Mattson, (2014), with the specific detection of mRNA products from the *BDNF* alleles containing and not containing the Val66Met polymorphism for the first time, and their responsivity to metabolic stress (**Paper 04:** de Assis et al., 2021).

To achieve that purpose, we submitted 13 healthy active adults to a VO_2max test (BRUCE protocol) and we collected blood samples from the antecubital vein and muscle samples from femoral quadriceps at the same time-points – 30-min resting (pre- test) and anaerobiosis conditions (post-test). Blood BDNF was analyzed by biochemistry – Elisa, while

the muscle samples were used for RNA isolation and analysis of full length BDNF mRNA transcript **(Table 3)**.

After complementary DNA (cDNA) extraction from the muscle samples, a 14-reference gene plate was ran with the samples cDNA in order to establish reference genes with stable expression in muscle tissue under resting and metabolic stress conditions. The beta-2-microglobulin (*B2M*) and the ribosomal protein S18 (*RPS18*) showed up to be the most stable among the known housekeeping genes. We then chose *B2M* as a baseline reference for the findings of *BDNF* expression. So, we used primers for the allele-specific identification of *BDNF* mRNA products specific for the Val66- and Met66- alleles (developed by Sheikh et al., 2010), in different fluorescent systems on Droplet Digital PCR which amplifies DNA molecules as small as 50 base pairs. This enable us to detect the absolute values of BDNF (Val66 and Met66 alleles) and B2M molecules in a same sample, for the first time **(Table 4)**.

We reported that *BDNF* expresses both alleles simultaneously. It was also shown that in heterozygotes, the Met66- BDNFmRNA quantified 76.1 ± 11.2% of Val66-coding mRNA values, regardless of metabolic conditions. Both the metabolic stress ($p < 0.0001$) and the Met66-allele presence ($p = 0.0001$) had an effect on BDNF expression (**Figure 5**).

Our results demonstrate that the anaerobic metabolism might decrease *BDNF* expression up to 1.8 ± 0.4-fold, regardless of the genotype. And that the expression of BDNF is negatively affected by the Met66- allele presence in individuals heterozygote. This suggests a partial dominance of the Val66-allele over the Met66-allele variant of *BDNF*. Importantly, the expression levels of BDNF do not correlate with circulating BDNF levels as analyzed from plasma (**Figure 6**).

Table 3. Subject characteristics and plasma BDNF levels.

ID (Age 34 ± 9)	BMI (22.85 ± 1.6 kg/m^2)	VO_2max (54.33 ± 6.2 ml/kg/min)	Genotype	Plasma BDNF-pre (pg/ml)	Plasma BDNF-post (pg/ml)	Δ BDNF (pg/ml)
1	21.97	60.00	Val/Val	9300.08	7547.9	−1752.18
2	22.35	62.00	Val/Val	8220.33	7848.24	−372.09
3	21.97	59.38	Val/Met	8522.45	7214.06	−1308.39
4	26.31	41.40	Val/Val	8088.49	7456.73	−631.76
5	23.10	45.62	Val/Met	10103.24	8898.5	−1204.74
6	22.74	51.38	Val/Val	6961.47	8314.07	1352.6
7	23.70	56.06	Val/Val	8558.53	6998.88	−1559.65
8	23.89	61.40	Val/Val	7276.23	8690.76	1414.53
9	20.67	54.24	Val/Val	7623.1	8630.74	1007.64
10	22.32	57.3	Val/Val	8511.54	8831.91	320.37
11	23.39	51.46	Met/Met	9803.92	8328.3	−1475.62
12	20.23	56.38	Val/Met	7779.02	8160.82	381.8
13	24.42	49.64	Val/Val	10351.91	9005.23	−1346.68

Val, Val66-coding; Met, Met66-coding BDNF allele; BMI, body mass index; VO_2max, the maximum rate of oxygen consumption; means are given at the top of the table. BDNF-pre, plasma BDNF concentrations at rest; BDNF-post, plasma BDNF concentrations after the VO_2max test; ΔBDNF, the BDNF-pre and BDNF-post difference.

Table 4. Allele-specific quantification of BDNF mRNA in human muscle tissue by ddPCR with B2M mRNA as a reference.

ID	Genotype	*Allele-specific BDNF mRNA**				*B2M/10 mRNA**		*BDNF mRNA/1000 B2M mRNA*			
	Condition ->	Pre-Val	Pre-Met	Post-Val	Post-Met	Pre	Post	Pre-Val	Pre-Met	Post-Val	Post-Met
1	Val/Val	1.09	0	3.27	0	83.8	444	1.305	0	0.736	0
2	Val/Val	5.48	0	2.95	0	279	235	1.962	0	1.255	0
3	Val/Met	1.85	1.17	2.08	1.43	220	406	0.840	0.530	0.512	0.346
4	Val/Val	0.62	0	2.64	0	49.6	500	1.253	0	0.528	0
5	Val/Met	1.55	1.11	1.35	1.2	170	268	0.913	0.655	0.503	0.376
6	Val/Val	7.02	0	2.59	0	367	368	1.912	0	0.704	0
7	Val/Val	2.64	0	1.40	0	155	163	1.701	0	0.860	0
8	Val/Val	3.04	0	6.43	0	101.9	435	2.982	0	1.478	0
9	Val/Val	5.17	0	8.47	0	245	714	2.109	0	1.186	0
10	Val/Val	2.70	0	1.80	0	224	302	1.205	0	0.596	0
11	Met/Met	0	1.53	0	1.97	60.8	130	0	2.522	0	1.513
12	Val/Met	0.90	0.63	6.33	5.03	115	1,201	0.783	0.551	0.527	0.419
13	Val/Val	4.40	0	5.43	0	230	428	1.913	0	1.269	0

**Expression values are disposed in (copies/μl of reaction mixture), 1 μl of reaction mixture corresponds to 0.625–1.25 mg of muscle tissue. Pre-Val, BDNF Val66-allele expression values in rest; post-Val, Val66-allele expression values after VO_2max test; pre-Met, BDNF Met66-allele expression values in rest; post-Met, Met66-allele expression values after VO_2max test. B2M, beta-2-microglobulin expression values, pre- and post.*

Figure 5. Allele-specific BDNF expression of heterozygotes pre-post GXT. Val, Val66-coding BDNF mRNA; Met, Met66-coding BDNF mRNA. **p < 0.05, ***p < 0.01, ****p < 0.001. Bars are given with ± SD.

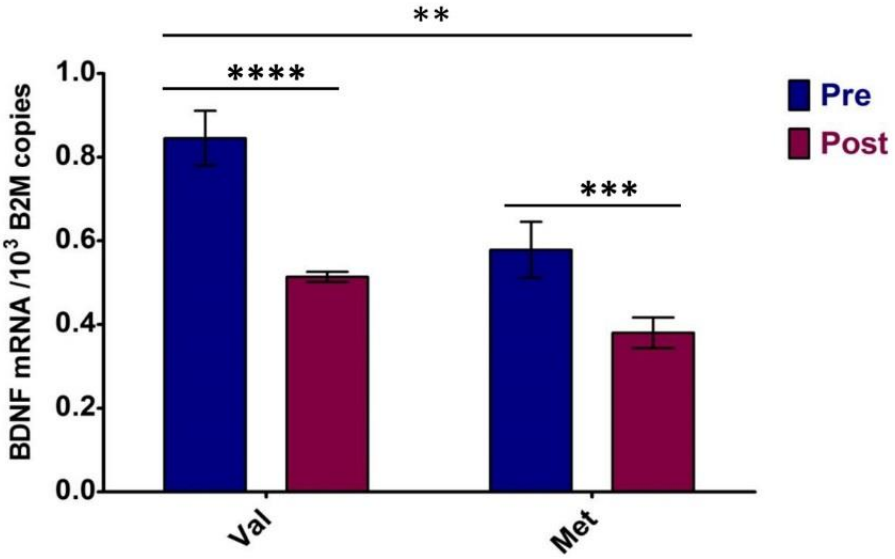
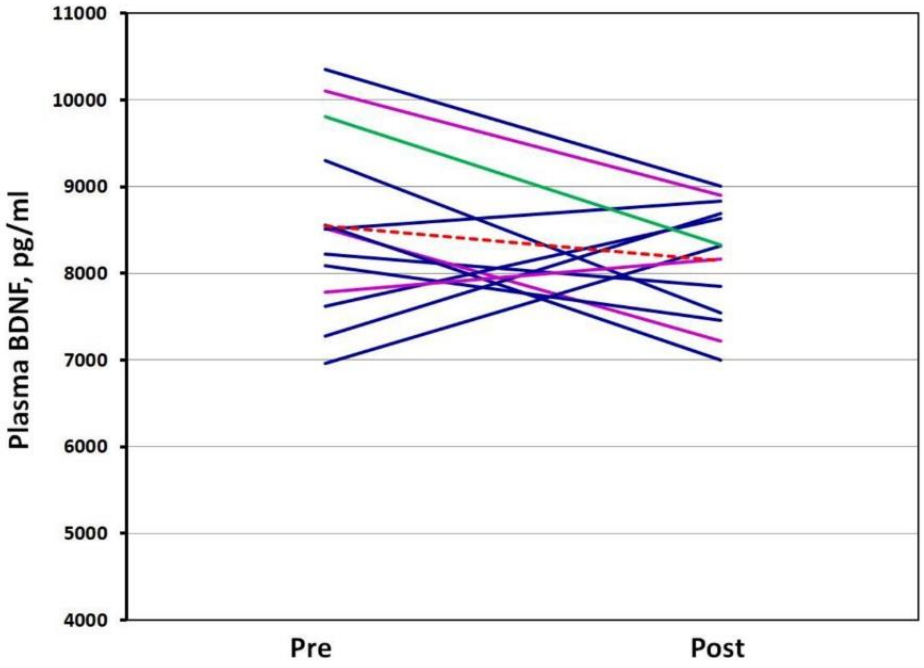


Figure 6. Spaghetti plot of plasma BDNF levels changes pre-post GXT. Val66Val; dark blue, Val66Met heterozygotes; magenta, Met66Met homozygote; green, Dotted red line: mean.



STUDY 03

The outstanding results have inspired us to contribute for the development of a new protocol that was sable to detect the allele-specific expression of *BDNF* including the Val66met polymorphism using RT-PCR (**Paper 05:** Gilmara Gomes de Assis et al., 2020). For that, we used once again the cDNA from the muscle samples of 10 genotyped individuals (1 female, for the reference gene protocol), from pre- post VO_2max test time-points. **(Table 5)**.

Table 5. Characteristic of Participants in ddPCRTM and qRT-PCR analyses

Subject	Age (years)	BMI (kg/m²)	VO_2max (ml·kg/min)	Genotype
Female	36	19.84	44.00	VAL/VAL
Male	24	21.97	60.00	VAL/VAL
Male	46	22.35	62.00	VAL/VAL
Male	28	21.97	59.38	**VAL/MET**
Male	43	26.31	41.40	VAL/VAL
Male	26	23.10	45.62	**VAL/MET**
Male	22	22.74	51.38	VAL/VAL
Male	46	23.70	56.06	VAL/VAL
Male	28	23.89	61.40	VAL/VAL
Male	36	20.67	54.24	VAL/VAL
Means ± SD	32.9 ± 10.3	23.25 ± 1.4	54.7 ± 7.7	

*VO_2max, maximal oxygen intake; BMI, body mass index.

The cDNA solutions were then applied to a RT-PCR protocol with the allele-specific BDNF assay (Sheikh et al. 2010), containing hydrolyzed probes (TaqMan) with both HEX (hexachloro- fluorescein) and FAM (6-carboxyfluorescein) systems of detection (for Val66- and Met66-coding *BDNF* allele's products, respectively). A HEX-based *B2M* Expression Probe Assay was ran in parallel and the melting curves were used as a product specificity control. Reactions were performed in triplicate and mean results were used in subsequent mathematical conversions. As reports of FAM and HEX probes of Met66- and Val66-coding BDNF mRNA levels from RT-PCR could not be compared directly by threshold cycles (C(t)s), probably due to a difference in FAM and HEX fluorescence detection sensitivity, after conversion of FAM to HEX detection, Met66-coding allele's expression was standardized to HEX detection **(Tables 6-7)**.

Table 6. qRT-PCR BDNF analysis. Threshold cycles, C(t)s, calculations

Genotype	Fluorescense detection	B2Mpre mean	B2Mpost mean	BDNFpre mean	BDNFpost mean	mRNApre mean	mRNApost mean	BDNFpre HEX/FAM correction	BDNFpost HEX/FAM correction	TotalBDNFpre expression in heterozygous	TotalBDNFpost expression in heterozygous
V/V	HEX	24.55	23.26	35.43	34.44	40.11	36.95	35.49	34.67		
	FAM	0	0	0	0	0	0	0	0		
V/V	HEX	21.34	22.46	32.33	34.10	38.22	41.00	32.35	34.11		
	FAM	0	0	0	0	0	0	0	0		
V/M	HEX	23.91	24.05	34.21	34.68	36.46	40.30	34.48	34.7	33.86	34.01
	FAM	0	0	29.98	30.83	31.50	35.78	35.37	35.39		
V/V	HEX	26.12	23.60	35.7	33.34	38.62	36.02	35.88	33.54		
	FAM	0	0	0	0	0	0	0	0		
V/M	HEX	23.92	23.8	34.42	34.98	36.30	36.56	34.76	35.39	33.96	34.66
	FAM	0	0	30.09	31.01	31.62	32.13	35.19	35.99		
V/V	HEX	23.21	23.17	32.29	33.37	35.29	35.63	32.45	33.64		
	FAM	0	0	0	0	0	0	0	0		
V/V	HEX	24.37	24.61	34.04	34.23	36.94	37.71	34.22	34.35		
	FAM	0	0	0	0	0	0	0	0		
V/V	HEX	24.6	22.95	33.19	32.19	36.65	36.65	33.32	32.25		
	FAM	0	0	0	0	0	0	0	0		

Pre, Analyses from the rest condition; **Post**, Analyses from after effort test

Table 7. qRT-PCR results validation by ddPCR. Condition- and allele-specific BDNF expression differences.

Genotype	qRT-PCR			ddPCR			qRT-PCR		ddPCR	
	Tpre BDNF-B2M	Tpost BDNF-B2M	T BDNF post /pre	Tpre BDNF /1000B2M	Tpost BDNF /1000B2M	T BDNF post /pre	Pre MetBDNF /ValBDNF	Post MetBDNF /ValBDNF	Pre MetBDNF /ValBDNF	Post MetBDNF /ValBDNF
V/V	10.94	11.41	0.721	1.444	0.703	0.487				
V/V	11.01	11.66	0.641	1.792	1.17	0.653				
V/M	9.950	9.960	0.996	1.427	0.926	0.649	0.541	0.621	0.707	0.614
V/V	9.760	9.950	0.877	0.793	0.498	0.628				
V/M	10.04	10.86	0.566	1.524	0.910	0.598	0.741	0.662	0.660	0.968
V/V	9.250	10.48	0.427	2.319	0.745	0.321				
V/V	9.850	9.740	1.077	2.045	1.110	0.543				
V/V	8.720	9.300	0.665	3.460	1.607	0.464				
Means ± SD	T BDNF post/pre, 0.736 ± 0.205			T BDNF post/pre, 0.543 ± 0.115			MetBDNF/ValBDNF, 0.641 ± 0.084		MetBDNF/ValBDNF, 0.737 ± 0.158	

Pre, Analyses from the rest condition; **Post**, Analyses from exercise protocol; Tpre and Tpost – total (both alleles) *BDNF* expression, ValBDNF, MetBDNF – allele-specific *BDNF* expression.

In order to validate the results of RT-PCR with reference on ddPCR analysis values, Paired Student's t-test was used to compare condition/changes description and a Spearman's rank test for determining the correlation between ddPCR and RT-PCR results.

Our results demonstrate and propose RT-PCR as a feasible method for the determination of the allele-specific expression of BDNF regarding the presence of Val66met polymorphism in DNA containing samples, with the sensibility for detecting metabolic stress conditions.

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Efekt polimorfizmu Val66Met genu BDNF na ekspresje BDNF a aktywność fizyczna

STRESZCZENIE

Neurotroficzny czynnik pochodzenia mózgowego (BDNF) to neurotrofina silnie eksprymowana w tkance nerwowej zarówno podczas rozwoju mózgu jak i potem, przez całe życie. Przypuszczalnie pełniąc role w synaptogenezie i neuroplastyczności, BDNF jest zaangażowany w podstawowe mechanizmy poznawcze i zachowanie, takie jak pamięć, nastrój, funkcje motoryczne i wykonawcze. Ostatnio zaobserwowano wpływ ćwiczeń na poziom BDNF w krwiobiegu oraz ich pozytywny wpływ na procesy poznawcze, co wykazano w zadaniach poznawczych.

Jednym z najczęściej badanych polimorfizmów w ludzkim genie *BDNF* jest Val66Met (rs 6265), mający wpływ na postać prekursorowej izoformy pro-BDNF, mającej kluczowe znaczenie dla funkcjonalności BDNF. Badania z zakresu genetyki psychiatrycznej wykazały większą częstość występowania genotypu Val/Met genu *BDNF* w subpopulacji z różnymi diagnozami neuropsychiatrycznymi w porównaniu z grupą kontrolną bez rozpoznania. Może to sugerować zwiększoną podatność na rozwój choroby psychicznej u heterozygot Val/Met genu *BDNF*.

Moje badania nad BDNF rozpoczęły się od obserwacji, pacjentów z zaawansowaną chorobą Parkinsona (stadium III i IV w skali Hohna i Yahra), którzy byli poddani terapii ruchem (4-tygodniowy program ćwiczeń aerobowych w wodzie). Przeprowadzone badania wykazały wzrost poziomu BDNF we krwi pod wpływem wykonywanego wysiłku. Fakt ten skłoniły mnie do podjęcia decyzji o kontynuowaniu badań nad BDNF i jego właściwościami fizjologicznymi, w tym potencjalnymi implikacjami polimorfizmu Val66Met *BDNF*, w ramach przewodu doktorskiego realizowanego w AWFiS.

Głównym celem realizowanego projektu było zbadania powiązań pomiędzy Val66Met *BDNF*, a poziomem reakcji adaptacyjnej organizmu na wykonywany wysiłek fizyczny.

Pierwsze z podjętych zadań badawczych polegały na zestawieniu doniesień naukowych dotyczących mechanizmów łączących wysiłek fizyczny i wydzielanie BDNF. Moją szczególną uwagę zwróciły mechanizmy wywołane stresem oksydacyjnym, gdzie cała kaskada procesów metabolicznych regulowaną po aktywacji rzez PGC-1α ekspresją klastra genów związanych z komórkową odpowiedzią adaptacyjną wywołaną deficytem tlenu w konsekwencji zwiększa

ekspresję *BDNF*. Mechanizmy te okazały się być jednak nie do końca wyjaśnione i co istotniejsze, przez wielu naukowców wciąż uznawane były za nie potwierdzone.

Początkowo realizowane przeze mnie badania miały na celu sprawdzenie hipotezy, czy wykonywany wysiłek fizyczny rzeczywiście wpływa na zwiększenie wydzielania BDNF. Grupa dorosłych ochotników realizowała specjalnie zaplanowany 9-tygodniowy program treningowy o wysokiej intensywności. Po zrealizowanym treningu przeprowadzono testy wydolności tlenowej (test VO_2max) i beztlenowej (test Wingate), które potwierdziły zwiększone wydzielanie BDNF pod wpływem wysiłków aerobowych. Co ciekawe, zaobserwowano wydzielanie BDNF zmniejsza się wraz z poprawą kondycji fizycznej osób poddanych ćwiczeniu (wydzielanie BDNF było największe u osób najmniej aktywnych). Przeprowadzone z kolei testy wydolności beztlenowej wykazały, że wydzielanie BDNF zmniejsza się w warunkach stresu oksydacyjnego.

Rozwinięciem opisanego powyżej projektu był kolejny protokół badawczy, który miał na celu sprawdzenie poziomu ekspresji BDNF w zależności od genotypowej postaci *BDNF*. Do badań przeprowadzonych wśród zdrowych, nietrenujących ochotników wykorzystano sondę molekularną do wykrywania produktów mRNA ze swoistych alleli *BDNF* (dodatnich lub ujemnych (+/-) dla polimorfizmu Val66Met). Wykazaliśmy, że do ekspresji *BDNF* dochodzi w przypadku obu alleli jednocześnie, przy czym u heterozygot Met66-*BDNF* ekspresja mRNA wynosi nie więcej niż 76,1±11,2% ekspresji mRNA jaką można przypisać Val66-*BDNF*. Stosunek ten był niezależny od warunków metabolicznych. Przeprowadzona analiza parametrów z osocza potwierdziła także wcześniejsze spostrzeżenia, że stres oksydacyjny wpływa negatywnie na poziom ekspresji *BDNF*. Wykazano także brak korelacji pomiędzy poziomem ekspresji *BDNF* a poziomem BDNF w krwiobiegu.

Realizowane kolejno eksperymenty były przesłanką opracowania zupełnie nowego protokołu umożliwiającego wykrycie specyficznej ekspresji *BDNF* zależnej od allelu Val66Met. Do opracowania protokołu wykorzystano cDNA z mięśni 10 ochotników (pobór wykonany przed i po teście VO_2max). cDNA było podstawą do analizy RT-PCR ze specyficzną dla allelu próbką *BDNF*, zawierającą zhydrolizowane sondy (TaqMan) z systemem detekcji barwników fluorescencyjnych zarówno HEX (heksachlorofluoresceina), jak i FAM (6-karboksyfluoresceina) (odpowiednio dla kodowania produktów alleli Val66- i Met66-*BDNF*). Testy sondy ekspresji *B2M* opartej na HEX prowadzono równolegle, a krzywe topnienia stosowano jako kontrolę specyficzności produktu. Reakcje przeprowadzono w trzech powtórzeniach. W konsekwencji otrzymano udało się opracować efektywniejszą i tańszą metodę oznaczania ekspresji *BDNF* specyficznej dla poszczególnych alleli Val66Met.

ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a type of neurotrophin that stands out for its high level of expression in the brain. This occurs both during and after the brain development, and the expression is lifelong. It is believed that by playing a role in synaptogenesis and neuroplasticity, BDNF is involved in basic cognitive abilities and behaviour such as memory, mood, motor and executive functions. Recently, it has been shown that blood BDNF level increases after exercise compared to resting conditions. Cognitive assays also highlight that exercising benefits cognitive functions.

One of the most studied polymorphisms in the human BDNF gene is Val66Met (rs 6265). The latter influences the pro-BDNF isoform precursor, which is significant for BDNF functional effects. Psychiatric genetics studies have revealed that Val/Met BDNF genotype is more frequent in subpopulations with neuropsychiatric disorders when compared to healthy controls. This might imply that Val/Met heterozygous carriers could me more susceptible to mental illness development.

My research on BDNF started off by observing patients with advanced Parkinson disease (stages III and IV on The Hoehn and Yahr scale). These patients underwent a movement-based therapy (4 week water aerobic training program). I found that blood BDNF level elevated in response to exercise. This provided an exciting opportunity to advance our knowledge of BDNF and its physiology, including potential BDNF Val66Met polymorphism implications. This work was carried out to fulfill the requirements for the doctoral degree at AWFiS.

The main objective was to examine the associations between Val66Met *BDNF* and adaptive response level upon exercise performance.

First, I reviewed the available literature in the context of the physical exercise and BDNF secretion. Oxidative stress mechanisms received my special attention. This was due to the fact that a multiple metabolic pathways are initiated upon the activation of a crucial metabolic node, PGC-1α linked to cellular adaptation to oxygen deficiency. The crosstalk of many signalling cascades result in increased BDNF expression. However, the mechanisms involved were not fully described and interestingly, they were not confirmed.

Therefore, I initially intended to test the hypothesis that physical activity increases blood BDNF concentration. A group of adult volunteers underwent a highly intense, 9 week training program. Aerobic (VO_2max) and anaerobic (Windgate) capacity tests were performed. An increased level of BDNF after Wingate test was recorded. The most striking result to emerge from the data was the following dependence: the better physical condition, the lower BDNF

secretion. The least active volunteers had the highest BDNF secretion. Moreover, anaerobic capacity tests revealed that BDNF secretion decreased in oxidative stress related conditions.

The above protocol was further developed with the aim of examining BDNF expression according to the *BDNF* genetic variants. Aapparently healthy and non-training volunteers participated in the study. A molecular probe was used to detect "BDNF mRNA" (positive or negative mRNA products (+/-) for Val66Met polymorphism). It was shown that BDNF was expressed for both alleles simultaneously, whereas heterozygous Met66-*BDNF* mRNA expression was no more than 76,1±11,2% ascribed to Val66-*BDNF* mRNA expression. This ratio was independent of metabolic conditions. Plasma analysis confirmed the former observations that oxidative stress negatively regulated the BDNF expression. No correlation between the BDNF expression and blood BDNF was found.

Further experiments were necessary to develop a new for the detection of Val66/Met66 allele-specific *BDNF* expression. To achieve that, muscle cDNA was obtained from 10 volunteers. The sampling occurred before and after the VO_2max. The cDNAs were subjected to a RT-PCR assay containing hydrolyzed probes (TaqMan) with both HEX (hexachloro-fluorescein) and FAM (6-carboxyfluorescein) systems of detection for the Val66- and Met66-coding *BDNF* allele's products, respectively. A HEX-based *B2M* expression probe assay was ran in parallel and the melting curves were used as a product specificity control. All the reactions were performed in triplicate and the mean was used in subsequent calculations. This was designed to develop cheaper and more effective method for the detection of Val66/Met66 allele-specific *BDNF* expression.

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REVIEW

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Exercise – A unique endogenous regulator of irisin, BDNF, leptin and cortisol against depression

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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abstract

Depression is the most prevalent stress-related disorder affecting the population world-wide with potential for an eminent increase after the 2020 pandemic. The mechanisms through which metabolism is involved in depression and stress disorders have been extensively investigated. However, their assessment using exogenous measures is a current limitation. Exercise, as is well reported in animal studies, exerts a critical regulatory influence on the main factors known to participate in these mechanisms. This overview describes the role of cortisol, leptin, irisin and BDNF in the exercise physiology and the known mechanism through which these factors act in anti-depressant mechanisms. Furthermore, the exercise is proposed as a clinical recommendation due to its effective and affordable character in treating depression for the sustainability of public health.

Key words: leptin, irisin, BDNF, metabolism, obesity.

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INTRODUCTION

In 2008, the World Health Organization projected that depression would rank as the first cause of burden of disease by 2030. The disease onset is inherently unpredictable, and the duration of episodes, the number of episodes over a lifetime, and the pattern in which they occur are variable. However, the recurrence of depression is high, and its risk increases with every episode. Yet, each episode and the outcome is less favorable with older age [1]. Reports from nine months since the outbreak of the current Covid-19 pandemic in 2020 show a substantial increase in depression and its related disorders, such as sleeplessness, anxiety, post-traumatic stress symptoms, and preexisting psychiatric disorders, all over the globe [2–5]. Facing this global crisis, with unprecedented repercussions in the healthcare systems, policies of preventative care, which are considerably less explored, seem to be a relevant solution for economies both with higher incomes and lower incomes [6, 7].

Initially, considered as therapeutic for cardiorespiratory conditions [8,9], the known benefits of exercise, beyond esthetics or performance, have expanded to a side support for treatment of obesity and depression. Depression is clinically characterized by atrophy or impairment in the functioning of cortico-limbic neuronal circuits that affect the regulation of mood and emotion [10–12]. Physiologically, the onset of depression is associated with a malfunction in the hypothalamus-pituitary-adrenal (HPA) axis and excessive cortisol levels [13]. By contrast, a regular practice of exercise is associated to a 'stabilizing effect' on the symptoms and progression of the depression condition and has been observed to positively affect response to its treatment [14, 15].

Hormones and cytokines converge in the physiological control of exercise and stress. Briefly, the corticotropin-releasing hormone released by the hypothalamus in response to a metabolic stress (a psychologically-derived metabolism response) induces adrenocorticotropin secretion which stimulates secretion of cortisol from adrenal cortex cells. Lower cortisol levels signal through membrane-associated receptors and second messengers (or non-genomic pathways), while high concentrations of cortisol bind to glucocorticoid receptor (GR) within the cell cytoplasm to initiate, in the nucleus, the expression of a groups of glucocorticoid-responsive genes that are involved in several metabolic functions under the regulation of transcription factors [16]. The chronic exposure to stressful events and high cortisol loads likely result in a GR resistance which, in turn, interferes with the appropriate regulation of inflammation. Therefore, stress and GR resistance conditions result in an HPA down-regulation of local proinflammatory cytokine response, which is the main risk factor for depression and stress-related metabolic disorders [17].

Key cytokines synergistically involved in the cell response to stress have been intensively investigated for their critical roles in the central mechanisms of depression and metabolic disorders. Reports on the role of brain-derived neurotrophic factor (BDNF) and irisin, which are produced by muscle and neuronal tissues, and on leptin, which is secreted by adipocytes under metabolic stress conditions, place these proteins as axil molecules for the understanding of the link between metabolism and the brain. Secreted leptin can cross the blood brain barrier (BBB) where in the brain, leptin binds to leptin receptors present in several thalamic, hypothalamic and midbrain regions that are associated with depression and energy homeostasis [18]. While irisin secreted by muscles and neural tissue has been demonstrated to play a role in depression disorders that are associated with obesity and metabolic syndrome [19, 20]. Finally BDNF, the main neurotrophin produced in the adult brain whose signal transduction is essential for neuroprotection and plasticity [21, 22], has been extensively studied for metabolic features [23, 24].

Not by coincidence, these proteins have been frequently targeted in research on therapeutic approaches. However, following the example of cortisol, the complexity and tight local

regulation of these cytokines represent an end line for the attempts of an exogenous manipulation [24–27]. Whereas the mechanisms associating depression and metabolic disorders are less clear, the systems underlying the onset of depression undeniably include cortisol dynamics, which is fundamentally involved in a systemic regulation of the above mentioned cytokines time-response and tissue-specific synthesis [28–30].

In a preventative perspective, it is reasonable to suggest that a physiological approach to the treatment of depression – regarding the exercise-induced endogenous regulation of these factors – is a feasible alternative for the sustainability of policies on public healthcare. Here, we will describe the physiological effects of the exercise metabolism on regulation of irisin, BDNF and leptin, and elucidate the neurobiological mechanisms by which the regulation of these factors affects depression disorders with a potential for prevention and treatment.

EXERCISE METABOLISM AND THE PHYSIOLOGICAL ROLE OF LEPTIN, IRISIN AND BDNF

Moving from resting to exercise states, there is an increase in the amount of energy demand by cells and speed of chemical reactions that provide this energy out of the assimilation of nutrient's substrates – also known as the metabolic rate (MR). This is possible due to a sympathoadrenal response with a rapid increase in the release of epinephrine in support of the ventilation demand. The acute rise of epinephrine then immediately elevates the heart rate and circulating blood pressure enhancing oxygen delivery to the requesting tissues [31]. Of the substrates available for the aerobic energy metabolism, glucose (GLU) is the most profitable and fast, and also the primary source required for the brain's supply. In the cells under normal aerobic conditions, GLU goes through nine enzymatic reactions to produce pyruvate which is converted into acetyl-coenzyme A that proceeds to oxidation inside mitochondria through a cycle chain of chemical reactions known as the Krebs cycle. As a result, cellular energy in the form of adenosine triphosphate (ATP) is produced while residual carbon dioxide is transported into the blood [32]. To a certain MR range, the cells can plead for the oxidation of glycogen and fatty acids as alternative sources as well, taking into account several intracellular events [33]. Whenever the oxygen is insufficient, pyruvate can be converted into lactate by one additional enzymatic reaction [34, 35]. Sidewise accumulated lactate thus is released into the circulation where it can be transported to the liver and goes through gluconeogenesis, or else it may cross the BBB and reach the neural tissue where it is also converted to pyruvate and enter the Krebs cycle. Lactate uptake by the brain might increase more than 2-fold from rest to exercise states [36].

Increase in the activity of 5' adenosine monophosphate-activated protein kinase (AMPK), a central enzyme involved in the oxidation cascades of GLU, glycogen and fatty acid, leads to induction of the expression of the co-transcription activator proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) via direct phosphorylation [37]. PGC-1α co-activates transcription factors that regulate the expression of several nuclear genes including FNDC5 and BDNF and those involved in the mitochondrial genesis and DNA duplication. Since that expression of mitochondrial genes also responds to metabolic stress-induced PGC1-α activity, this 'reward effect' enables an immediate response and a long lasting adaption against oxidative stress and degradation [38,39]. PGC-1α upregulation also mediates the acute blood pressure-induced vasodilatory boost and is involved the microvascular adaptions to exercise [40].

First identified as a PGC1-α -dependent cytokine, FNDC5 named after the gene FNDC5, is cleaved and secreted from muscle cells as irisin and acts preferentially on subcutaneous

fat tissue through an increase in the adipocytes expression of thermogenesis-related genes. While a specific receptor for irisin has not been discovered, its activity-dependent effects of irisin have also been observed in the bone tissue through the promotion of skeletal remodeling via integrin receptors. The autocrine and paracrine actions of irisin confer an inhibitory effect on adipogenesis [41–43]. Conversely, the neuronal expression of BDNF in response to metabolic stress is also associated to the PGC1-α/FNDC5 pathway [44–46]. BDNF plays multiple roles in the activity-dependent regulation of neuronal synapses structure and function. The mechanism by which BDNF expression and signaling promotes neurogenesis, synaptogenesis, long-term potentiation directly influence on memory, executive function and other cognitive processes have been greatly reported [21,47–49]. In exercise, the circulating levels of BDNF, whose main source is attributed to the brain, increase in accordance to demands on aerobic metabolism [50–52].

Meanwhile, a pulsatile release and well-synchronized tonic increase in cortisol levels occurs along with the metabolic stress, which then penetrates into the cells binding to glucocorticoid receptors within the cytoplasm, in a complex that translocates to the nucleus and starts catabolic and anti-anabolic actions by regulating expression of specific genes [53]. The actions of cortisol revolve around providing a pool of free branched chain amino acids that are used either as building blocks for the synthesis of the ultimately needed proteins expressed during metabolic stress or as an additional substrate for oxidation. All of which thus inhibits inflammation. Such process remains up to the recovery period ensuring the destruction of physiologically exhausted protein structures in order to make possible their substitution by newly synthesized ones [54].

Finally, secreted by adipocytes, an increase in the circulating levels of leptin during the exercise serves as a signal report of the energetic reserves status to the CNS and occurs in an inverse proportion to the fitness conditions. Leptin activation of the long form of leptin receptor located especially in the hypothalamus, midbrain and brainstem is thus considered a mechanism of central control of the energy homeostasis [55]. Increases in leptin levels ultimately regulate the food intake and energy storage, whereas the serum levels of leptin correlate with the individual's body fat mass [56–59].

THE ANTI-DEPRESSANT EFFECT OF THE EXERCISE

Research on neurobiology of exercise and experimental models have helped to build the knowledge on the importance of an endogenous regulation of factors with critical roles in the mechanisms involved in the physiology of stress and depression that still have been a challenge for pharmacologic approaches. Experimental reports suggest that a decrease in leptin receptors which are found in subcortical structures of the brain involved in the limbic system, associated with increased activation of the HPA axis can lead to an obesity-related depression disorder [26, 60]. Consistently, an increase in leptin levels as a reflection of leptin receptors resistance, gathered with an uncontrolled eating, is observed in obese and overweight subjects that display inflammatory states [61]. On the other hand, the role of leptin in neuronal growth in association with BDNF has been pointed as a positive effect of leptin signaling in these brain regions [18, 62]. For instance, BDNF-deficient animals present obesity and cognitive impairment which can be reversed by exercise through an improvement in leptin sensitivity induced synaptic plasticity [55]. Furthermore, in well-trained individuals who display of physiological levels of leptin lower than the average range, these levels are more stable in accordance to the fitness conditions [56, 58].

Similarly, increases in cortisol levels with consequent glucocorticoid receptors (GR) resistance is a common cause of stress-induced depression. While leptin appears to be able to reverse some of the negative effects of GR resistance [63, 64], the excessive cortisol-

induced GR resistance implicates in different types of depression and a variety of stress-related disorders [65–67]. Moreover, the down-regulation of GR signaling observed in stress disorders is associated with impairment in the downstream cascades of BDNF signaling which interferes in the neuronal capability of plasticity (as reviewed by De Assis and Gasanov, 2019 [68]). It is conclusive that a critical endogenous balance in the circulating levels of both leptin and cortisol is essential for their delivery of pro-neuroplasticity and homeostasis control actions, which are the key elements linking metabolism and stress-related disorders.

Additionally, the exercise-induction of irisin and BDNF expression via up-regulation of PGC1-α has shown to modulate neuroplasticity in the hippocampal and cortex areas as an anti-depressant effect [69,70]. Optimal levels of these two cytokines are thus associated with neuroprotection and preservation against cognitive decline [71–73]. Specifically for depression, the exercise-induced increases in BDNF helps as a generic contributor to neuronal plasticity affecting the related brain regions in a morphological and functional manner [74–76].

CONCLUSION

Considering the advances in explaining the molecular mechanisms connecting the brain and the body metabolism into an integrated system in the development of mental and metabolic illness. Furthermore, recognizing the practical limitations of an exogenous regulation of this integrate system, it seems to be the time to assume exercise as a clinical recommendation for depression and various stress-induced disorders, as a way of minimizing the overburden of the primary healthcare system. This overview suggests inclusion of exercise as a prescription in the guidance protocols for the patients with depression and stress-related metabolic disorders.

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Mini Review

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"BDNF and Neurodegeneration: The Rise of the Exercise as a Preventive Care"

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Mini Review

Since early 90's, the protein named brain-derived neurotrophic factor (BDNF) structure, functioning, gene code, location and specificities, as one of the most abundantly expressed growth factors of the Neurotrophins' family, have been massively explored in neuroscience fields [1,2]. With multiple functions in the formation and function of the central nervous system, all of which are lethal, the gene encoding for BDNF (named BDNF) abides highly stable throughout all vertebrates, including human [3]. From an evolution perspective, BDNF is reported as one of the key neurobiological molecule portraying the urge in the homo sapiens brain's grow after their needs for conquering new territories, acquiring memory, and navigation skills that implied in changes in the metabolism profile [4]. Nowadays, as the accumulated scientific knowledge on BDNF has fairly explained the association between brain and metabolism, the concept of neuroplasticity has so crossed boundaries from the molecular through clinical research fields, reaching the everyday conversation of individuals engaged into physical activity and healthy life style [5]. The sense that the brain is built of plastic matter and in so doing it is to a certain point "healable" comes together with an immediate preoccupation of how to protect the brain from malfunctions or else, how to preserve the brain from the losses of neurological diseases progress. Here I will elucidate the importance of BDNF for mechanisms involved in the functional brain's health and introduce the aerobic exercise, more specifically the aerobic energy metabolism, as main player in this scenario.

BDNF and the Brain's Health

Expressed mainly by neural tissue, BDNF participates in orientating cells differentiation into neurons during development processes and also in the adult brain processes as synaptogenesis, dendrite genesis, long-term potentiation and depreciation (memory formation) [6-8]. Corroborant, impairments in brain functions such as in Mild Cognitive Disorder disorders, Parkinson's, Alzheimer's, Levy's Body's and less frequent types of dementia are accompanied by lowered BDNF levels [9]. The very neuronal ability of functional regeneration which is based on the construction and strengthening of connections between neurons is compulsorily supported by BDNF in a vary of ways [10]. The release of BDNF occur after a multi-stage processing that primarily produces a precursor form of the neurotrophin (named pro-BDNF) which is later cleaved into the mature BDNF both before or during the release process. Binding of either the mature BDNF with its high-affinity Tyrosine kinase receptors B or of the released pro-BDNF form with low-affinity to p75 neurotrophin receptors mediate either pro-survival or pro-apoptotic processes, respectively, in different neuronal cell populations, in a highly coordinate dynamics of lethal importance [11].

In blood, the levels of BDNF are believed to be a plausible report of changes in the brain's cognitive performance associated with aging and natural neurodegeneration or neurodegenerative

disorders [12-14]. While reports of studies involving older individuals show a natural decrease in BDNF levels in time; a noticeable reduction in BDNF levels is observed in those individuals with neurodegenerative disorders. Moreover, decreases in BDNF levels appear to be associated with disease progress and cognitive impairment degrees [15]. Although a great effort has been spent into experimental attempts of finding an exogenous manner of manipulating BDNF in the past decades [16,17], the precise dynamics of BDNF release and reuptake differentially orchestrated in different brain regions and cell populations has led the pharmacological research to exhaust the possibilities, before advances to human studies.

The Exercise as a Preventive Care

At a physiological perspective, regulation of BDNF expression under the hub of a main energy metabolism regulator – peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [18,19] – is thus highly responsive to challenges in aerobic energy demands (see more at De Assis GG, 2018 5). In compliance, studies in healthy populations have revealed that individuals with higher fitness conditions display of higher circulating BDNF levels [20,21]. In the same sense, clinical approaches using the aerobic exercise as intervention show an exercise effect on blood levels of BDNF increase even in aging individuals at the course of cognitive disorders [22]. Likewise, physically active individuals show off better results in executive tasks when compared to sedentary individuals in same social environment [23]. Additionally, the BDNF increases related to exercise have been confirmed to exert a positive impact on the benefits observed in cognition. Furthermore, such BDNF- mediated improvement in cognitive processes can be found in individuals of all ages, when submitted to exercise [24]. Regarding the world's populations are aging as a result of an enhancement in the quality of life which, among other factors, contributes to increases in the prevalence of dementia, the aerobic exercise shall be considered not only as a healthy practice but, and more importantly, as an affordable long-term preventive Health care support, with potential for reducing public expenses.

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Effect of four different forms of high intensity training on BDNF response to Wingate and Graded Exercise Test

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This study examined the effects of a nine-week intervention of four different high-intensity training modalities [high-intensity functional training (HIFT), high-intensity interval training (HIIT), high-intensity power training (HIPT), and high-intensity endurance training (HIET)] on the resting concentration of brain-derived neurotropic factor (BDNF). In addition, we evaluated the BDNF responses to Graded Exercise Test (GXT) and Wingate Anaerobic Test (WAnT) in men. Thirty-five healthy individuals with body mass index 25.55 ± 2.35 kg/m^2 voluntarily participated in this study and were randomly assigned into four training groups. During nine-weeks they completed three exercise sessions per week for one-hour. BDNF was analyzed before and after a GXT and WAnT in two stages: (stage 0—before training and stage 9—after nine weeks of training). At stage 0, an increase in BDNF concentration was observed in HIFT (33%; $p < 0.05$), HIPT (36%; $p < 0.05$) and HIIT (38%; $p < 0.05$) after GXT. Even though HIET showed an increase in BDNF (10%) this was not statistically significant ($p > 0.05$). At stage 9, higher BDNF levels after GXT were seen only for the HIFT (30%; $p < 0.05$) and HIIT (18%; $p < 0.05$) groups. Reduction in BDNF levels were noted after the WAnT in stage 0 for HIFT (– 47%; $p < 0.01$), HIPT (– 49%; $p < 0.001$), HIET (– 18%; $p < 0.05$)], with no changes in the HIIT group (– 2%). At stage 9, BDNF was also reduced after WAnT, although these changes were lower compared to stage 0. The reduced level of BDNF was noted in the HIFT (– 28%; $p < 0.05$), and HIPT (– 19%;$p < 0.05$) groups. Additionally, all groups saw an improvement in VO_{2max} (8%; $p < 0.001$), while BDNF was also correlated with lactate and minute ventilation and selected WAnT parameters. Our research has shown that resting values of BDNF after nine weeks of different forms of high-intensity training (HIT) have not changed or were reduced. Resting BDNF measured at 3th (before GXT at stage 9) and 6th day after long lasting HITs (before WAnT at stage 9) did not differed (before GXT), but in comparison to the resting value before WAnT at the baseline state, was lower in three groups. It appears that BDNF levels after one bout of exercise is depended on duration time, intensity and type of test/exercise.

It is well established that exercise is beneficial to the health and functioning of the body[1]. Moreover, exercise results in beneficial adaptive changes (remodeling) observed at many levels, including molecular systems[2,3]. This 'remodeling' promotes the improvement of general physical fitness as well as the efficiency of energy processes, increases muscle mass, and improves neuromuscular coordination, which has a positive effect on brain function[4]. In addition, cognitive and memory-related functions are improved thanks to physical effort[5].

Substances secreted by various cells are responsible for tissue remodeling. After entering the bloodstream, they participate in tissues/organs crosstalk. These substances are classified as growth factors, with multidirectional activity, affecting many essential biological processes[6]. These auto-, para- and hemocrine compounds are

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secreted by the skeletal muscles (myokines), adipose tissue (adipokines) or nervous system cells (neurokines)[7–10]. An example of such substances, which can communicate through different organs is brain derived neurotrophic factor (BDNF)[8].

BDNF is a member of neurotrophic factors family and acts via the specific tyrosine kinase receptor B (TrkB)[11]. According to Rasmunsen et al.[12] BDNF is widely expressed and produced in the brain. BDNF is also a contraction inducible protein[13,14]. Its expression is also observed in immune cells[15] and endothelium cells[16]. BDNF crosses the blood-brain barrier in both directions and communicates with different brain structures, skeletal muscle, adipose tissue or immune system while taking part in homeostasis maintenance[17–19]. Although BDNF is mainly expressed and secreted in the brain, it circulates in platelets, which serve as the main storage of BDNF in the blood[20]. BDNF plays a significant role in neurogenesis by stimulating neuronal plasticity and facilitating neurons development, differentiation and survival. In addition, it takes part in synaptogenesis and dendritogenesis[20,21]. Moreover, BDNF participates in hippocampus cells differentiation, strengthening signal transmission inducing and maintaining long-term potentiation (LTP) of synaptic enhancement[22,23], which are the molecular bases of cognition, emotional processes, spatial orientation or learning[5,20–27].

Matthews et al.[13] reported that the mRNA of BDNF and the protein expression were increased in skeletal muscles after exercise, yet BDNF was not secreted into the circulation. It is thought that BDNF produced in skeletal muscles is utilized locally during muscle's fibers and nerve regeneration as a supporting factor of motor neurons' survival, and promotor of growth of motor and sensory neurons[27]. It participates also in regulation of satellite cells differentiation and skeletal muscles regeneration[28,29].

According to Pedersen et al.[30] the main source of this myokine in skeletal muscles are probably neurons. BDNF and its receptor have a key role in central regulation of the energy balance and several reports suggest the possibility that the BDNF/TrkB axis in adipose tissue may have a role in the regulation of systemic metabolism[19,31–33]. BDNF via AMP- activated protein kinase (AMPK) is capable to enhance fatty oxidation[13,19,30]. In addition, it is reported that BDNF acts in an autocrine or paracrine fashion with strong influence on energy metabolism and plays important role in fat oxidation[33]. In addition, it is believed to also regulate weight loss, appetite suppression and modifiy the size of adipose tissue through a central mechanism[19,30]. According to Sornelli et al.[31,33] BDNF is a new adipokine that is expressed in both white and brown adipose tissue in mice and rats during experimental stress and in type 1 diabetes.

Physical activity is known to provide many health benefits and is thought to be as a strong stimulus of brain health by increasing blood supply resulting in improvement of brain function, and prevention of nervous system diseases[4,8–10,30,34–37]. Physical exercise is a kind of activity that requires the brain to constantly monitor movement patterns, especially at the stage of learning of new motor activities[9,34,37]. BDNF is secreted, among others under the influence of physical exercise and is implicated in satellite cells stimulation that play an important role in muscle regeneration[28,29]. Different molecular mechanisms have been proposed to explain how aerobic exercise can impact BDNF synthesis in brain and peripheral tissue[34–39].

High intensity functional training (HIFT) has gained a significant following over the last several years[40]. HIFT is multimodal type of training that includes multi-joint movements to create movement patterns that are consistent with everyday movements, and commonly known as "functional movements," which engage the entire kinematic chain. These movements are performed as quickly as possible with the workload adjusted to the individual's abilities, in a limited time period or with a limited number of repetitions, with the main goal to target different fitness domains – cardiorespiratory endurance, muscle strength, speed, coordination or anaerobic power, agility, flexibility[40–43].

High-intensity interval training (HIIT) include short and intense elements of work alternated with low-intensity recovery periods. According to Buchheit and Laursen[44], HIIT can include repeated short (<45 s) to long (2–4 min) bouts of high- but not maximal-intensity exercise, or short (≤10 s) repeated-sprint sequences [RSS] or long (20–30 s, sprint interval session [SIT]) all-out sprints, interspersed with recovery periods. These varying-length efforts combine to create training sessions that last a total of 5–40 min. Time of workouts is depends most of all on a participant's current fitness level as well as of the session intensity. The main goal of HIIT is to improve cardiorespiratory and metabolic function, as well as overall physical performance.

High intensity power training (HIPT) is a variation of the more popular HIIT, and incorporates high intensity resistance training by using varied multiple – joint movements and focused on high power output. In this type of training there is no defined recovery period and incorporates functional lifts such as the squat, deadlift, clean, snatch, and overhead press. The main goal of HIPT is to shape skeletal muscle mass, yet it can also be used as a stimulus for cardiorespiratory fitness and body composition[45,46].

High intensity endurance training (HIET) is an endurance type of training with the intensity of 85–100% HR_{max}. The time of this type of training is proportional for its intensity. Taking into account the age of athletes and physical fitness, HIET lasts from several minutes up to several hours[47–49] and it is considered a continuous form of exercise. The best cardiorespiratory effects are observed when the intensity is about 85% VO_{2max} (~ 90% HR_{max}). Continuous endurance training may be realized with long, slow distance (LSD) and long, high distance (LHD) or variable intensity. LSD training is characterized by constant pace of low to moderate intensity over an extended distance or duration. Heart rate varies in range 60 – 80% HR_{max} (~140 – 160 bpm). The goal of LSD method is energy expenditure maximizing and reduction of body mass by fat mass utilization. The intensity of LHD method is high and varies between 85 and 95% HR_{max}. The volume of this type of training is low. The main goal of endurance training with high intensity is to shape the cardiorespiratory fitness by effective improving maximal oxygen uptake in individuals[49].

Several studies have reported BDNF levels in blood increase after one bout of exercise[12,34–37,50]. Both acute and chronic exercise stimulate BDNF production and improve memory and mood[3,26,34,36,37,50–52]. Huang, et al.[53] showed a correlation between VO_{2max} and the magnitude of BDNF changes. According to Schmidt-Kassow et al.[54] and Rojas Vega et al.[50], BDNF concentrations increases with the duration of exercise and return to baseline after

Groups	Age (years)	Weight (kg)	Height (cm)	BMI (kg/m^2)	FMI (kg/m^2)	WHR
HIFT	26.9 ± 4.20	83.12 ± 7.30	1.78 ± 0.04	26.19 ± 2.48	4.58 ± 0.93	0.87 ± 0.04
HIPT	28.9 ± 3.70	79.93 ± 6.78	1.81 ± 0.07	24.48 ± 2.03	4.96 ± 1.46	0.88 ± 0.03
HIIT	26.5 ± 3.30	83.99 ± 6.50	1.81 ± 0.06	25.59 ± 4.38	3.81 ± 0.65	0.85 ± 0.06
HIET	28.9 ± 3.10	81.42 ± 6.50	1.77 ± 0.04	26.07 ± 2.04	4.31 ± 1.03	0.91 ± 0.02

Table 1. Anthropometric characteristics of volunteers before the experiment. Data are presented as mean ± SD. *BMI* body mass index; *FMI* fat mass index; *WHR* waist to hip ratio.

Groups	FAT (kg) Stage 0	FAT (kg) Stage 9	*p*	FAT (%) Stage 0	FAT (%) Stage 9	*p*	LBM (kg) Stage 0	LBM (kg) Stage 9	*p*	LBM (%) Stage 0	LBM (%) Stage 9	*p*
HIFT	14.48 ± 3.47	12.14 ± 3.56	0.02	17.27 ± 3.00	14.67 ± 3.09	0.001	68.65 ± 4.85	69.63 ± 5.03	0.102	82.73 ± 3.00	85.33 ± 3.09	0.001
HIPT	12.36 ± 2.25	11.12 ± 1.39	0.02	15.47 ± 2.55	14.02 ± 1.78	0.091	67.58 ± 6.2	68.44 ± 5.71	0.219	84.53 ± 2.55	85.98 ± 1.70	0.086
HIIT	16.39 ± 6.88	13.36 ± 5.31	0.02	18.64 ± 5.38	15.60 ± 4.13	0.001	67.60 ± 9.77	68.97 ± 9.51	0.176	81.36 ± 5.38	84.40 ± 4.13	0.001
HIET	13.48 ± 3.62	12.03 ± 2.34	0.04	16.45 ± 3.68	14.85 ± 2.47	0.087	67.89 ± 5.19	68.83 ± 5.24	0.198	83.47 ± 3.74	85.15 ± 2.47	0.088

Table 2. Changes of FAT (kg and %) and LBM (kg and %) mass at stage 0 and 9 in all groups. Data are presented as mean ± SD.

few minutes of recovery. Saucedo Marquez et al.[55] and Renteria et al.[56] observed much higher levels of BDNF after a single session of HIT than in continuous exercise. Additionally, Yarrow et al.[57] and Rojas Vega et al.[58] reported an increase in BDNF after resistance training, while Goekint et al.[59] did not observe BDNF changes either after an acute bout or after a 10-week strength training intervention. In another study, Correira et al.[60] reported that acute strength exercise did not induce alterations in the BDNF level among healthy individuals.

Despite the number of studies demonstrating increases in BDNF secretion as a result of an exercise sessions and/or exercise training program, there are several studies opposing these reports. Rojas Vega et al.[50] reported that BDNF during recovery was reduced in comparison to resting values. Figueiredo et al.[61] also reported lower BDNF levels after training compared to pre-training values. As well as Nafuji et al.[62,63] who reported lower levels of BDNF among trained athletes. Hebisz, et al.[64] showed that BDNF did not change after six-months of SIT and decreased after intensive sprint interval exercise test (SIET). Murawska–Ciałowicz et al.[65] did not report changes in resting BDNF after three-months of CrossFit training among men. As a result of these findings scientists believed that the type of exercise program may be a decisive factor in altering BDNF levels, as well as intensity of the exercise or the individual level of physical performance and physiological adaptation[62,63].

Considering the contradictory accounts in the literature and taking into account the insufficient reports about the influence of different types of HIT on BDNF secretion, and the limited reports describing the BDNF response to standard aerobic and anaerobic performance tests, it would be of significant interest for practitioners to explain the dynamics of BDNF changes after several weeks of high-intensity training. To the best of our knowledge, no other study has compared changes of BDNF level after different types of HIT over nine-weeks among men of similar fitness levels.

Therefore, the aim of the study was to determine (1) if resting BDNF concentration change after nine-weeks of high intensity training of various types –HIFT, HIPT, HIIT and HIET; (2) examine BDNF changes after graded exercise test (GXT) and Wingate anaerobic test (WAnT); and (3) changes in performance and anthropometric parameters after nine-weeks of different types of HIT.

Results

Prior to the experiment there were no differences in anthropometrical parameters noted among all measured groups (Table 1).

After nine-weeks of training a significant reduction of fat mass (kg) and percentage of body mass were observed in all groups (Table 2). The greatest changes were noted in the HIIT group (18.5%) with the other groups changing as follows: 16.2% in HIFT group, 10% in HIPT and in endurance group in 10.8%. Changes in absolute LBM (kg) were not observed (Table 2), however, when expressed as a percentage of body mass the differences between the two stages were noted.

After the nine-week intervention VO_{2max} improved in all groups except in the HIET group (Table 3). Additionally, the nine-week intervention had no impact on resting BDNF levels before the GXT. The levels of BDNF did not change after this time or was lower whencompared to the rest value before WAnT in the stage 0 (Table 3). The results of BDNF before and after the GXT and WAnT at baseline and after nine-weeks of training are presented in Figs. 1, 2, 3 and 4.

Figure 1 shows the BDNF concentrations at baseline, before and after GXT. No statistically significant differences were observed between the groups for resting BDNF concentration ($F = 2.267$; $p = 0.101$; $\eta^2 = 0.185$) while after the GXT the differences in BDNF concentration between the groups were statistically significant ($F = 3.389$; $p < 0.05$; $\eta^2 = 0.253$). In addition, statistically significant differences were observed within the groups before and

	Resting BDNF					
	VO_{2max} (ml/kg/min)		BDNF (pg/ml) GXT		BDNF (pg/ml) WAnT	
	Baseline (Stage 0)	9 weeks (Stage 9)	Baseline (Stage 0)	9 weeks (Stage 9)	Baseline (Stage 0)	9 weeks (Stage 9)
HIFT	46.89 ± 5.79	50.97 ± 5.43**	278.99 ± 142.7	178.30 ± 121.7	349.96 ± 186.2	196.83 ± 89.38*#
HIPT	47.85 ± 3.73	50.77 ± 3.60*	369.26 ± 241.2	272.03 ± 148.9#	441.65 ± 285.4†	241.02 ± 104.6*#†
HIIT	44.81 ± 3.81	49.89 ± 5.62**	170.50 ± 97.46	162.23 ± 54.76	178.55 ± 60.41	134.19 ± 44.32
HIET	48.15 ± 5.99	50.81 ± 6.48	118.46 ± 33.92	83.80 ± 15.79	175.43 ± 76.18	55.74 ± 17.84*

Table 3. VO_2max and resting value of BDNF before GXT and WAnT at the baseline and after the 9 weeks. Data are presented as mean ± SD. *p < 0.05; **p < 0.01 in comparison to the baseline values. #p < 0.05 in comparison to HIET. †p < 0.05 in comparison to HIIT and HIET.

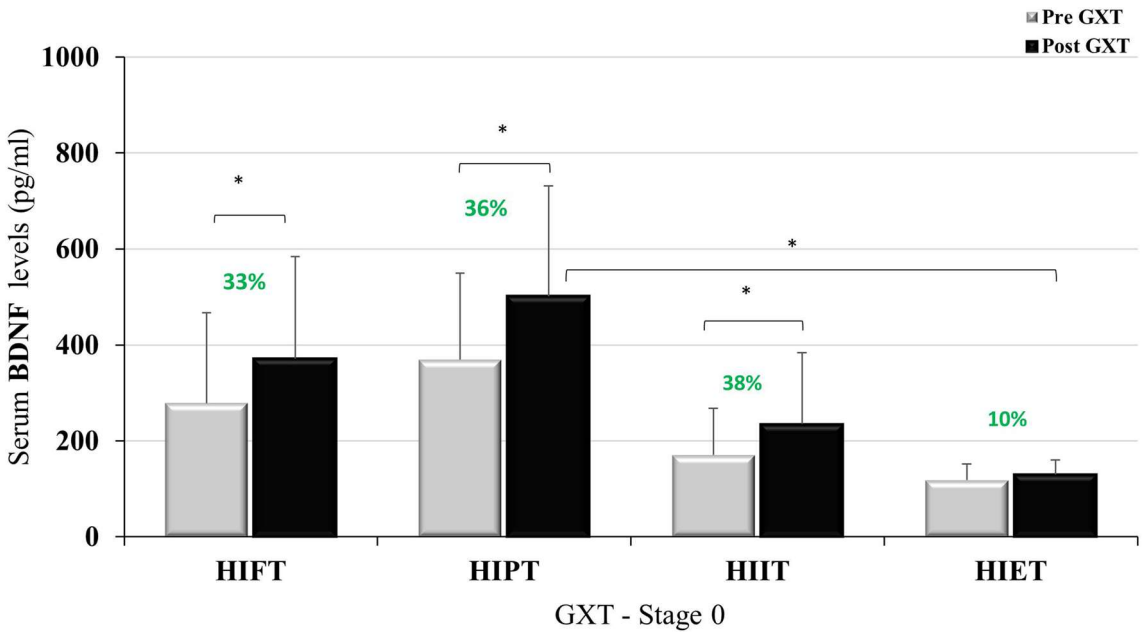

Figure 1. BDNF level at baseline, pre- and post GXT; *p < 0.05.

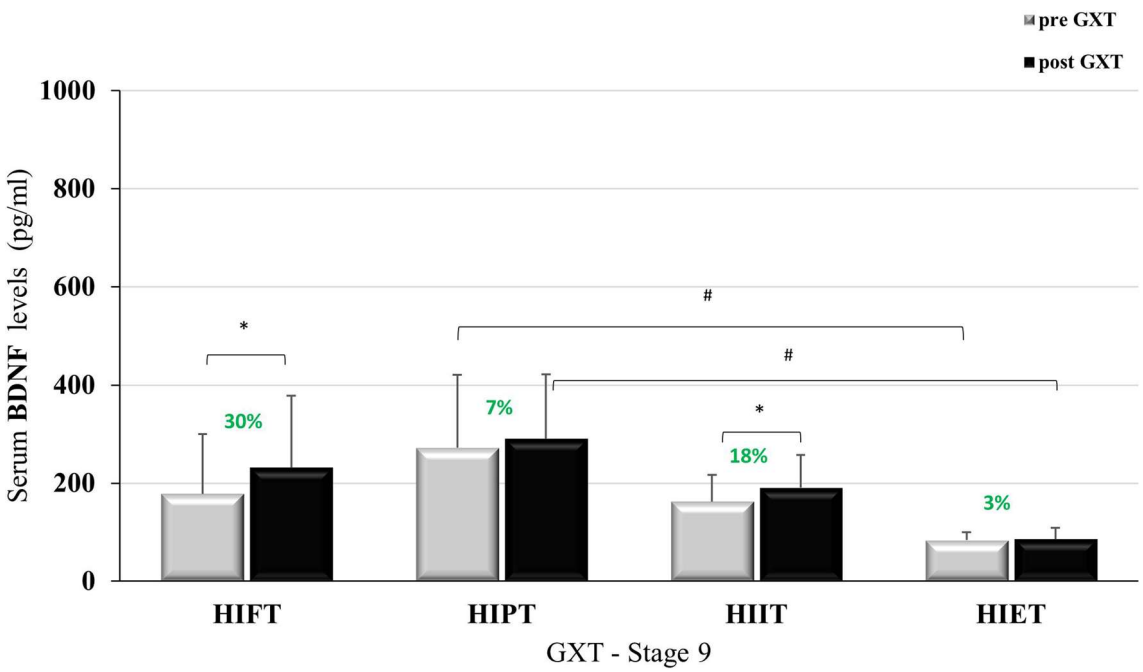

Figure 2. BDNF level after nine weeks training, pre- and post GXT; #p < 0.01; *p < 0.05.

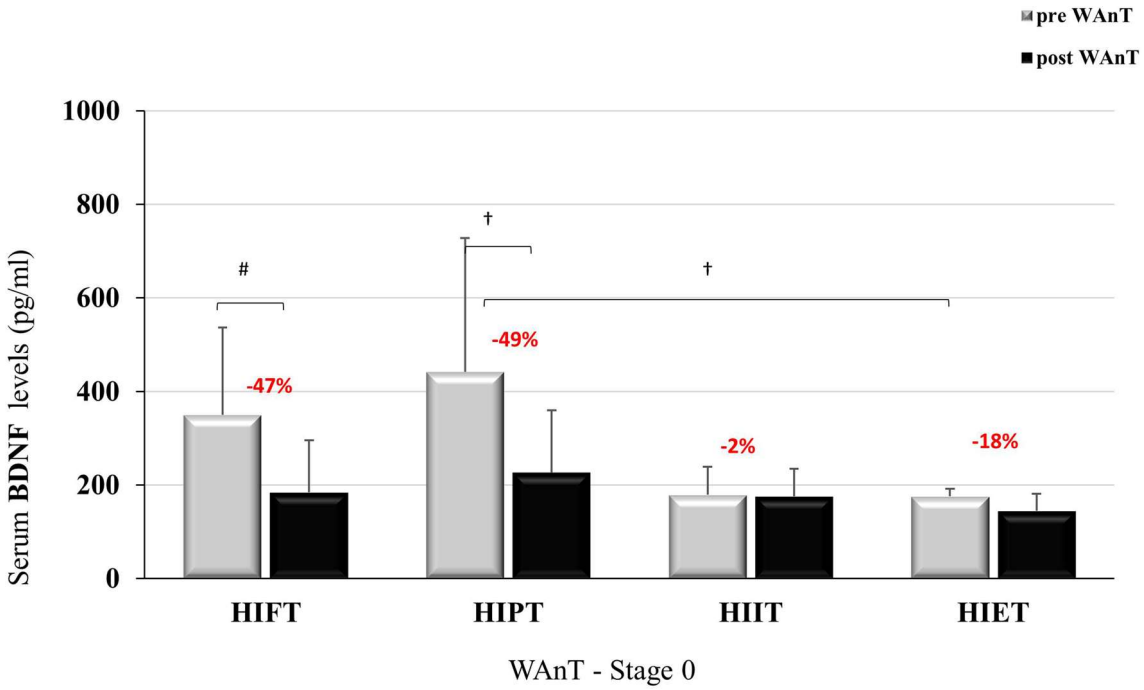

Figure 3. BDNF level at stage 0, -pre and post-WAnT; *p < 0.05; #p < 0.01; †p < 0.001.

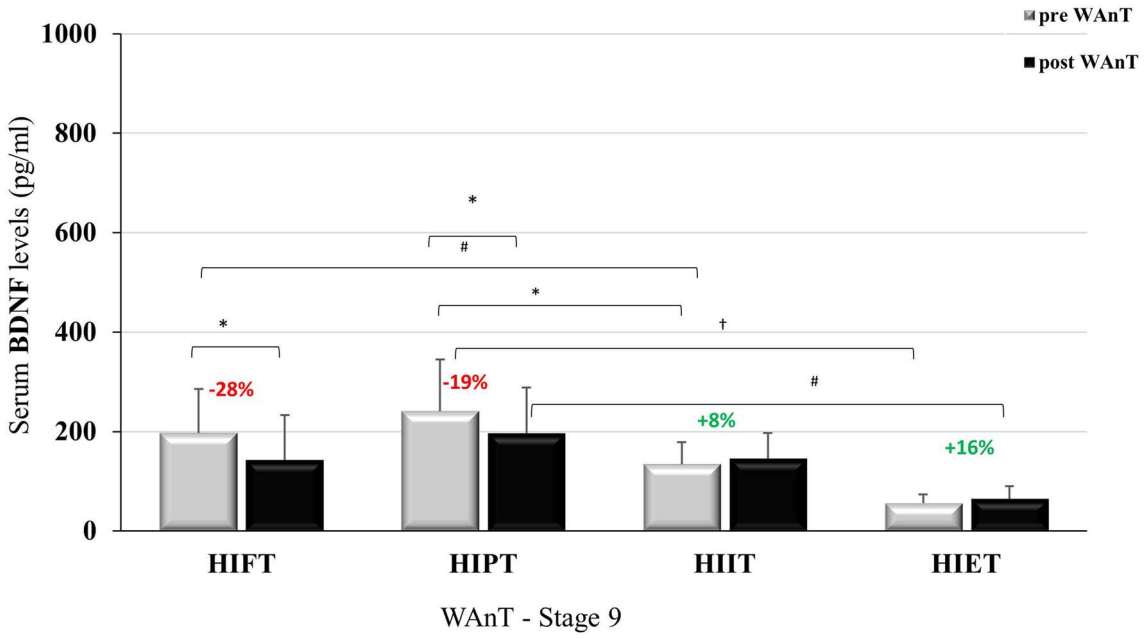

Figure 4. BDNF level in stage 9, -pre and post-WAnT; *p < 0,05; #p < 0,01; †p < 0,001.

after the GXT ($F = 17.39$; $p < 0.001$; $\eta^2 = 0.367$), without noting significant differences between the groups when analyzing the effect size of the BDNF changes before and after the GXT ($F = 1.964$; $p = 0.141$; $\eta^2 = 0.164$).

Changes of BDNF concentration before and after GXT at stage 9 are shown in Fig. 2. The resting values were statistically significantly different between the groups ($F=4.978$; $p<0.01$; $\eta^2=0.332$), as were the BDNF values after the GXT ($F=5.672$; $p<0.01$; $\eta^2=0.362$). Statistical analysis also showed a difference in the results before and after the GXT ($F=4.370$; $p<0.05$; $\eta^2=0.127$). BDNF in the HIPT group were higher than in the HIET group ($p<0.01$).

Figure 3 shows BDNF concentrations at stage 0, before and after the WAnT. Statistically significant differences in BDNF concentration were observed between the groups at rest ($F = 4.888$; $p < 0.01$; $\eta^2 = 0.328$). After the WAnT, differences between the groups disappeared ($F = 1.102$; $p = 0.364$; $\eta^2 = 0.099$). Comparing differences within groups showed differences in BDNF concentration before and after the WAnT ($F = 28.686$; $p < 0.001$; $\eta^2 = 0.489$). The magnitude of these changes was statistically significant ($F = 7.270$; $p < 0.001$; $\eta^2 = 0.421$). The

Groups	LA After GXT	LA After WAnT	FAT (%)	LBM (%)	FAT (kg)	LBM (kg)
BDNF before GXT	0.1411	–	− 0.3035	0.3050	− 0.2353	0.0656
	p = 0.426	–	p = 0.081	p = 0.079	p = 0.180	p = 0.712
BDNF after GXT	0.2019	–	− 0.3876	0.3896	− 0.3105	0.0571
	p = 0.252	–	p = 0.024	p = 0.023	p = 0.074	p = 0.749
BDNF before WAnT	–	0.1321	− 0.3689	0.3709	− 0.2744	0.1906
	–	p = 0.457	p = 0.032	p = 0.031	p = 0.116	p = 0.280
BDNF after WAnT	–	0.1908	− 0.4146	0.4172	− 0.3145	0.0820
	–	p = 0.280	p = 0.015	p = 0.014	p = 0.070	p = 0.645

Table 4. BDNF correlation with LA and anthropological parameters at stage 0 (Person's coefficient).

Groups	LA After GXT	LA After WAnT	FAT(%)	LBM (%)	FAT (kg)	LBM (kg)
BDNF before GXT	0.5398	–	− 0.3060	0.3060	− 0.2287	0.0333
	p = 0.001	–	p = 0.078	p = 0.078	p = 0.193	p = 0.852
BDNF after GXT	0.3618	–	− 0.1251	0.1251	− 0.0921	− 0.0325
	p = 0.035	–	p = 0.481	p = 0.481	p = 0.605	p = 0.855
BDNF before WAnT	–	0.3821	− 0.1473	0.1473	− 0.1001	0.0439
	–	p = 0.026	p = 0.406	p = 0.406	p = 0.573	p = 0.805
BDNF after WAnT	–	0.4438	− 0.2259	0.2259	− 0.1751	− 0.0232
	–	p = 0.009	p = 0.199	p = 0.199	p = 0.322	p = 0.896

Table 5. BDNF correlation with LA and anthropological parameters at stage 9 (Person's coefficient).

Groups	VO_{2max} (ml/kg/min) Stage 0	VO_{2max} (ml/kg/min) Stage 9	VO_{2max} (ml/min) Stage 0	VO_{2max} (ml/min) Stage 9	VE_{max} (L/min) Stage 0	VE_{max} (L/min) Stage 9
BDNF before GXT	0.1859	0.2268	0.1037	0.1376	0.2021	0.4739
	p = 0.293	p = 0.197	p = 0.560	p = 0.438	p = 0.252	p = 0.005
BDNF after GXT	0.2333	0.1334	0.1027	0.0532	0.1515	0.3077
	p = 0.180	p = 0.452	p = 0.563	p = 0.765	p = 0.392	p = 0.077

Table 6. Correlation BDNF with VO_{2max} and VE_{max} measured in GXT parameters at stage 0 and stage 9.

reduction of BDNF concentration after the WAnT in the HIFT group (↓ 47%) and in the HIPT group (↓ 49%) were significantly greater than in the HIIT group (↓ 2%) and HIET (↓ 18%).

Figure 4 presents BDNF concentration in stage 9 before and after WAnT. Statistically significant BDNF concentration differences were observed between the groups at rest ($F = 10.087$; $p < 0.001$; $\eta^2 = 0.50$), as well as after WAnT ($F = 4.849$; $p < 0.01$; $\eta^2 = 0.327$). Significant BDNF concentration differences were observed within the groups before and after WAnT, ($F = 7{,}796$; $p < 0.01$; $\eta^2 = 0.206$). BDNF concentrations after WAnT in the HIFT group decreased (↓28%) and were statistically significantly higher than in the HIET group.

When analyzing the entire sample at the stage 0, a noted negative correlation between BDNF and FAT (%) was observed. A positive correlation was seen between BDNF with LBM (%) before and after the GXT and WAnT (Table 4). In addition, at stage 9 a positive correlations between BDNF level before and after GXT was observed, as well as with lactate before and after WAnT (Table 5).

We also noted a correlation of BDNF before GXT with VE_{max} after 9 weeks of training (Table 6). After the training it was noted that BDNF correlated with WAnT parameters. The positive correlation of BDNF with P_{max} and negative correlation with T2 time of P_{max} maintenance (Table 7) was noted.

The intensity of each training session was monitored by HR measurement, energy expenditure (EE) of the work during the main part and lactate level (LA) measured 10 min after the session completion. The average values of these parameters for all sessions are presented in Table 8. Moreover, in this table the average internal load for all sessions evaluated based on the volunteers RPE is presented.

Discussion

In this study we investigated the effectiveness of nine-weeks of several high-intensity training protocols on resting BDNF level. Moreover, we wanted to evaluate BDNF response to GXT and WAnT pre and post training intervention. Three variant directions of changes in BDNF serum concentration were observed.

Groups	P_{max} (W) Stage 0	P_{max} (W) Stage 9	P_{max} (W/kg)Stage 0	P_{max} (W/kg) Stage 9	T1 (s) Sage 0	T2 (s) Stage 9
BDNF before WAnT	0.3119	0.3135	0.2887	0.4100	0.2926	– 0.3623
	p = 0.073	p = 0.071	p = 0.098	p = 0.016	p = 0.093	p = 0.035
BDNF after WAnT	0.1099	0.2134	0.2255	0.3769	0.2814	-0.3981
	p = 0.536	p = 0.226	p = 0.200	p = 0.028	p = 0.107	p = 0.020

Table 7. Correlation BDNF with WAnT parameters before and after 9 week training.

	Lactate (mmol/L)	EE (kcal/min)	HRmax (b/min)	%HRmax (%)	RPE (AU)
HIFT	9.94 ± 2.42†	12.52 + 1.82†	179.67 ± 11.75	92.76 ± 5.46	958.00 ± 87.53$^{\#}$
HIPT	4.07 ± 2.02*	9.57 ± 1.97*	166.06 ± 13.54*	88.31 ± 5.55*	1013.20 ± 66.53
HIIT	12.79 ± 3.42	10.27 ± 1.60	184.55 ± 8.12	95.30 ± 52.64	1001.85 ± 51.69
HIET	11.21 ± 2.63	11.35 ± 1.09	180.19 ± 7.59	94.81 ± 2.43	1126.69 ± 58.31

Table 8. Markers of training intensity and internal load. Data are presented as mean ± SD of all sixteen sessions. *$p \leq 0.01$ in comparison to all groups. $^{\dagger}p \leq 0.05$ in comparison to all groups. $^{\#}$ $p \leq 0.05$ in comparison to HIET.

Resting BDNF post training before GXT did not change in any of the groups. Before WAnT resting values were significantly lower in HIFT, HIPT and HIET in comparison to pre training values. Between GXT and WAnT two days of recovery were applied in both stages. The high fluctuation of BDNF levels has been observed in BDNF resting values before GXT and WAnT in stage 0; however, no significant differences were noted between both tests-days. In stage 9, differences in BDNF were statistically significant between groups before GXT and WAnT but without changes between both tests.

We speculated that it is an effect of individual variability[66], or daily variation in BDNF secretion[67,68]. Several studies have already demonstrated that BDNF is implicated in the regulation of circadian pacemaker function in the central nervous system. The highest level of BDNF is noted in the morning (at 08:00 h) and decreasing throughout the day. Plasma BDNF levels is significantly lower at 12:00 h in comparison to BDNF levels in blood at 08:00 h[68]. perhaps the fluctuations in our BDNF values is due to diurnal variations as blood sample were collected between 08:00 h and 13:00 h.

In our study the BDNF response to GXT was not the same as it was noted for BDNF response to WAnT. The BDNF was increased after the GXT pre and post training intervention, although the magnitude of the change was lower post training in comparison to pre values. Before training, an increase of over 30% was recorded. At stage 0 the greater variation of BDNF between groups was also observed after GXT than in post training. In contrast to the result observed after GXT were results of BDNF after WAnT pre and post training intervention. In both stages BDNF decreased or did not change in comparison to the resting values. With this test, more intense changes were also found at stage 0 than in stage 9.

The improvement of VO_{2max} in all groups except the HIET we also observed. According to many studies very high-intensity workouts, as HIFT, HIIT[40,44,69–72], provoke anaerobic metabolism, high lactate concentration and oxygen deficit. Such conditions can stimulate BDNF secretion which can be mediated by PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator-1α) as well as by lactate. PGC-1α is a key regulator of BDNF secretion and lactate metabolism. Between PGC-1α and BDNF the positive loop exists[35,38,73]. Moreover lactate, is an energy fuel for brain[74] as well as a key mediator of neuroplasticity and BDNF regulation[38,39,73]. It can induce PGC1α/FNDC5/BDNF pathway through the silent information regulator 1 (SIRT1) activation[38,75–77]. It is postulated that PGC-1α is also the main factor influencing the biogenesis of mitochondria and is highly expressed in tissues rich in mitochondria and active oxygen metabolism such as brain, brown adipose tissue or skeletal muscles[69,70]. Oxygen deficiency during exercise and increases in LA concentration promote mitochondrial biogenesis[70–73]. Unfortunately, despite maximal oxygen uptake improvement reported in our study we have not observed correlation between BDNF and $VO_{2max.}$ It is also interesting that in the HIET group the average of LA level after all sessions was the highest among training groups; however, no improvement in VO_{2max} was noted after 9 weeks. This evidence suggests that not only training length, intensity, but also interrupted type of modalities is important in maximal oxygen uptake modification.

Reduction or no BDNF level changes observed after WAnT or high intensity training were noted by several authors[62–64,78–80]. Hebisz et al.[64] found no changes in BDNF at baseline, as well as after two and six months' of SIT training. Figueiredo et al.[61] also reported lower BDNF values after eight-weeks of HIET training combined with strength training. In our study, the HIET group had reduced resting BDNF levels after nine-weeks of training in comparison to baseline values. In another study it was observed that BDNF returned to baseline during recovery in a sedentary group while in the trained group were reduced below baseline[62]. Moreover, after a maximal test BDNF increase much more in sedentary group (30%) than in trained athletes (only 11%). This observation suggests that greater dynamics of changes of BDNF levels can be observed in sedentary participants in comparison to the physically active individuals[63].

We can speculate several possible mechanisms in this BDNF reduction. One of them could be a body mass and fat mass. Glud et al.[81] measured BDNF in obese or overweight men and women after physical training and recorded a reduction of BDNF in men and women. The significant reduction of body mass with subsequent decrease of BDNF level was also reported by Lee et al.[82] after a 12-weeks program for weight reduction in overweight men. Taking into account our study included normal weight men, and we have observed a negative correlation between BDNF level and percentage of body fat, as well as the significant reduction in the body mass, it is quite possible that body fat mass may explain the lack of BDNF changes or reduction observed in our study. Lommatzsch et al.[83] also reported that body weigh negatively during recovery BDNF levels. It could be explained by the physiological/metabolic stress during long lasting high intensity trainings. According to Sornelli et al[31,33] BDNF is present in adipose tissue and has potential anorexigenic effects. Moreover, BDNF can act via PGC1α/FNDC5/BDNF pathway and stimulates irisin secretion during skeletal muscle contraction. Irisin is produced by fibronectin type III domain containing 5 (FNDC5) cleavage[75] and is an exercise hormone capable of increasing energy expenditure by fat oxidation, browning of white fat tissue and promoting weight loss[35,38,84,85].

Another possible explanation could be seasonal variations observed in BDNF secretion. Bus et al.[86] and Molendijk[87] observed a high correlation of BDNF with amount of sunlight. The nadir values of BDNF were noted in the early spring and the peak serum levels were observed in the early autumn. In our study, the first round of measurements (stage 0) were performed in the autumn (October/November) and the second round of measurement in early spring (February/March). In line with this seasonal variation in BDNF we can suppose that the weaker respond of BDNF after both tests post-training may be explained by this mechanism.

It is also postulated that cAMP-response element binding protein (CREB) is an important regulator of BDNF secretion[88,89]. However, CREB activity is under influence of serotonin, which is dependent on amount of light[90], and is higher during long days. Thus, serotonin is the most essential players involved in BDNF signaling. According to Martinovich[91] and Jin[92] a specific synergy between BDNF and serotonin signaling systems exists where a feedback loop between the two molecules exist. As it was previously mentioned BDNF can cross blood-brain-barrier[12,19,21,91]. In line with this it could be supposed that the peripheral level of BDNF can reflect the BDNF level in the brain and any seasonal and circadian BDNF changes in brain can reflect the BDNF in periphery.

The other possible biological mechanisms related to the reduction of BDNF could be explained by usage and consumption of BDNF in the regeneration of nerve fibers and miofibers and the inflammatory process that could occur in damaged tissues. Skeletal muscle damage is often observed during strength training. Resistance exercises performed with high intensity as a part of HIFT, HIIT or HIPT training could provoke mechanical muscle damage especially in the eccentric phase of movement during weightlifting[93]. The training programs included in our study are considered very intense. The average intensity exceeded 90% HR_{max}. It may be hypothesized that BDNF circulating in the blood was consumed by muscles in the process of repairing damaged structures and more intense neuroprotection.

According to several studies[94,95] in several conditions associated with adrenergic stimulation, an increase in peripheral count of larger platelets has been observed. Such conditions are always present during exercise[95,96]. Exercise stimulates thrombocytosis and megakaryocytes release from the liver and spleen[95–99]. Platelets' α-granules are the main BDNF storage site in the blood (~99%). Only a small amount of free BDNF circulates in the plasma[99,100]. Platelet's α-granules contain many different growth factors. When an injury occurs, growth factors secretion by platelets and macrophages is induced and the inflammation-healing process is initiated[99,100]. According to Nofuji, et al.[63] regular exercise facilitates the utilization of circulating BDNF after acute exercise with maximal intensity.

Metabolic/physiological stress can also decrease BDNF secretion. It has been reported that cytokines and chemokines are secreted in both central and peripheral nervous systems during psychological stress and that BDNF is thought to be involved in the neuroimmune axis regulation[101,102]. In addition, Jin et al.[90] report that expression of BDNF is strongly affected by immune cells and the immune factors they secrete. It is well known that very intensive physical effort is a great stress for the body[103,104]. Increased stress levels affect BDNF mRNA and significantly reduce BDNF expression[69]. According to Rasmussen et al.[12], 70–80% of BDNF circulating in the blood is produced in the brain both in restitution and after exercise. According to de Assis and Gasanov[105] BDNF negatively correlates with the level of cortisol a catabolic hormone. Garcia- Suarez et al.[106] also observed no changes of BDNF which was accompanied by higher level of cortisol and cortisol/BDNF ratio. Intensive training and the accompanying strong physiological stress reduce the concentration of testosterone with the associated increase in cortisol. It was also shown that testosterone administration increases BDNF protein levels in castrated male rats[107]. Unfortunately, we did not determine the concentration of hormones and cytokines, however, the level of lactate, energy expenditure, RPE and HRmax noted after each sessions always were high and may be evidence of high metabolic load/stress.

The genetic bases of our finding should also be considered. Human BDNF genes has been identified as a single nucleotide polymorphism (SNP). It results in valine (Val) for methionine (Met) substitution in position 66 in pre-pro-BDNF which is a precursor protein of BDNF[108]. It is quite possible that this mutation has important influence on BDNF probably by impairment of the secretion and function of this protein. According to Leraci et al.[109] the BDNF Val66Met polymorphism impairs the beneficial behavioral and neuroplasticity effects induced by physical exercise and moderate the exercise response.

Even though several studies exist evaluating the influence of exercise on BDNF, to the best of our knowledge, this study is the first to compare BDNF changes as a result of four different high-intensity exercise programs. Even though no study is without limitations, we have attempted to reduce these by enlisting a homogenous participant pool, who exercised at the same facility, using the same equipment during testing and training sessions.

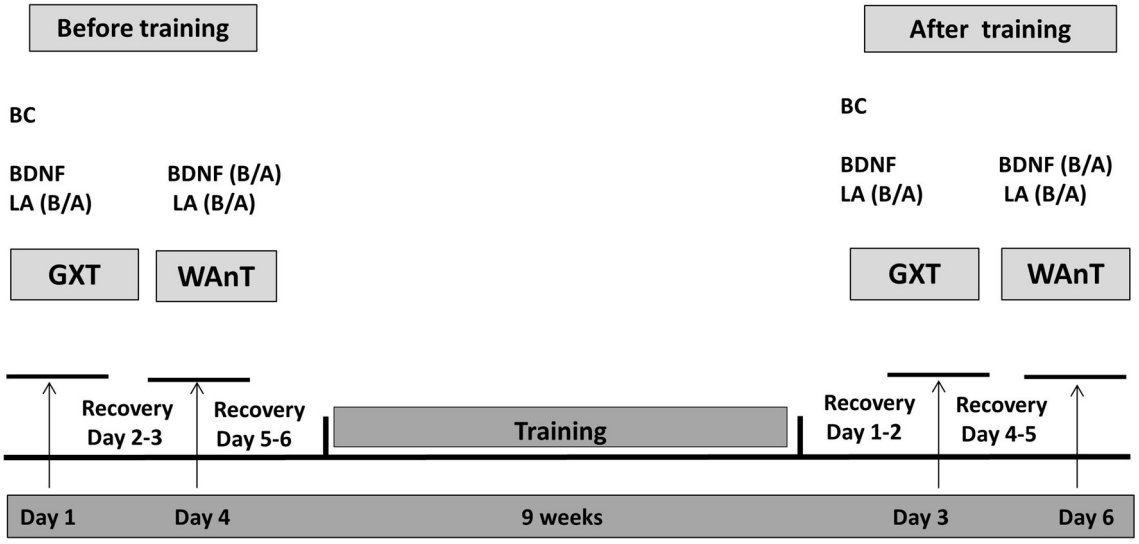

Figure 5. Scheme of GXT and WAnT tests before and after 9-week training.

Conclusions

Our research shows that very intensive forms of training with anaerobic metabolism lasting nine-weeks does not affect BDNF levels at rest and 10 min after WAnT. Exercise performed in anaerobic conditions reduces BDNF levels. Even though the mechanisms that explain these changes are not easily understood, we speculate that it can be connected with diurnal variation or seasonal changes negatively affected by secretion of cortisol and by anabolic/catabolic hormones disruption, which in turn leads to a significant decrease in BDNF concentration, which were not measured in this study. Therefore, future research should expand our findings to include hormonal and immunological aspects. As a results, our findings and their implications should be discussed in the broadest context possible.

Materials and methods

Study design. A pre-post study design comparing the effects of four high-intensive interventions on the BDNF concentration, maximal oxygen uptake (VO_{2max}) and anaerobic power was conducted. The interventions lasted nine-weeks with three sessions per week. Participants were randomly assigned to four groups. Before and after the intervention, participants were tested using a graded exercise test (GXT) with progressive intensity performed on a treadmill. The Wingate anaerobic test (WAnT) was also completed and performed on a cycloergometer. BDNF concentration was assessed before and 10 min after the GXT and WAnT. Both measures were completed at baseline and after nine-weeks of trainings. The study design is presented in Fig. 5.

Participants characteristic. The study was initiated with sixty men randomly classified into four groups (15 participants per group). However, during the course of the nine-weeks several participants withdrew as a result of injury (n = 8), and others as a result of the intensity of the program (n = 5). The study was designed to maintain the highest level of rigor and required participants to participate in the greatest number of sessions (no more than 10% absence was accepted). Seven individuals were excluded as a result of missing the baseline testing session. The final sample consisted of thirty-five men (27.80 ± 3.59 years; 25.55 ± 2.88 kg/m^2 body mass index (BMI); 4.41 ± 1.14 kg/m^2 fat mass index (FMI)], with participants divided into four training groups: HIFT (n = 8), HIPT (n = 9), HIIT (n = 9), HIET (n = 8). The inclusion criteria for the study included healthy man, between 20–35 years, and recreationally active.

Physical activity level was established based on the interview with the research team and on frequency of declared participation in the voluntary exercise per week (1–3/week about 1 h with low to moderate intensity (e.g., running, cycling, fitness gym, swimming, climbing). All participants engaged in physical only for fun and their own satisfaction, and were not considered athletes. All participants were in good health, and approved by the research team based on medical examinations. Study participants were approved to engage in HIT exercise after written agreement with a physician. Conversely, the exclusion criteria included: circulatory and respiratory system disorders, cigarette smoking, diabetes mellitus, thyroid diseases, hypertension, joint pain, and musculoskeletal injuries. Individuals who completed the study declared they only participated in the training classes provided by the research team and adhered to the training cycles. All participants were asked, and declared not

to use the supplements, ergogenic aids and specific diets. They individually (for the personal use only) controlled their diet for energy and basic energetic elements.

Before testing and training, all participants were informed about the purpose of the tests, the procedures for performing biochemical and performance tests, and the possible effects during post—performance discomfort. A research protocol was presented to them and safety principles were discussed. Each participant provided informed written consent to participate in the study. Moreover, they also were informed that they could resign from the study at any stage without reason given. The research was conducted in accordance with the Declaration of Helsinki. The study was approved by and performed in accordance with the recommendation of the Bioethics Committee of Scientific Research at University School of Physical Education in Wrocław, Poland (resolution of 13/03/2017, No. 4/2017).

Anthropometrics and body composition. Body composition and body mass were measured at baseline (stage 0) and after 9-weeks of training (stage 9) just before the GXT test. Body composition analysis was performed by use of a BodyMetrix BX 2000 device (*Intel Matrix, USA*). All procedures of body composition analysis were made in accordance with previously stablished protocols[110]. We measured fat mass (FAT) and percentage of body fat (%FAT) as well as lean body mass (LBM). Moreover, the BMI was calculated based on body mass (kg) and height (m) of volunteers. FMI was calculated based on the body fat mass (kg) and body height, and the waist-to-hip ratio (WHR) on the basis of circumferences of waist and hip (cm).

Biochemical analysis. Participants were given a meal 2 h before GXT and WAnT. The blood samples for biochemical measurements were collected between 08:00 h and 13:00 h. Before each test and 10 min after the blood was collected from the basilic vein to determine BDNF concentration. The blood was than centrifuged and the serum was frozen at – 85 °C. When the serum from all volunteers had been collected all samples were thawed and BDNF was measured. Lactate level (LA) was collected from the fingertip and was also measured before and 10 min after both tests completion.

The Nori Human BDNF ELISA Kit (*Genorise, USA*) was used for the determination of BDNF concentration in the serum. Detection range of this method was 15–1000 pg/ml, sensitivity of this methods was 3 pg/ml, intra-assay coefficient was 5% and inter-assay coefficient was 9%.

The colorimetrical method was used for lactate concentration in the capillary blood. The Lactate Cuvette Test kit (*Dr Lange, Germany*) was used for this purpose and the Mini Photometer Plus LP20 (*Dr Lange, Germany*). The normal range of this parameter was established on 0.6–0.9 mmol/l.

The enzymatic amperometric methods with chip-sensor technology was used to evaluate the lactate level after each session completed in the fitness gym. The Lactate Scout 4 (*EFK Diagnostics for Life, UK)* was used for this purpose. Measuring range of this method is 0.5–40 mmol/L with inter assay coefficient ≤ 1.5%.

Tests protocols. The Graded Exercise Test (GXT) and Wingate Anaerobic Test (WAnT) are very popular tests and very often used to physical performance evaluation. The GXT is used for maximal oxygen uptake (VO_{2max}) measurement and the WAnT for maximal anaerobic power and capacity. The detailed procedures of GXT and WAnT tests were described previously[108].

Briefly, the GXT was performed on a treadmill (*SEG-TA7720 treadmill InSportLine, Czech Republic*), with the initial belt speed set at 6 km/h, with 2 km/h increments every 3 min until exhaustion. All participants used a face mask using a one-way mask where the exhalated air was directed to a spirometer (Quark b^2; *Cosmed, Italy*). Ventilatory parameters were recorded 'breath by breath' and then averaged every 30 s. VO_{2max} (ml/min/kg), respiratory exchange ratio (RER), maximal pulmonary ventilation (VE_{max}) and lactate were also analyzed. The maximal oxygen uptake reaching in the GXT test ought to be established by the plateau in oxygen uptake. Because none of the volunteers met plateau criterion, the VO_2max was confirmed by at list two end criteria: heart rate (HR) ≤ 10 b/min or ≤ 5% of age-predicted maximum (220-age); RER >1.00–1.15; blood lactate concentration after test ≥ 8 mmol/L[111,112]. During the entire test the HR was recorded using POLAR m400 sportester (*Kempele, Finland*). 10 min after the test completion the LA concentration was measured.

The WAnT was conducted using a Monark 828E cycloergometer (*Monark Lidingo, Sweden*). The procedure required participants to pedal as fast as possible for 30 s. The goal of the test was to generate the highest velocity possible and maintain it for the duration of the session (30 s). The external load was set at 7.5% of the individuals body mass. The warm-up before the test consisted of pedaling for 5 min with a 50W load. During the warm-up the heart rate should also correspond to 150 beats/min. During the test there are recorded such parameters as: maximum power (P_{max} - W; W/kg), time to reach maximal power (T1), time of maximal power maintenance (T2), minimal power (P_{min}), index of fatigue (IF), total work (TW—kJ; J/kg). 10 min after the WAnT completion LA concentration was measured. Between GXT and WAnT two days of recovery was used in stage 0 and stage 9.

Training protocols. The training protocols differed in the amount of intensity used and always included a general warm-up and a cooldown (Table 9). Each training session was monitored by HR using the Polar 400 data and energy expenditure and lactate measurements were evaluated 10-min after the session was completed. Participants evaluated their effort and were asked their exertion 30-min after the session using the ten points Borg Rating of Perceived Exertion (RPE) scale[113]. The score provided was then multiplied by the time of the session in minutes to determine the internal load of the session[114]. The answers were provided individually, and the participants were previously familiarized with the scale.

Protocol of HIPT training. In the HIPT group, all training sessions lasted 59 min, with the conditioning phase lasting 24 min (Table 9). For this training, all participant completed eight sets, each lasting 30 s, of strength

Summary of TRAINING SESSION								
Groups	**Warm-up**	**Work**	**Total work**	**Rest**	**Total rest**	**TC /EL**	**Cool-down**	**Σ time/session**
HIPT	10 min	8 rep × 30 s	4 min	8 rep × 150 s	20 min	20 min	5 min	59 min
HIIT	10 min	6 rep × 90 s	9 min	6 rep × 90 s	9 min	20 min	5 min	53 min
HIET	10 min	12 min	12 min	–	–	20 min	5 min	47 min
HIFT	10 min	8 rep × 30 s	4 min	8 rep × 150 s	20 min	20 min	5 min	59 min
Summary of MICROCYCLE								
Groups	**Warm-up (Σ)**	**Work**	**Total work**	**Rest**	**Total rest**	**TC /EL**	**Cool-down (Σ)**	**Σ time /week**
HIPT	3 × 10 min (30 min)	3 × 4 min	12 min	3 × 20 min	60 min	3 × 20 min (60 min)	3 × 5 min (15 min)	177 min
HIIT	3 × 10 min (30 min)	3 × 9 min	27 min	3 × 9 min	27 min	3 × 20 min (60 min)	3 × 5 min (15 min)	159 min
HIET	3 × 10 min (30 min)	3 × 12 min	36 min	–	–	3 × 20 min (60 min)	3 × 5 min (15 min)	141 min
HIFT	3 × 10 min (30 min)	4 + 9 + 12 min	25 min	20 + 9 min	29 min	3 × 20 min (60 min)	3 × 5 min (15 min)	159 min
Summary of the 9 week MESOCYCLE								
Groups	**Warm-up (Σ)**	**Work**	**Total work**	**Rest**	**Total rest**	**TC /EL**	**Cool-down (Σ)**	**Σ time/9 weeks**
HIPT	9 × 30 min (270 min)	9 × 12 min	108 min	9 × 60 min	540 min	9 × 60 min (540 min)	9 × 15 min (135 min)	1593 min (26 h 33 min)
HIIT	9 × 30 min (270 min)	9 × 27 min	243 min	9 × 27 min	243 min	9 × 60 min (540 min)	9 × 15 min (135 min)	1431 min (23 h 51 min)
HIET	9 × 30 min (270 min)	9 × 36 min	324 min	–	–	9 × 60 min (540 min)	9 × 15 min (135 min)	1269 min (21 h 09 min)
HIFT	9 × 30 min (270 min)	9 × 25 min	225 min	9 × 29 min	261 min	9 s × 60 min (540 min)	9 × 15 min (135 min)	1431 min (23 h 51 min)

Table 9. General overview of training regimen. TC/LC—technique correction and learning of the exercises performed.

Exercise during training session of HIPT/CrossFit			
Bench press	Box jumps with a load	Clean	CLEAN AND JERK
Deadlift	Front suat	Kettlebell clean	Kettlebell swings
Lunges with barbells	Overhead squat	Snatch	
Exercise during training session of HIIT/ CrossFit			
Box Jumps	Box jumps with a load	Burpees	Clean
Clean and jerk	Deadlift	Dips	double unders
Front squat	Jump rope	Kettlebell swings	Knees to elbows
Lunges	Lunges with barbells	Overhead squat	Push-ups
Rope climb	Row	Sit-ups	Toes to Bar
Exercise during training session of HIET/CrossFit			
Air squat	Box jumps	Box jumps with a load	Burpees
Clean	Clean and jerk	Dips	Double unders
Front squat	Hand stand push-ups	Jump rope	Kettlebell swings
Lunges	Lunges with barbells	Overhead squat	Push-ups
Rope climb	Row	Sit-ups	Toes to bar

Table 10. Type of exercises used during the main part of the training sessions.

exercises (Table 10). The load in each exercise was 75–95% 1RM (established as: weight/[(1.0278 – (.0278*reps)], the number of repetitions were from 3 to 12, and the intensity of the exercises performed was to be greater than 85% HR_{max}. HR during training was measured by POLAR m400 sport tester. Each time after work, there was a 150-s break. Training units were completed as many rounds as possible (AMRAP). The main assumption of the training was to perform each repetition with maximum intensity. The total weekly microcyle time in the HIPT group was 177 min, and the 9-week mesocycle 26 h and 33 min. The training unit scheme corresponded to day 1 for the HIFT group.

Protocol of HIIT training. In the HIIT group, the training session lasted 53 min (Table 9), including the main part of the training of 18 min. It consisted of 6 sets each lasting 90 s, of exercise separated by a 90 s break. Endurance and strength exercises for the HIIT type were used here (Table 10). The load in individual exercises could

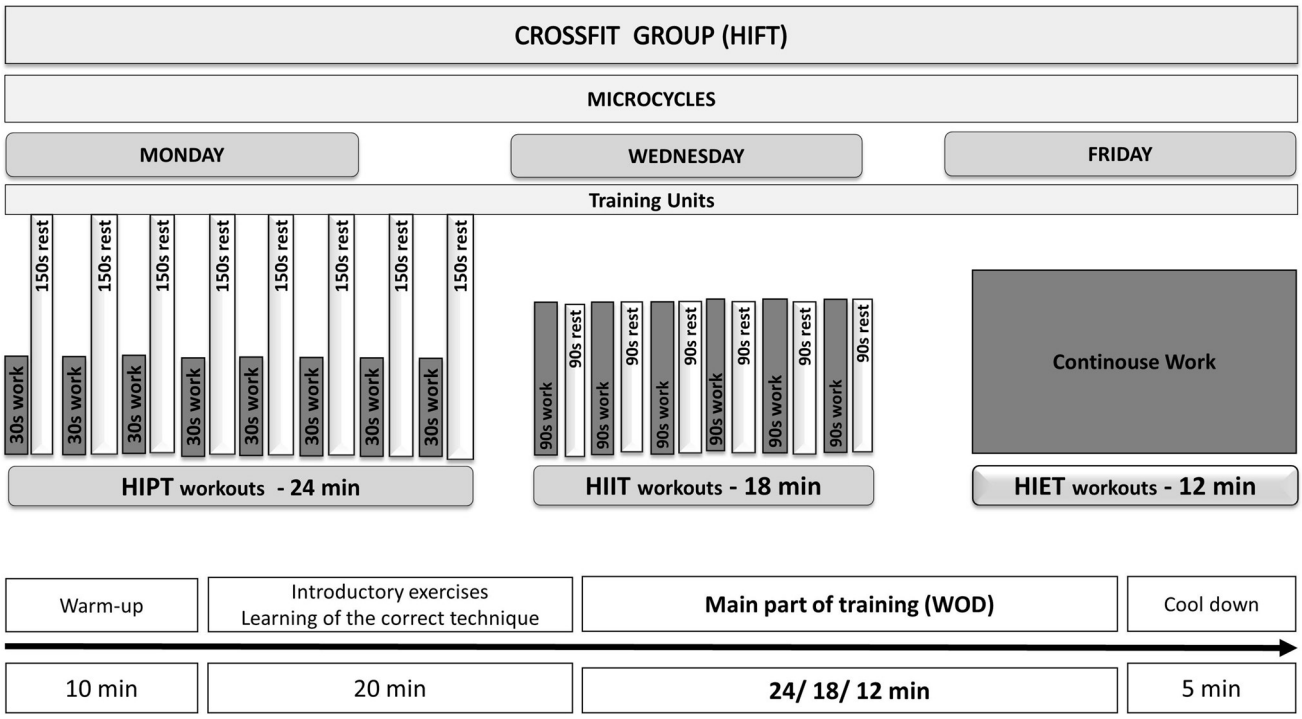

Figure 6. Scheme of HIFT microcyles.

not exceed 60% 1RM (established as: weight/[(1.0278 – (.0278*reps)], the number of repetitions was not determined, and the task of the person performing the exercise was to perform as many repetitions of the given exercise as possible during 90 s, or two exercises in a closed cycle with the number of repetitions given. The intensity of the performed exercises was to be greater than 85% HR_{max}. Each time after work, there was a 90-s break. Training units were conducted, among others in the form of AMRAP and "You Go I Go". The main assumption of the training was to perform each repetition with maximum intensity. The total weekly microcyle time in the HIIT group was 159 min, and the 9-week mesocycle 23 h and 51 min. The training unit scheme corresponded to the training environment from the HIFT group.

Protocol of HIET training. Training session in the form of HIET lasted 47 min and was the shortest among all the session in the project (Table 9). The main part of the unit was carried out in a continuous form for 12 min using endurance type of exercises (Table 10). The load in individual exercises could not exceed 40% 1RM, the number of exercises from 4 to 8, the number of repetitions from 10 to 20, and the intensity of performed exercises were to be greater than 85% HR_{max}. Training units were conducted in the form of AMRAP. The main assumption of the training was to perform each repetition with maximum intensity. The total weekly microcyle time in the HIPT group was 141 min, and the 9-week mesocycle 21 h and 9 min. The training unit scheme corresponded to the Friday training from the HIFT group.

Protocol of HIFT training. In the HIFT group, the training units had a different duration depending on each training day of the training microcyle. On day 1 (Monday), classes were conducted in the form of HIPT training and lasted 59 min. The second classes in the training microcyle took place on Wednesday, were conducted in the form of HIIT training and lasted 53 min (Table 9). Training in the form of HIET lasted 47 min and was carried out as the last in a weekly microcyle (on Friday). The total weekly microcyle time in the HIFT group was 159 min, and the 9-week mesocycle 26 h and 33 min The scheme of workouts used for the HIFT group is presented on Fig. 6.

During the training, the participants of the HIPT group did the same exercises three times a week as the HIFT group during classes on Monday (Table 9). The HIIT group did the same exercises during each workout as the HIFT group did on Wednesdays, while the HIET group did the same exercises that the HIFT group did on Friday. In all groups, the main goal of the training was to perform each repetition during the training unit with maximum intensity. The intensity of the exercises was to be greater than 85% HR_{max} and it was monitored using a POLAR m400 sportester *(Kempele, Finland)* and LA concentration 5 min after training.

Statistical analysis. Statistica version 13.1 (*StatSoft, Cracow, Poland*) was used to perform statistical analysis. All values were presented as a mean ± SD. All analyses of variance for the system with repeated measurements were performed using the ANOVA/MANOVA test and were verified based on normality of the distribution (Shapiro-Wilk) and homogeneity of variance (Levene test). In each case when the Levene test showed non-compliance with the assumption of homogeneity of variance (small groups below 30 observations), the

inference was confirmed by nonparametric tests. According to Kruskal-Wallis ANOVA tests, comparisons between 4 groups or Wilcoxon pair order test for tests repeated in each group separately. If the interpretation of differences obtained by both methods was consistent, it was assumed in a given case that the parametric analysis of variance is resistant to failure to meet the assumption of homogeneity of variance. The size of the effects of the observed variables was determined by partial eta square (η^2). The values of η^2 between 0.01–0.05 were evaluated as a low effect, 0.06–0.13 as medium effect and above 0.14 as a high effect. The Bonferroni parametric post-hoc test was used to determine the differences between the groups. In all the tests used, a statistically significant level was set at $p \leq 0.05$.

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The Val66 and Met66 Alleles-Specific Expression of BDNF in Human Muscle and Their Metabolic Responsivity

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Brain-derived neurotrophic factor (BDNF) plays an essential role in nervous system formation and functioning, including metabolism. Present only in humans, the "Val66Met" polymorphism of the BDNF gene (*BDNF*) is suggested to have a negative influence on the etiology of neurological diseases. However, this polymorphism has only been addressed, at the molecular level, in nonhuman models. Knowledge about Val66- and Met66-variant differences, to date, has been achieved at the protein level using either cell culture or animal models. Thus, the purpose of our study was to analyze the impact of the Val66Met polymorphism on *BDNF* expression in healthy humans and compare the allele-specific responses to metabolic stress. Muscle biopsies from 13 male recreational athletes (34 ± 9 years, 1.80 ± 0.08 m, 76.4 ± 10.5 kg) were obtained before and immediately following a VO_2max test. Allele-specific BDNF mRNA concentrations were quantified by droplet digital PCR (ddPCR) in heterozygous and homozygous subjects. The results indicated that *BDNF* expression levels were influenced by the genotype according to the presence of the polymorphism. *BDNF* expression from the Met66-coding alleles, in heterozygotes, was 1.3-fold lower than that from the Val66-coding alleles. Total BDNF mRNA levels in these heterozygotes remained below the whole sample's mean. A partial dominance was detected for the Val66-coding variant on the Met66-coding's. *BDNF* expression levels decreased by an average of 1.8-fold following the VO_2max test, independent of the individual's genotype. The results of this study indicate that metabolic stress downregulates *BDNF* expression but not plasma BDNF concentrations. No correlation between expression level and plasma BDNF concentrations was found.

Keywords: Val66Met polymorphism, BDNF, allele-specific gene expression, mRNA level, metabolism, VO_2max test

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a neurotrophin, a small secretory dimeric protein that appears in all vertebrates with a highly conserved structure (Rafieva and Gasanov, 2016). Showing the most pervasive and abundant expression throughout the brain among the neurotrophins, BDNF plays a pivotal role in cell proliferation and differentiation, and supports neural tissue formation, muscle–neuron interaction, and the organization of the central nervous system (CNS) (Friedman, 2010). In addition, BDNF is essential for neuronal outgrowth and continuous regulation of processes such as synaptic transmission, long-term potentiation, and depreciation (Lamb et al., 2015; Sasi et al., 2017). BDNF is largely expressed in neurons and neural tissue and is also produced by muscle (Matthews et al., 2009), although the role of muscle production has yet to be elucidated in humans to date.

In humans, a single nucleotide polymorphism resulting in a substitution of an adenine (A) base by guanine (G) in the BDNF gene (*BDNF*) has been identified in ∼20% of the population (Maisonpierre et al., 1991; Shimizu et al., 2004). This polymorphism is named "Val66Met" due to a Valine to Methionine substitution in the 66th amino-acid position of the synthesized protein (BDNF) with respect to the A or G genotype (Rafieva and Gasanov, 2016; Hunt et al., 2018). Considering the pivotal function of BDNF, it has been suggested that the *BDNF* Val66Met may have a role in the etiology of several neurological diseases (Egan et al., 2003; Notaras and van den Buuse, 2018; Shen et al., 2018) and psychiatric disorders (Kishi et al., 2018).

The BDNF protein (mature form, referred from here on as BDNF) is synthesized by cells as a precursor, pro-BDNF, that undergoes a multistage processing, which includes the loss of the pro-domain part containing the polymorphic 66th amino-acid position. Therefore, the Val66Met variance does not appear in the structure of released BDNF. However, a change in the pro-domain part of pro-BDNF, the Val66Met substitution, could influence the secretory pathway direction and release of BDNF (Chen et al., 2004) affecting the efficiency of BDNF synthesis and function (Bath and Lee, 2006; Pruunsild et al., 2007).

In humans, the influence of the Val66Met polymorphism has been observed through the association between Met66 genotypes and pathological changes in the morphology of specific areas of the brain (Egan et al., 2003). Importantly, the polymorphism fails to parcel out as a determinant for the development of neuropathological conditions among other factors such as ethnicity, gender, and other influences (Shimizu et al., 2004; Kishi et al., 2018; Shen et al., 2018). When examining various clinical subpopulations, there are no reliable associations between the genotype and plasma BDNF concentrations (Yoshimura et al., 2011), suggesting that the association of this polymorphism with risk for disease, based on circulating BDNF concentrations, is limited (Shen et al., 2018). Nevertheless, there appears to be a pattern in the acute stress response of individuals carrying the Met66 allele in *BDNF* that results in an upregulation in their cortisol response to stress, which may provide support for the influence of the Val66Met polymorphism in the development of stress-related disorders including neurological disease (Kishi et al., 2018; de Assis and Gasanov, 2019).

The present understanding of Val66- and Met66-variant differences, to date, has been achieved at the protein level using either cell culture or animal models (Egan et al., 2003; Chen et al., 2004; Lou et al., 2005; Anastasia et al., 2013; Uegaki et al., 2017). However, BDNF production could also be affected at the level of gene function. Serving as a template for the pro-BDNF translation, mRNA initiates BDNF production, reflecting gene activity, and is the first level of BDNF regulation. In regard to gene expression, Val66- and Met66-coding alleles may be transcribed either equally or not at all, being in competition for the mRNA synthesis machinery and potentially interfering with their expression levels. However, to the best of our knowledge, the influence of the Val66Met polymorphism on *BDNF* functioning (i.e., transcription) has never been previously analyzed.

In consideration of previous observations of BDNF responsivity to aerobic exercise (Dinoff et al., 2016; de Assis et al., 2018), we have developed a method for analyzing the gene expression of human *BDNF* regarding the Val66Met polymorphism *in vivo* using a maximal endurance exercise protocol. In order to reveal the relation between the Met66 and Val66 alleles of *BDNF*, and its impact on *BDNF* functioning (i.e., transcription), we have analyzed the quantities of BDNF mRNA in the muscle of healthy subjects at rest and following a maximal aerobic capacity (VO_2max) test.

MATERIALS AND METHODS

In accordance with the European Union regulation 2016/679 of 27 April 2016 and the Ethical Committee of the Central Clinic Hospital of the MSWiA in Warsaw, Poland, 25 physically active healthy volunteers (37.5 ± 9.9 years, among them five women) following an explanation of all procedures, risks, and benefits, provided his or her informed consent to participate in this study. All participants were genotyped for the *BDNF* Val66Met polymorphism. Thirteen of the participants, all males (age: 34.5 ± 9 years, BMI: 22.85 ± 1.6, and VO_2max: 54.3 ± 6.2 ml/kg/min), were included in the experiment (**Table 1**).

Genotyping

All study participants reported to the Human Performance Laboratory in the Mossakowski Medical Research Centre, Polish Academy of Science, Warsaw, Poland. During the visit, all participants were provided with a brief medical exam that included anthropometric measurements. A blood draw (3–4 ml) was obtained by a nurse from a cubital vein, divided to aliquots, and immediately embedded in dry ice and stored at −80°C until analysis. A 0.5 ml aliquot from a blood sample was used for genomic DNA (gDNA) extraction by a NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co., KG, Germany) according to the manufacturer's procedures. The resulting DNA concentrations were determined by a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, United States).

TABLE 1 | Subject characteristics and plasma BDNF levels.

ID (Age 34 ± 9)	BMI (22.85 ± 1.6 kg/m^2)	VO_2max (54.33 ± 6.2 ml/kg/min)	Genotype	Plasma BDNF-pre (pg/ml)	Plasma BDNF-post (pg/ml)	Δ BDNF (pg/ml)
1	21.97	60.00	Val/Val	9300.08	7547.9	−1752.18
2	22.35	62.00	Val/Val	8220.33	7848.24	−372.09
3	21.97	59.38	Val/Met	8522.45	7214.06	−1308.39
4	26.31	41.40	Val/Val	8088.49	7456.73	−631.76
5	23.10	45.62	Val/Met	10103.24	8898.5	−1204.74
6	22.74	51.38	Val/Val	6961.47	8314.07	1352.6
7	23.70	56.06	Val/Val	8558.53	6998.88	−1559.65
8	23.89	61.40	Val/Val	7276.23	8690.76	1414.53
9	20.67	54.24	Val/Val	7623.1	8630.74	1007.64
10	22.32	57.3	Val/Val	8511.54	8831.91	320.37
11	23.39	51.46	Met/Met	9803.92	8328.3	−1475.62
12	20.23	56.38	Val/Met	7779.02	8160.82	381.8
13	24.42	49.64	Val/Val	10351.91	9005.23	−1346.68

Val, Val66-coding; Met, Met66-coding BDNF allele; BMI, body mass index; VO_2max, the maximum rate of oxygen consumption; means are given at the top of the table. BDNF-pre, plasma BDNF concentrations at rest; BDNF-post, plasma BDNF concentrations after the VO_2max test; ΔBDNF, the BDNF-pre and BDNF-post difference.

The gDNA was used as a template in a one-step amplified refractory mutation system PCR according to Sheikh et al. (2010). In this protocol, two primers (P1 and P2) surrounding the *BDNF's* area of interest (polymorphism bearing ∼400 bp) and two primers (P3 and P4) corresponding to Val- and Met-coding sequences, respectively (internal to the first pair and in opposite orientation to each other) were added together with a PCR Mix Plus mixture (A&A Biotechnology, Poland) according to the manufacturer's procedures. The reaction was performed in a C1000 Touch Thermal Cycler (Bio-Rad, United States). PCR results were analyzed in a 6% polyacrylamide or 2.5% agarose gel-electrophoresis with ethidium bromide staining. Images were generated by Bio-Rad Gel Documentation System. Results showed the full-length *BDNF*-specific product (∼400 bp) and the corresponding products to the presence of Val (∼250 bp) and Met (∼200 bp) in *BDNF* variants (see **Figure 1**).

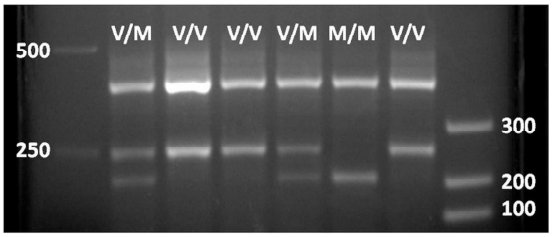

FIGURE 1 | Agarose gel electrophoresis of PCR *BDNF* polymorphism analysis. 400 bp, BDNF gene full-length fragment (internal positive PCR control); 250 bp, Val66-coding BDNF gene fragment; 200 bp, Met66-coding BDNF gene fragment.

Exercise Protocol

Participants reported to the laboratory and rested comfortably in a supine position for 30 min. Heart rate and blood pressure were obtained prior to the initial muscle biopsy. Following the biopsy (∼15 min), participants performed a maximum aerobic capacity (VO_2max) test using the Bruce treadmill protocol (Bruce et al., 2004). Change in both the gradient and the speed of the treadmill occurred every 3 min (seven stages) that began at 2.7 kph/10% gradient and progressed up to 8.9 kph/20% grade, or until the participants' volitional exhaustion. Immediately (30–60 s) following the conclusion of the VO_2max test, a second muscle biopsy was obtained. Blood samples were collected from an antecubital vein at the same time-point of the muscle biopsies.

Muscle Biopsy Procedure

Approximately 50 mg (wet weight) of skeletal muscle from *Vastus lateralis* was obtained per sampling by suction using a semiautomatic needle biopsy (14 g, 100 mm). The sample was immediately embedded in dry ice and stored at −80°C until analysis. All procedures occurred between 3 p.m. and 5 p.m. (GMT+2).

RNA Extraction

Muscle samples were manually homogenized in Eppendorf tubes and mixed with 0.5 ml of a TRIzol reagent (T9424, Sigma-Aldrich). The TRIzol-chloroform RNA extraction was performed according to the manufacturer's protocol, and the total RNA was dissolved in 0.03 ml of RNAse-free water; RNA concentrations were determined by a NanoDrop™ 2000 Spectrophotometer, and the RNA solutions were stored at −80°C. One microgram of RNA was later used for cDNA synthesis in 0.02 ml of the reaction mixture using the iScript™ Reverse Transcription kit (Bio-Rad) according to the manufacturer's protocol. Subsequently, 1 μl of the cDNA solution was used as a template for ddPCR analyses.

Droplet Digital PCR (ddPCR) Analysis

A housekeeping gene was primarily established as a reference for the patterns of *BDNF* expression in the ddPCR™ system (de Assis et al., 2020). For this, the cDNA of three subjects' samples (one woman) both at rest and post VO_2max were applied

in 20 µl reactions for the Bio-Rad PrimePCR Pathway Plate—Reference Genes H96, Human—containing 14 housekeeping gene candidates recommended for muscle tissue analysis, internal PCR, reverse transcription, RNA quality, and gDNA admixture controls. RT-PCRs were performed in triplicate according to the manufacturer's protocol (Bio-Rad, United States). The beta-2-microglobulin (*B2M*) and the ribosomal protein S18 (*RPS18*) were among the three of the most reliable candidate genes applicable for ddPCR^{TM} analysis [inter- and intraindividual threshold cycle, C(t), difference did not exceed 2.5 cycles]. The 13 cDNA samples were then applied to the ddPCR^{TM} system with an allele-specific BDNF assay containing hydrolyzed probes (TaqMan) with both HEX (hexachlorofluorescein) and FAM (6-carboxyfluorescein) detection of Val- and Met-coding alleles, respectively (ddPCR^{TM} Mutation Assay: rs6265, dHsaMDS320493890), using PrimePCR^{TM} ddPCR^{TM} Supermix for Probes (no dUTP), as well as the PrimePCR^{TM} ddPCR^{TM} Expression Probe assay, *B2M*, Human (HEX-probe detection, qHsaCPE5053101) in parallel (all designed by Bio-Rad). RNA samples without a reverse transcription step were also used in parallel as a template, in the same quantity according to the RNA template in cDNA samples as a genomic DNA (gDNA) admixture control. The quantities of reaction products in these samples were subtracted from the corresponding samples analyzed for *BDNF* expression (de Assis et al., 2020). The gDNA of Val66Met heterozygotes was used as a control of the *BDNF* allele ratio and as a positive control of the reaction. The ratios between Val and Met-alleles of *BDNF* in gDNA samples were 1 ± 0.03.

To exclude the reference gene influence, the second part of mRNA samples were treated by DNase I (M0303, NEB, United States) according to the manufacturer's protocol and used for cDNA synthesis as described above. One microliter of cDNA solution was used in ddPCR for the allele-specific *BDNF* expression analysis with the RPS18 gene as a reference using the PrimePCR^{TM} Probe assay, *RPS18*, Human (FAM-detection, qHsaCEP0040177, Bio-Rad). *B2M* and *RPS18* expression was also compared in the same reaction using FAM- and HEX-fluorescence detection. All reactions were performed in duplicate.

Plasma BDNF Analysis

To measure the BDNF concentration in plasma, blood samples were collected and drawn into tubes containing ethylene-diaminetetraacetic acid (EDTA) and stored at 4°C for 2–3 h. Then samples were centrifuged at 2,500 × *g* for 15 min at 4°C, and the supernatants were stored at −80°C until analysis. During analysis, thawed samples were centrifuged in Eppendorf tubes at 10,000 × *g* for 10 min. BDNF concentrations in plasma samples were analyzed based on the manufacturer's guidance by the sandwich enzyme-linked immunosorbent assay (ELISA) Human BDNF PicoKine kit (Boster Bio, United States). The absorbance at 450 nm was measured with a Spark multimode microplate reader (Tecan, Switzerland) to determine BDNF values using the lyophilized human BDNF (Boster Bio, United States) dilutions as a standard. The ELISA kit sensitivity was <15 pg/ml, the coefficient of variation was <7.5%, and all blood samples were analyzed in duplicate.

Statistical Analysis

Pearson product-moment correlations were applied to the pre-post VO_2max values of BDNF/B2M*10^3 expression and BDNF plasma concentrations, as well as for their concentration deltas. The association of age, BMI, and VO_2max with BDNF expression and plasma BDNF was also verified by Pearson correlation analysis. Paired Student's *t*-tests were used to verify pre-post differences in BDNF expression and BDNF blood concentrations. Two-way analysis of variance (ANOVA) was applied to verify for significance in Val66Met genotype influence on pre-post VO_2max *BDNF* expression changes and allele-specific expression difference in heterozygotes. In the event of a significant *F* ratio, Bonferroni *post hoc* analysis was used to examine pairwise differences.

To compare the allele-specific expression in the heterozygotes regardless of conditions, the means of single allele mRNA levels were calculated in both pre- and post-VO_2max test (pre- and post-means for each allele). Next, the levels of Val66- and Met66-coding alleles' expression in pre- and post-VO_2max conditions were standardized by calculating their ratios with their pre- and post-means, respectively. The standardized numbers of Val66- and Met66-coding alleles' expression were then analyzed by a paired *t*-test. An alpha level of $p \leq 0.05$ was used to determine statistical significance. All the data are presented as mean ± SD.

RESULTS

Characteristics and genotypes of the study participants including plasma BDNF concentrations are presented in **Table 1**. Comparison between *B2M* and *RPS18* showed that these genes' expression patterns were fully coherent, with no difference in pre-post VO_2max test results (**Supplementary Figures 1**, **2**). Therefore, all the calculations of *BDNF* expression quantities were related to B2M mRNA quantities. The results of BDNF mRNA/B2M*10^3 expression analyses from skeletal muscle samples showed a high homogeneity at rest (1.78 ± 0.53 BDNF/10^3 B2M mRNA molecules) and even higher homogeneity after the VO_2max test conditions (0.98 ± 0.33 BDNF/10^3 B2M mRNA molecules) (**Table 2**). The BDNF mRNA concentrations ranged from 0.528 to 2.982 molecules of BDNF per 10^3 molecules of B2M. Neither the individual differences nor the changes evoked by the VO_2max test exceeded a threefold change in BDNF mRNA levels.

The BDNF mRNA expression was significantly higher at rest compared to post-VO_2max test conditions ($p < 0.001$). A 44 ± 9.7% decrease in BDNF mRNA levels following the VO_2max test was observed (**Table 3**). There was a strong positive correlation between resting- and post-VO_2max test values of BDNF mRNA ($r = 0.859$; $p < 0.001$). Participants whose *BDNF* expression levels were above the group average at rest maintained *BDNF* expression levels above the group mean following the VO_2max test, whereas those whose expression levels were below the mean remained below the average *BDNF* expression levels of the group. A strong negative correlation was detected between resting *BDNF* expression levels and the pre-post changes in *BDNF* expression (pre/post Δ) ($r = -0.838$; $p < 0.001$), indicating

TABLE 2 | Allele-specific quantification of BDNF mRNA in human muscle tissue by ddPCR with B2M mRNA as a reference.

ID	Genotype	*Allele-specific BDNF mRNA**				*B2M/10 mRNA**		*BDNF mRNA/1000 B2M mRNA*			
	Condition ->	Pre-Val	Pre-Met	Post-Val	Post-Met	Pre	Post	Pre-Val	Pre-Met	Post-Val	Post-Met
1	Val/Val	1.09	0	3.27	0	83.8	444	1.305	0	0.736	0
2	Val/Val	5.48	0	2.95	0	279	235	1.962	0	1.255	0
3	Val/Met	1.85	1.17	2.08	1.43	220	406	0.840	0.530	0.512	0.346
4	Val/Val	0.62	0	2.64	0	49.6	500	1.253	0	0.528	0
5	Val/Met	1.55	1.11	1.35	1.2	170	268	0.913	0.655	0.503	0.376
6	Val/Val	7.02	0	2.59	0	367	368	1.912	0	0.704	0
7	Val/Val	2.64	0	1.40	0	155	163	1.701	0	0.860	0
8	Val/Val	3.04	0	6.43	0	101.9	435	2.982	0	1.478	0
9	Val/Val	5.17	0	8.47	0	245	714	2.109	0	1.186	0
10	Val/Val	2.70	0	1.80	0	224	302	1.205	0	0.596	0
11	Met/Met	0	1.53	0	1.97	60.8	130	0	2.522	0	1.513
12	Val/Met	0.90	0.63	6.33	5.03	115	1,201	0.783	0.551	0.527	0.419
13	Val/Val	4.40	0	5.43	0	230	428	1.913	0	1.269	0

**Expression values are disposed in (copies/μl of reaction mixture), 1 μl of reaction mixture corresponds to 0.625–1.25 mg of muscle tissue. Pre-Val, BDNF Val66-allele expression values in rest; post-Val, Val66-allele expression values after VO_2max test; pre-Met, BDNF Met66-allele expression values in rest; post-Met, Met66-allele expression values after VO_2max test. B2M, beta-2-microglobulin expression values, pre- and post.*

a greater fall in *BDNF* expression levels for those displaying higher basal levels of *BDNF* expression. ANOVA performed between *BDNF* Val66Met homo- and heterozygotes indicated a significant effect of the VO_2max test on gene expression [$F(1,22) = 12.02$, $p = 0.002$, part. eta sq. = 0.33] with no influence of the genotype ($p = 0.1656$). *Post hoc* analysis confirmed the significant effect of the VO_2max test on *BDNF* expression ($p < 0.001$) and the absence of significant genotype influence ($p > 0.05$) (**Figures 2**, **3**).

Regarding allele specificity, Met66-coding BDNF mRNA levels in heterozygotes quantified 76.1 ± 11.2% of Val66-coding mRNA level values independent of metabolic conditions, which means 1.3-fold lower. This was confirmed by a comparison of the allele-specific expression levels standardized with the condition-specific expression means ($p < 0.001$) (**Table 4**).

Two-way ANOVA performed between the Val66- and Met66-coding allele expression levels in *BDNF* heterozygotes pre- and post-VO_2max test confirmed both the metabolic stress effect [$F(1,8) = 82.35$, $p < 0.0001$, part. eta sq. = 0.58] on *BDNF* expression and the *BDNF* allele expression difference [$F(1,8) = 47.05$, $p = 0.0001$, part. eta sq. = 0.33] (see **Figure 4**). In addition, a significant interaction [$F(1,8) = 5.19$, $p = 0.052$, part. eta sq. = 0.04) was noted between Val66 and Met66 allele expression differences in response to the VO_2max test.

The pre-/post-VO_2max test changes in *BDNF* expression, as well as the Val66-/Met66-coding allele relations, were confirmed in independent measurements with *RPS18* expression as a reference (**Table 5**). A 38.3 ± 12.5% decrease in BDNF mRNA levels following the VO_2max test was observed (**Supplementary Figure 2**). Met66-coding BDNF mRNA levels in heterozygotes quantified 78.5 ± 9.2% of Val66-coding mRNA level values (1.29 ± 0.14-fold lower) independent of metabolic conditions (**Table 5**).

The differences between resting and post-VO_2max test plasma BDNF concentrations were not significantly different. In addition, the genotype did not appear to influence plasma BDNF concentrations. However, a strong negative correlation ($r = -0.821$; $p < 0.001$) was noted between plasma BDNF concentrations pre-VO_2max test and the changes evoked by the test—the ΔBDNF (plasma post-BDNF – pre-BDNF). This suggests that participants with higher resting plasma BDNF concentrations experienced a decrease in post-test BDNF concentrations, while those with lower plasma BDNF concentrations experienced an increase in post-exercise BDNF concentrations (**Figure 5**). A moderate negative correlation was noted between the *BDNF* expression level changes (pre/post Δ) and plasma BDNF ones (ΔBDNF) ($r = -0.531$, $p = 0.062$). This

TABLE 3 | Pre-Post VO_2max test differences in *BDNF* expression related to B2M mRNA.

ID	*Allele-specific BDNF expression decrease (%)*				
	Val	*Met*	*Val* Mean, SD	*Met* Mean, SD	Total mean, SD
1	−43.6		−45.5 ± 9.4%*	−35.3 ± 8.4%*	−44.5 ± 9.7%
2	−36.0				
3	−39.9	−34.7			
4	−57.9				
5	−44.9	−42.6			
6	−63.2				
7	−49.4				
8	−50.4				
9	−43.8				
10	−50.6				
11		−40.0			
12	−32.6	−23.7			
13	−33.6				

*Val, Val66-coding BDNF allele; Met, Met66-coding BDNF allele. *p-value of Val/Met mean difference is 0.09.*

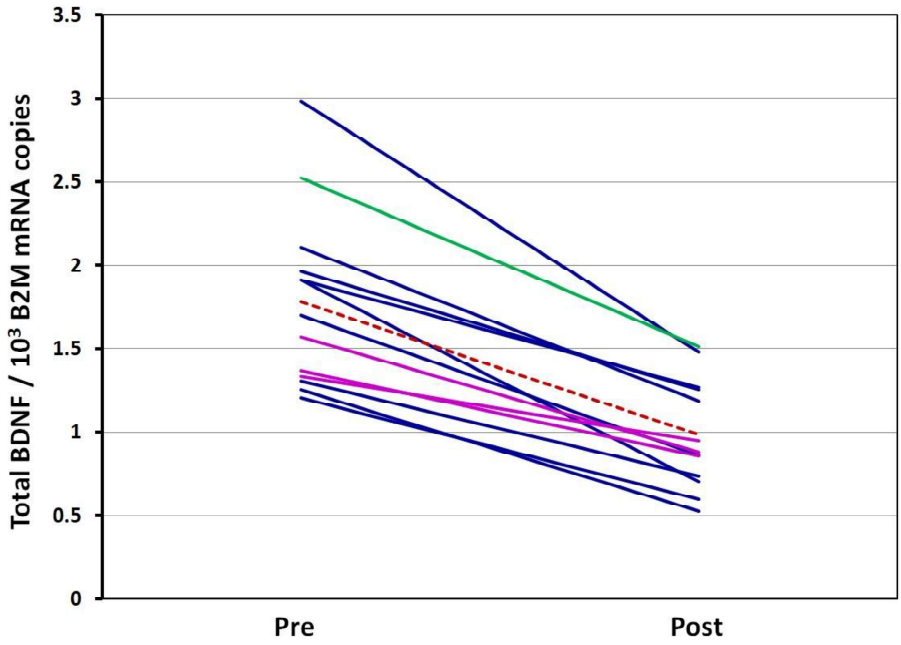

FIGURE 2 | Spaghetti plot of *BDNF* expression level change. Pre—Total *BDNF* expression in rest conditions; Post—Total *BDNF* expression after VO_2max test. Val66Val homozygotes are colored dark blue, Val66Met heterozygotes: magenta, Met66Met homozygote: green, Mean: the dotted red line. All BDNF expression values are related to B2M mRNA.

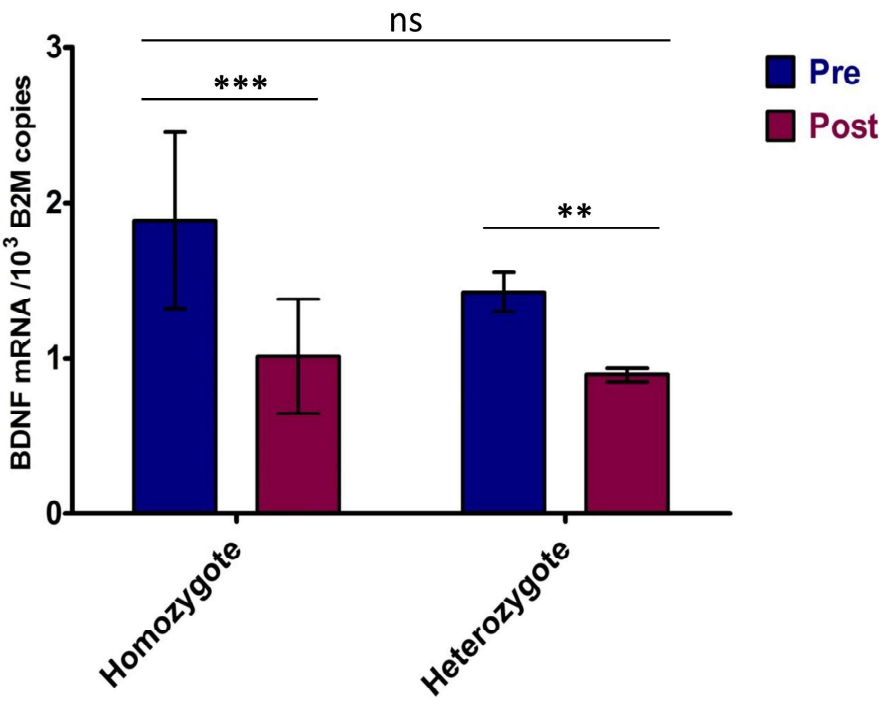

FIGURE 3 | *BDNF* expression in homo- and heterozygotes in response to maximum effort test. Pre—Total *BDNF* expression in rest conditions; Post—Total *BDNF* expression after VO_2max test. Significance levels marked by asterisks, where $^{**}p < 0.05$, $^{***}p < 0.01$. Bars are given with ± SD.

means that a greater decrease in the *BDNF* expression level was associated with an increase in the plasma BDNF concentrations resulting from the VO_2max test, while a lighter decrease in the *BDNF* expression tended to reflect a decrease in plasma BDNF concentrations. No association was noted between BDNF mRNA and plasma concentrations of BDNF at rest ($r = -0.15$,

TABLE 4 | Val66-Met66 allele *BDNF* expression ratio in heterozygotes.

ID	*BDNF* expression, Val/Met ratio (Met, % of Val)			Normalized to pre- and post-means *BDNF* allele expression*			
	Pre	Post	Mean, SD	Pre-Val	Pre-Met	Post-Val	Post-Met
3	1.58 (63.3%)	1.04 (96.1%)	1.33 ± 0.18 (76.1 ± 11.2%)	1.18	0.74	1.15	0.77
5	1.39 (71.9%)	1.30 (76.9%)		1.28	0.92	1.12	0.84
12	1.42 (70.4%)	1.26 (79.4%)		1.10	0.77	1.18	0.94

*Pre, post, rest, and post-VO_2max test conditions. *p-value of normalized Val/Met mean difference is 0.0002. BDNF expression values related to B2M mRNA.*

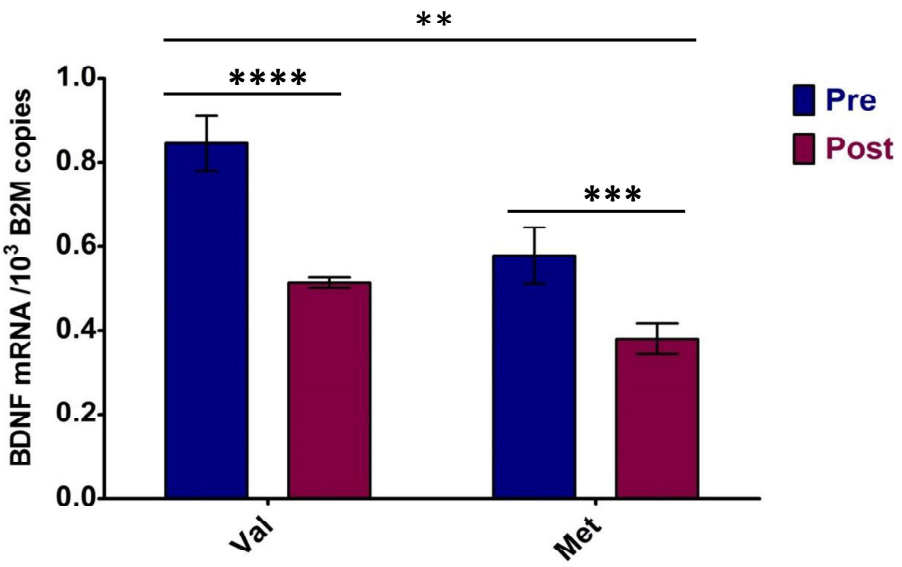

FIGURE 4 | Allele-specific *BDNF* expression of heterozygotes in response to maximum effort test. Pre—Total *BDNF* expression in rest conditions; Post—Total *BDNF* expression after VO_2max test. Val, Val66-coding BDNF mRNA; Met, Met66-coding BDNF mRNA. Significance levels marked by asterisks, where $^{**}p < 0.05$, $^{***}p < 0.01$, $^{****}p < 0.001$. Bars are given with ± SD.

$p = 0.634$) nor post-VO_2max assessments ($r = 0.36$, $p = 0.221$), as well as between any of the participants' physiological parameters (VO_2max, height, weight, and age).

DISCUSSION

Our results indicated that BDNF expression in muscle was within a narrow range among all participants, regardless of the genotype and conditions (rest and metabolic stress), suggesting that muscle *BDNF* expression is very stable. The absolute quantification of muscle *BDNF* expression by ddPCR provided an opportunity to detect relatively small changes resulting from different metabolic conditions and genotype-induced expression variations. The effect of exercise on changes in *BDNF* expression levels was not dependent on the participants' age or genotype suggesting a consistent response. This is supported by previous research of Matthews et al. (2009) who reported an increase in *BDNF* expression from 2 to 24 h following endurance exercise (120 min cycling at 60% of VO_2max) using quantitative RT-PCR. However, immediately after the bout of exercise, *BDNF* expression levels were shown to decrease at the same time-point as our results. It is likely that an initial decay in *BDNF* expression is seen during an acute metabolic stress, which is followed by a compensatory rise during recovery. The results of the present study indicated that the higher the pre-exercise *BDNF* expression levels, the more pronounced their decline during exercise. This perhaps is suggestive of a stability feature for *BDNF* expression, as well as an immediate sensitivity to metabolic changes.

Plasma BDNF concentrations failed to correlate with *BDNF* expression levels at both rest and post-exercise, with no one-directional changes noted in plasma BDNF following the exercise stress. Instead, an increase in plasma BDNF concentrations was noted in the participants with lower resting BDNF concentrations in response to the VO_2max test, while the participants with higher plasma BDNF concentrations at rest experienced a decrease following maximal effort exercise. This bidirectional response, which appears to be related to resting BDNF concentrations, may reflect a stabilizing tendency to maintain circulating BDNF concentrations within a certain biological range during metabolic stress, such as exercise. Another potential explanation is also related to the exhaustive exercise protocol in which participants have a specific switch point that is activated when the metabolic system moves from predominantly aerobic to anaerobic. This is supported by

TABLE 5 | Allele-specific quantification of BDNF mRNA in human muscle tissue by ddPCR with RPS18 mRNA as a reference, pre-post VO_2 max test *BDNF* differences, and Val66-Met66 allele *BDNF* expression ratio in heterozygotes related to RSP18 mRNA.

ID	Genotype	*Allele-specific BDNF mRNA**				*RPS18/10 mRNA**		*BDNF mRNA/10,000 RPS18 mRNA*				*BDNF expression decrease (%)*		*BDNF expression, (Met, % of Val)*	
Condition ->		Pre-Val	Pre-Met	Post-Val	Post-Met	Pre	Post	Pre-Val	Pre-Met	Post-Val	Post-Met	Val	Met	Pre	Post
1	Val/Val	3.4	0	1.4	0	1,440	1,036	2.36	0	1.35	0	−42.8			
2	Val/Val	3.3	0	5	0	1,008	2,566	3.27	0	1.95	0	−40.5			
3	Val/Met	2.6	1.7	2.1	1.5	1,006	1,057	2.58	1.69	1.99	1.42	−23.1	−16.0	65.4	71.4
4	Val/Val	3.1	0	0.8	0	1,253	764	2.47	0	1.05	0	−57.7			
5	Val/Met	1.7	1.3	1.6	1.4	1,453	1,994	1.17	0.89	0.80	0.70	−31.4	−21.5	76.5	87.5
6	Val/Val	7.9	0	3.4	0	2,322	2,024	3.40	0	1.68	0	−50.6			
7	Val/Val	2.2	0	3.3	0	1,088	2,516	2.02	0	1.31	0	−35.1			
8	Val/Val	6.5	0	3.1	0	1,203	1,017	5.40	0	3.05	0	−43.6			
9	Val/Val	7.5	0	5.7	0	2,361	2,452	3.18	0	2.32	0	−26.8			
10	Val/Val	6.4	0	5	0	1,620	1,924	3.95	0	2.60	0	−34.2			
11	Met/Met	0	6.1	0	8.8	1,454	3,200	0	4.20	0	2.75	0	−34.5		
12	Val/Met	3.8	3.1	2.7	2.4	1,507	2,396	2.52	2.06	1.13	1.00	−55,3	−51.3	81.6	88.9
13	Val/Val	4.1	0	2.9	0	2,275	3,095	1.80	0	0.94	0	−48,0			
Mean												−38.3 ± 12.5		78.5 ± 9.2	

**Expression values are disposed in (copies/μl of reaction mixture), 1 μl of reaction mixture corresponds to 0.625–1.25 mg of muscle tissue. Pre-Val, BDNF Val66-allele expression values in rest; post-Val, Val66-allele expression values after VO_2max test; pre-Met, BDNF Met66-allele expression values in rest; post-Met, Met66-allele expression values after VO_2max test; RPS18, ribosomal protein S18 gene expression values, pre- and post.*

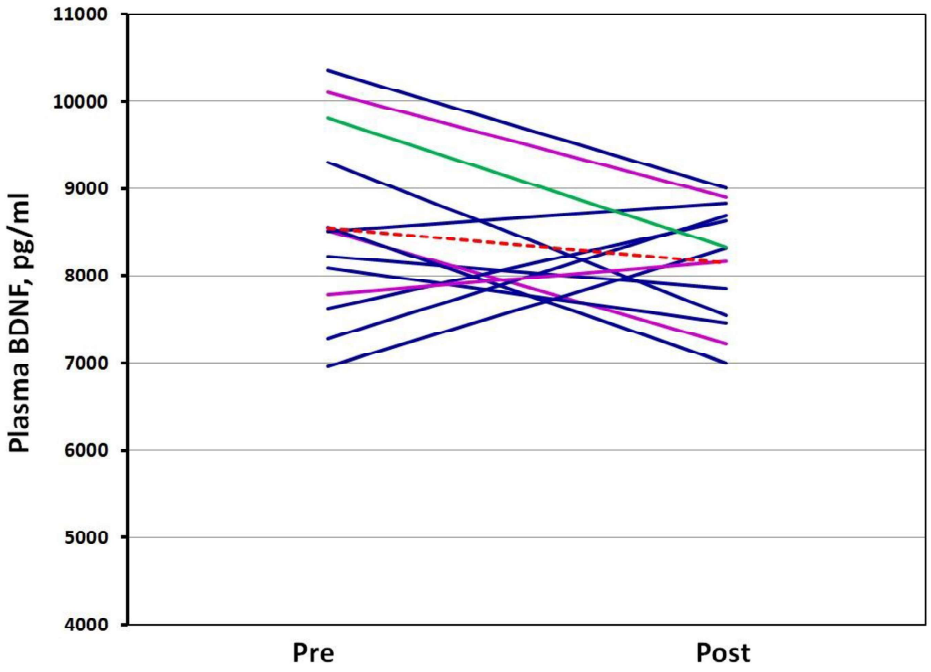

FIGURE 5 | Spaghetti plot of plasma BDNF level change. Pre—plasma BDNF concentrations in rest conditions; Post—plasma BDNF concentrations after VO_2max test. Val66Val homozygotes are colored dark blue, Val66Met heterozygotes: magenta, Met66Met homozygote: green, Mean: the dotted red line.

previous studies that reported a difference in the circulating BDNF response to aerobic vs. anaerobic exercise (Dinoff et al., 2016; de Assis et al., 2018).

A weak correlation was observed between changes in both expression and circulating levels of BDNF. Those participants showing greater changes in gene expression experienced smaller changes in plasma BDNF concentrations. This suggests negative regulatory feedback from circulating BDNF concentrations to its gene expression, suggesting an indirect or delayed connection between *BDNF* expression and blood concentrations. The discrepancy between *BDNF* expression and secretion may be related to possible differences in the response of BDNF producing blood and muscle cells to a metabolic stress, especially if the latter are not a source of circulating BDNF (Matthews et al., 2009). Furthermore, the appearance of BDNF in the circulation requires a considerable time lag from mRNA translation to dimeric active protein secretion. BDNF released by cells into the circulation involves secretory vesicle trafficking and a complex multistage process that includes the cleavage of pro-BDNF with the pro-domain removal. Increases in BDNF expression leads to an increase in pro-BDNF synthesis with a slow accumulation in secretory vesicles where pro-BDNF conversion to BDNF begins (Chen et al., 2004; Lou et al., 2005; Pruunsild et al., 2007; Rafieva and Gasanov, 2016). Thus, changes in *BDNF* expression levels are unlikely to immediately affect BDNF production.

The release of BDNF in response to metabolic stress and other types of signals is characteristic of the regulated pathway of neurotrophin secretion (Sasi et al., 2017). It represents a stimulus-dependent liberation by cells of secretory vesicles containing pre-synthesized BDNF that results in increases in circulating BDNF concentrations (Chen et al., 2004; Lou et al., 2005; Rafieva and Gasanov, 2016). The temporal gap between BDNF mRNA synthesis and BDNF release is dependent on the stimulus but not on the current level of *BDNF* expression. This likely contributes to the lack of an association between BDNF gene expression levels and changes in circulating concentrations.

The discrepancy between *BDNF* expression and circulating BDNF concentrations should be addressed with respect to the efficiency in the conversion of pro-BDNF to BDNF. Secretory vesicles contain unprocessed pro-BDNF; once secreted, it is subsequently cleaved to BDNF by blood and tissue proteases, increasing the gap between gene expression and mature factor appearance (Friedman, 2010; Rafieva and Gasanov, 2016). The portion of secreted unprocessed pro-BDNF may potentially increase when metabolically stimulated. This has been supported by investigators reporting an increase in the pro-BDNF/BDNF ratio during an acute aerobic stress (Brunelli et al., 2012). Thus, relying solely on blood BDNF concentrations may not provide the full spectrum of BDNF production. Measuring *BDNF* expression levels, as well as pro-BDNF circulating concentrations, may provide a more complete picture.

To the best of our knowledge, this appears to be the first investigation to demonstrate that the two alleles of human *BDNF* are expressed simultaneously in both non-polymorphic and polymorphic individuals, carrying Val66- and Met66-coding alleles. By comparing the total *BDNF* expression in all participants and detecting the allele-specific expression in the heterozygotes, we were able to conclude that both alleles of

human *BDNF* are active. At the same time, our data showed an inequality between Val66 and Met66 *BDNF* allele activity. Despite the low number of heterozygotes occurring in healthy volunteers (3 out of 25 participants), the difference between the Val66-coding and the Met66-coding BDNF mRNA levels was detected in each one of the heterozygotes, at rest or during a metabolic stress (e.g., maximal exercise).

Gene activity (i.e., effectiveness of transcription) depends on the *cis*-regulatory elements represented in the gene and its neighboring DNA sequence, as well as on *trans*-regulatory elements represented by a set of regulatory genes. In the cellular environment of a heterozygous organism, all the *trans*-acting elements are shared between the two alleles of a gene so that their interaction with the gene's alleles (in our case, *BDNF*) is determined by the relative effectiveness of the alleles' *cis*-regulatory elements. By this, the heterogeneity in the gene alleles' expression depends on its sequence only. Analyzing the transcription effectiveness of two different *BDNF* alleles in one subject, excluding differences in non-genetic and *trans*-acting factors, we compared the alleles' relative activity, i.e., dominant/recessive relations (Yan and Zhou, 2004). Our results suggested that the Val66Met polymorphism is a *cis*-acting element affecting the gene expression in which the Val66-coding allele variant exhibits a partial dominance on the Met66-coding allele variant.

To exclude the metabolic stress factor, a mathematical averaging of *BDNF* expression at rest and post-exercise in heterozygotes was performed. A difference in Val66 and Met66 BDNF gene alleles was confirmed. The dominant-recessive relation of *BDNF* Val66 and Met66 BDNF alleles was also supported by the expression pattern of the Met66-homozygote subject, where in the absence of Val66-coding allele suppression, it demonstrated one of the highest levels of *BDNF* expression.

The total expression of *BDNF* in Met66-heterozygotes remained below the mean expression level of the whole sample. A trend for a stabilizing shape was also observed for total *BDNF* expression—the higher the resting expression levels, the greater the decrease following maximal exercise. Interestingly, this effect was also attributed to an allele-specific reaction. The Val66-allele, which exposed higher levels of expression in comparison to the Met66 one, demonstrated the more pronounced exercise-induced decrease, while the Met66-allele's expression decline after exercise was milder.

There are several limitations to our study. Our observations are based on comparing three heterozygotes to 10 homozygote participants. Although results are consistent, further investigations are needed in larger samples of genotyped populations. As allele interplay is possible in heterozygotes only, this group should be analyzed apart from the homozygotes where no such *BDNF* expression suppressive effect is possible. Therefore, association studies dividing populations traditionally into groups of Val66-homozygotes and Met66-carriers (with Met66-homozygotes and Val66Met-heterozygotes inside) can be led to a diminishment of significant findings and/or incorrect interpretations. The results of our study support the hypothesis that the Val66Met polymorphism impacts the BDNF gene function (Egan et al., 2003; Chen et al., 2004; Bath and Lee, 2006; Kishi et al., 2018; Notaras and van den Buuse, 2018; Shen et al., 2018; Skibinska et al., 2018; Toh et al., 2018). However, alteration in *BDNF* expression levels does not appear to directly impact BDNF release/secretion. It appears that the post-translation processes prior to BDNF release can be differentially affected by the presence of Methionine, which might imply the Val66Met polymorphism influence on BDNF release and functions (Egan et al., 2003; Chen et al., 2004; Yoshimura et al., 2011; Uegaki et al., 2017).

CONCLUSION

Our study showed that *BDNF* expresses both alleles simultaneously. Results indicated that the expression of *BDNF* immediately after maximal exercise decreases 1.8 $\pm$ 0.4-fold, regardless of the genotype. Resting *BDNF* expression levels positively correlate with post-exercise levels and negatively correlate with the magnitude of exercise-induced changes. Furthermore, *BDNF* expression levels do not correlate with plasma BDNF. The levels of *BDNF* expression appear to be modulated by the Met66 allele presence in heterozygotes. The Val66-allele variant exhibits a partial dominance over the Met66-allele variant. We believe that these findings further our understanding on the mechanisms of BDNF production and function. To elucidate the physiological implications of Val66Met polymorphism on nervous systems-related pathologies, further studies on *BDNF* expression and protein release must be addressed to specific genotyped populations.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available in the Zenodo repository, https://zenodo.org/record/3633677#X8EAyc1KiUk.

ETHICS STATEMENT

This study was performed in accordance with the European Union regulation 2016/679 of 27 April 2016 and the 96 Ethical Committee of the Central Clinic Hospital of the MSWiA in Warsaw, Poland.

AUTHOR CONTRIBUTIONS

GA, PC, and EG: conceptualization. GA, JB, and EG: sample collection and analysis, and resources. GA and EG: data curation and project administration. GA, JH, and EG: writing—original draft preparation. EM-C and PC: writing—review and editing, and funding acquisition. PC: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.638176/full#supplementary-material

Supplementary Figure 1 | *B2M* and *RPS18* expression levels in samples. cDNA samples numbered; mRNA samples represent the gDNA admixture, and reaction specificity control.

Supplementary Figure 2 | *B2M* and *RPS18* expression level ratio stability in Pre- and Post-VO_2max test samples.

Supplementary Figure 3 | Spaghetti plot of *BDNF* expression level change related to RPS18 mRNA. Pre—Total *BDNF* expression in rest conditions; Post—Total *BDNF* expression after VO_2max test. Val66Val homozygotes are colored dark blue, Val66Met heterozygotes: magenta, Met66Met homozygote: green, Mean: the dotted red line.

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Research Paper

BDNF Val66Met Polymorphism, the Allele-Specific Analysis by qRT-PCR – a Novel Protocol

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Abstract

Background: Alteration in brain-derived neurotrophic factor (BDNF) production is a marker of neuropathological conditions, which has led to the investigation of Val66Met polymorphism occurring in the human BDNF gene (*BDNF*). Presently, there are no reported methods available for the analysis of Val66Met impact on human *BDNF* functioning.

Purpose: To develop a qRT-PCR protocol for the allele-specific expression evaluation of the Val66Met polymorphism in *BDNF*.

Methods: Using RNA extracted from muscle samples of 9 healthy volunteers (32.9 ± 10.3 y) at rest and following a maximal effort aerobic capacity exercise test, a protocol was developed for the detection of Val66/Met66 allele-specific *BDNF* expression in Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) - relative to housekeeping genes - and validated by absolute quantification in Droplet Digital Polymerase Chain Reaction (ddPCR).

Results: Differences in the relative values of BDNF mRNA were confirmed by ddPCR analysis. *HPRT1* and *B2M* were the most stable genes expressed in muscle tissue among different metabolic conditions, while *GAPDH* revealed to be metabolic responsive.

Conclusion: Our qRT-PCR protocol successfully determines the allele-specific detection and changes in *BDNF* expression regarding the Val66Met polymorphism.

Key words: Brain-derived Neurotrophic Factor; Allele-specific detection, ddRT-PCR, Gene expression, Exercise metabolism, Reference gene

Introduction

Brain-derived neurotrophic factor (BDNF) is a bioactive protein from the family of neurotrophins, neurotrophic growth factors, with the most abundant expression in the neural system development and maturity throughout the mammalian lifespan [1,2]. With compulsory roles in neurogenesis, synaptogenesis, neuronal repair and protection, alteration in BDNF dynamics may result in various neurological disorders and neurodegenerative diseases [3,4,5,6].

Predominantly expressed in neural and skeletal muscle cells as a protein signaler [7], BDNF synthesis results in the formation of several precursor isoforms in a multistage process involving cleavage and storage [2,8]. The BDNF gene (*BDNF*) transcript encodes a precursor protein (pre-pro-BDNF isoform) which loses its secretory signal peptide (pre-region) and dimer into the pro-BDNF isoform. Pro-BDNF is subsequently cleaved by proprotein convertases in either the Golgi apparatus or in secretory vesicles to form mature BDNF [2,9]. Released BDNF acts locally via both autocrine and paracrine mechanisms [10,11].

A single nucleotide polymorphism (SNP) has been identified in the human *BDNF* gene, resulting in a valine (Val) for methionine (Met) substitution in the 66th position of pre-pro-BDNF (Val66Met). This SNP

in the *BDNF* gene is thought to have important implications for the gene and protein functioning. The *BDNF* Val66Met has been suggested to impair activity dependent release of BDNF [12], play a role in BDNF/pro-BDNF secretion ratios [13], and is also associated with increased susceptibility to cognitive deficits and neuropsychiatric disorders [14,15]. Implications for the Met66-allele presence have been evidenced in experimental models [16;17] in which the complexity of the post-translational mechanisms was unable to determine impairments in the neurotrophin's function [18]. In a clinical perspective, circulating BDNF concentration is likely to be associated with a greater acute response of cortisol to stress, especially in individuals with a psychiatric disorder [19]. Although circulating blood concentrations of BDNF may provide some indication of BDNF production [20], it provides little understanding of the *BDNF* Val66Met polymorphism expression.

The differential expression of *BDNF* Val66Met polymorphic alleles to physical exercise was first detected in a recent study [21]. Most clinical studies have reported changes in circulating BDNF concentrations during aerobic activities [22], but inconsistent in regards to resistance training [23]. Increases in BDNF concentrations appear to be associated with the intensity of aerobic exercise [24,25], but may decrease during the recovery period if the exercise stimulus created a significant elevation in oxidative stress [20]. Considering the increasing interest in the Val66Met polymorphism [26,27], and its possible implications in a variety of neuropathological processes, the purpose of this study was to determine the validity of assessing human *BDNF* expression *in vivo* by developing a Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) protocol using a standard maximum aerobic capacity test. Based upon the more consistent response of BDNF to higher intensity endurance exercise protocols, we believed that this protocol would best provide for the differential analysis of *BDNF* expression regarding the Val66Met polymorphism validated by absolute quantification in QX200™ Droplet Digital™ Polymerase Chain Reaction (ddPCR).

Materials and methods

Participants

Nine apparently healthy and physically active volunteers participating in medium and long distance Polish race circuits (10 and 21 km), with known genotypes for the *BDNF* gene agreed to participate in this study. Descriptive characteristics for all participants can be observed in Table 1. After an explanation of all procedures, risks, and benefits, each participant completed a Par-Q questionnaire [28] and provided his or her written informed consent to participate in the study. This study was conducted in accordance to Regulation 2016/679 of the European Union Parliament Council of 27 April 2016 on the protection of individuals with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46 / EC (general data protection regulations) approved by the Ethical Committee of the Central Clinic Hospital of the MSWiA in Warsaw, Poland.

Table 1. Characteristic of Participants in ddPCR™ and qRT-PCR analyses

Subject	Age (years)	BMI (kg/m²)	VO_2max (ml·kg/min)	Genotype
Female	36	19.84	44.00	VAL/VAL
Male	24	21.97	60.00	VAL/VAL
Male	46	22.35	62.00	VAL/VAL
Male	28	21.97	59.38	**VAL/MET**
Male	43	26.31	41.40	VAL/VAL
Male	26	23.10	45.62	**VAL/MET**
Male	22	22.74	51.38	VAL/VAL
Male	46	23.70	56.06	VAL/VAL
Male	28	23.89	61.40	VAL/VAL
Male	36	20.67	54.24	VAL/VAL
Means ± SD	32.9 ± 10.3	23.25 ± 1.4	54.7 ± 7.7	

*VO_2max, maximal oxygen intake; BMI, body mass index.

Experimental Design

All study participants reported to the Human Performance Laboratory in the Mossakowski Medical Research Centre, Polish Academy of Science, Warsaw, Poland. Prior to the testing session, participants reported to the laboratory 2-hours post-prandial and were instructed not to consume any caffeine or alcohol; they were also instructed not to perform any lower body physical activity within 48 hours of testing. During the visit, all participants were provided with a brief medical exam that included anthropometric measurements. In addition, two muscle biopsies were obtained from participants prior to, and following performance of a maximal aerobic capacity test.

Muscle Biopsies

Participants were required to rest in a supine position for 30 min. A muscle biopsy (50 mg wet weight) from the middle portion of the *Vastus lateralis* was obtained by a medical doctor, using local anesthesia and a semi-automatic 14-gauge biopsy needle (Guillotine needle double shoot DSGBL 14/10, Tsunami, Italy). Following the first sampling, participants were required to perform a maximal aerobic capacity test. Following the conclusion (within 1- 2 min) of the aerobic capacity test, a second muscle

sample was obtained. Muscle samples were immediately placed in dry ice before storage at -80° C. All tests were performed between 3 p.m. and 5 p.m. (GMT+2).

Maximal Aerobic Capacity Test

The maximal aerobic capacity test was conducted using the standard Bruce Protocol [29]. The treadmill protocol began with each participant performing a 3-min walk at 2.7 km·h^{-1} and at a 10% incline. A progressive increase in both speed and inclination occurred every 3-min (stages), until participant's volitional fatigue. VO_2max (maximum rate of oxygen consumption) was determined to be the highest 30-s VO_2 value recorded during the test and coincided with at least two of the following three criteria: (a) 90% of age-predicted maximum heart rate; (b) respiratory exchange ratio > 1.1; and/or (c) a plateau of oxygen uptake (less than 150 mL/min increase in VO_2 during the last 60 s of the test). Ventilation parameters were monitored and recorded from rest and throughout the protocol. Heart rate (bpm) and respiratory gases were assessed using Vmax29-Sensor Medics (CareFusion, USA) gas analyzer, and CardioSys software, version 4.1 (Marquette Hellige GmbH, Germany) (Data are presented in Table 1).

Muscle RNA extraction

The muscle samples (50 mg) were manually homogenized in Eppendorf tubes with 0.5 ml of TRIzol reagent (T9424, Sigma-Aldrich, USA) and TRIzol-chlorophorm RNA extraction was performed according to the manufacturer's protocol. The total RNA was dissolved in 0.03 ml of RNAse-free water and concentrations were determined by NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, USA). RNA samples were stored at -80°C. One µg of RNA was used for cDNA synthesis in 0.02 ml of reaction mixture using the iScript™ Reverse Transcription kit (BioRad, USA) according to the manufacturer's protocol. Samples of 1 and 3 µL of this cDNA solution was used as a template for qRT-PCR and ddPCR analyses.

Reference Gene Establishment

To quantify BDNF mRNA expression in muscle we first established a gene of reference - which is expressed in a constant and stable rate both during homeostasis and stressed (e.g. exercise) conditions – i.e., housekeeping gene. To select the most applicable reference gene we used the muscle samples of three participants from the set, at both resting and post-exercise conditions, and performed a qRT-PCR analysis using the BioRad Prime PCR Pathway assay and Reference gene panel, and SsoAdvanced™ Universal SYBR® Green Supermix. Participant samples from both sexes with a maximal discrepancy in age were used for the reference gene establishment in order to address the highest heterogeneity.

The Reference gene panel included the known candidate genes frequently recommended for muscle tissue analysis [30], and the internal PCR performance, reverse transcription, RNA quality, and genomic DNA (gDNA) admixture controls. Fourteen eligible housekeeping genes were tested for comparison in qRT-PCR analysis: *ACTB, B2M, G6PD, GAPDH, GUSB, HMBS, HPRT1, PGK1, RPL13A, RPLPO, RPS18, TBP, TFRC, YWHAZ*. CFX Connect Real-Time PCR System analysis revealed that the *B2M* was the most stable gene enabled for further usage in ddPCR, and that *HPRT1* was the most stable gene during the conditions of our intervention (see Fig. 1). On the base of stability in gene candidates' expression, *B2M* was included into the qRT-PCR and ddPCR analysis of the *BDNF* as a reference gene.

qRT-PCR *BDNF* polymorphism specific assay

One µl of cDNA solution from the same participant's samples, excluding the female participant, were used in a qRT-PCR with allele-specific *BDNF* assay containing hydrolyzed probes (TaqMan) with both HEX (hexachloro-fluorescein) and FAM (6-carboxyfluorescein) systems of detection for the Val66- and Met66-coding *BDNF* allele's products, respectively (ddPCR Mutation Assay: rs6265, dHsaMDS320493890, BioRad, USA). The *BDNF* allele-specific primers were designed according to Sheikh and colleagues [31]. A HEX-based *B2M* Expression Probe Assay (qHsaCPE5053101, BioRad, USA) with the same SsoAdvanced Universal Probes Supermix (BioRad, USA) was ran in parallel and the melting curves were used as a product specificity control. To count the genomic DNA admixture contribution in the mRNA, samples of the participant's total RNA solution were used as a template in concentrations equalized to the residual in cDNA samples. All the reactions were performed in triplicate and the mean was used in subsequent calculations.

Unlike the absolute quantities achieved by ddPCR, reports of FAM and HEX probe detection corresponding to Met66- and Val66-coding BDNF mRNA levels from qRT-PCR could not be compared directly by threshold cycles (C(t)s), probably according to difference in FAM and HEX fluorescence detection sensitivity. Therefore, *BDNF* allele content in genome DNA admixture of RNA samples was used for the mathematical conversion of FAM to HEX detection, then Met66-coding allele's expression was standardized to HEX detection.

PCR program used was: 95°C - 10 min and 40 cycles of 94°C - 30 sec, 55°C - 60 sec with fluorescence detection. The C(t)s - number of cycles that takes to reach threshold - were determined on the HEX and FAM channels by CFX Connect Real-Time PCR System (BioRad, USA). *BDNF* expression levels were calculated by 'delta-delta C(t)' method [32]. BDNF C(t)s of each sample was corrected according to the contribution of gDNA admixture using C(t) meanings achieved for the corresponding RNA samples ($C(t)_{RNA}$) by the following formula:

$$C(t)_{correctBDNF} = C(t)_{BDNF} + \log_2((1 + 2^{(C(t)BDNF - C(t)RNA)}) / 1)$$

C(t) for samples where RNA did not give a registered signal were accepted without correction. Corrections of C(t) were performed for FAM and HEX detection independently.

Values of *BDNF* Val66- and Met66-coding alleles present in RNA samples (the alleles' ratio in genome DNA admixture = 1:1) were used as controls for equalizing HEX and FAM detection. The C(t)s of the Met-coding allele were unified to HEX detection afterward in the following formula:

$$C(t)unifMetBDNF = C(t)_{FAM}MetBDNF + (C(t)_{HEX}RNA - C(t)_{FAM}RNA)$$

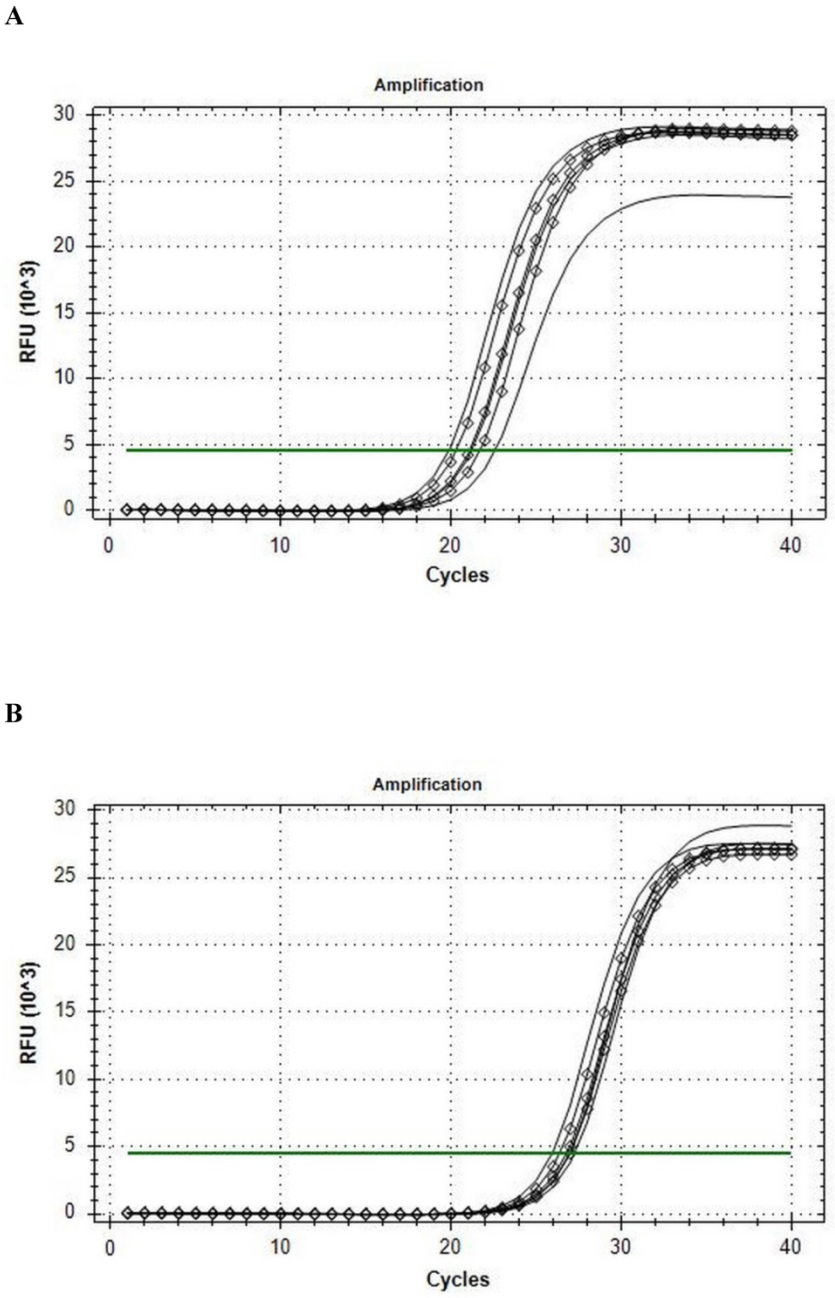

Figure 1. Amplification curves of in rest (line) and post-exercise (diamond) conditions. A, *B2M*; B, *HPRT1*.

Total *BDNF* expression in heterozygous subjects [C(t) - $C(t)_{sumBDNF}$] was calculated with the formula:

$$C(t)_{sumBDNF} = C(t)_{HEX}BDNF - \log_2((1 + 2^{(C(t)HEXBDNF - C(t)unifMetBDNF)}) / 1)$$

Statistics

Normality of values was determined by a Kolmogorov-Smirnov test. A paired Student's t-test was used to compare condition/changes description and Spearman's rank test for ddPCR to qRT-PCR correlation coefficient in SPSS. An α level of $p \leq 0.05$ was used to determine statistical significance. All data are reported as means ± SD.

Results

Among tested gene candidates as a reference the best stability in the metabolic stressed conditions in muscle was detected for *B2M* (less than 2.5 C(t) range inter- and intra-individually) (Fig. 1a) and *HPRT1* (less than 2 C(t) range inter- and intra-individually) (Fig. 1b) while the least stable for qRT-PCR analysis of stress conditions in the muscle was found to be *GAPDH,* (more than 4 C(t) range inter- and intra-individually). The other analyzed genes also showed low range of variability (less than 3.5 C(t) range inter- and intra-individually) and could possibly be used as a reference gene as well. The results of qRT-PCR Val66Met allele-specific *BDNF* expression analysis with the all mathematical proceeding can be observed in Table 2.

Real-time qPCR analysis detected the relative levels of *BDNF* expression with specificity for the Val66Met polymorphism, according to the absolute quantification performed by ddPCR (see Table 3). The *BDNF* expression showed small variability - often less than 1 C(t) in both the inter-individual differences (K-S(8) = 0.172, $p > 0.05$) and in regard to testing condition (Rest mean = 1.84 ± 0.79; Stress mean = 0.95 ± 0.34, $t(8) = 4.64$, $p = 0.002$) and genotype. Values detected by ddPCR were reported by qRT-PCR with a moderate negative correlation ($\rho = -0.456$), suggesting the higher BDNF mRNA copy number, the lower C(t).

Table 2. qRT-PCR BDNF analysis. Threshold cycles, C(t)s, calculations.

Genotype	Fluorescense detection	B2Mpre mean	B2Mpost mean	BDNFpre mean	BDNFpost mean	mRNApre mean	mRNApost mean	BDNFpre HEX/FAM correction	BDNFpost HEX/FAM correction	TotalBDNFpre expression in heterozygous	TotalBDNFpost expression in heterozygous
V/V	HEX	24.55	23.26	35.43	34.44	40.11	36.95	35.49	34.67		
	FAM	0	0	0	0	0	0	0	0		
V/V	HEX	21.34	22.46	32.33	34.10	38.22	41.00	32.35	34.11		
	FAM	0	0	0	0	0	0	0	0		
V/M	HEX	23.91	24.05	34.21	34.68	36.46	40.30	34.48	34.7	33.86	34.01
	FAM	0	0	29.98	30.83	31.50	35.78	35.37	35.39		
V/V	HEX	26.12	23.60	35.7	33.34	38.62	36.02	35.88	33.54		
	FAM	0	0	0	0	0	0	0	0		
V/M	HEX	23.92	23.8	34.42	34.98	36.30	36.56	34.76	35.39	33.96	34.66
	FAM	0	0	30.09	31.01	31.62	32.13	35.19	35.99		
V/V	HEX	23.21	23.17	32.29	33.37	35.29	35.63	32.45	33.64		
	FAM	0	0	0	0	0	0	0	0		
V/V	HEX	24.37	24.61	34.04	34.23	36.94	37.71	34.22	34.35		
	FAM	0	0	0	0	0	0	0	0		
V/V	HEX	24.6	22.95	33.19	32.19	36.65	36.65	33.32	32.25		
	FAM	0	0	0	0	0	0	0	0		

Pre, Analyses from the rest condition; **Post**, Analyses from after effort test

Table 3. qRT-PCR results validation by ddPCR. Condition- and allele-specific *BDNF* expression differences.

Genotype	**qRT-PCR**			**ddPCR**			**qRT-PCR**		**ddPCR**	
	Tpre BDNF-B2M	Tpost BDNF-B2M	T BDNF post /pre	Tpre BDNF /1000B2M	Tpost BDNF /1000B2M	T BDNF post /pre	Pre MetBDNF /ValBDNF	Post MetBDNF /ValBDNF	Pre MetBDNF /ValBDNF	Post MetBDNF /ValBDNF
V/V	10.94	11.41	0.721	1.444	0.703	0.487				
V/V	11.01	11.66	0.641	1.792	1.17	0.653				
V/M	9.950	9.960	0.996	1.427	0.926	0.649	0.541	0.621	0.707	0.614
V/V	9.760	9.950	0.877	0.793	0.498	0.628				
V/M	10.04	10.86	0.566	1.524	0.910	0.598	0.741	0.662	0.660	0.968
V/V	9.250	10.48	0.427	2.319	0.745	0.321				
V/V	9.850	9.740	1.077	2.045	1.110	0.543				
V/V	8.720	9.300	0.665	3.460	1.607	0.464				
Means ± SD	T BDNF post/pre, 0.736 ± 0.205			T BDNF post/pre, 0.543 ± 0.115			MetBDNF/ValBDNF, 0.641 ± 0.084		MetBDNF/ValBDNF, 0.737 ± 0.158	

Pre, Analyses from the rest condition; **Post**, Analyses from exercise protocol; Tpre and Tpost – total (both alleles) *BDNF* expression, ValBDNF, MetBDNF – allele-specific *BDNF* expression.

Discussion

BDNF and its gene polymorphism Val66Met have taken on a greater focus of attention regarding its crucial role in neural system development and functioning. Whereas alteration in BDNF production has been suggested to be a marker of neuropathological conditions [3,11,19,33]. Investigations examining the effect of exercise on changes in circulating BDNF concentrations have gained much interest as a possible target for the therapy and/or prevention of stress and neurodegeneration-related disorders [22,34,35]. Therefore, a precise and accessible method proposed for the laboratory analysis of the allele-specific expression of *BDNF* is a valuable tool for a broader assessment of the *BDNF* Val66Met polymorphism in a clinical and research scenario. By assessing relatively small, but detectable by absolute quantification changes in mRNA levels, we were able to design a protocol for the identification of *BDNF* expression in human tissue regarding to the allele specificity and the metabolic condition by qRT-PCR.

Our results showed that amongst the genes expressing constant patterns (stability) in the human muscle regardless of sex and physiological conditions, the most stable one is the gene of hypoxanthine phosphoribosyl transferase (*HPRT1*). The establishment of a tissue-specific reference gene can help discriminate both distinctive characteristics, and genetic-based changes in cell functioning among different physiological conditions [36,37,38,39;40]. Precision in the determination of a gene of reference for comparative analysis of the expression of specific proteins is crucial to enable the assessment of factors that are expressed in low quantities in tissue. These results contribute to the establishment of the *HPRT1* as the most stable gene expressed in muscle tissue, and can be used as reference for comparative analyses of gene expression in humans, regardless of sex and metabolic conditions.

Furthermore, our results indicate that the *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase) gene shows unacceptable parameters (the lowest among the genes tested) as a reference gene for comparisons in different metabolic conditions. Although initially considered a housekeeping gene and widely used as an internal control in experiments on proteins, mRNA, and DNA [41], recent evidence has shown that *GAPDH*, besides having a role in energy metabolism, participates is a more complex regulatory mechanism [42]. Nonetheless, the changes in expression patterns of this gene noted during the exercise protocol suggest that *GAPDH* should not be considered as a reference gene when testing different metabolic conditions.

Using the absolute quantification of ddPCR analysis as a reference, we have demonstrated that the identification of the allele-specific expression of *BDNF* by qRT-PCR is feasible. The differential detection of *BDNF* alleles' expression enables the objective analysis of the Val66Met polymorphism role on BDNF functioning, supporting the previous speculations [43,44,45]. Moreover, this methodology has proven to be applicable even in small samples, in spite of the small variability showed for the human *BDNF* expression. The qRT-PCR analysis was able to report the results of the ddPCR analysis of *BDNF* expression both in allele- and condition-specific manner (Table 3).

In conclusion, our results indicated that the qRT-PCR protocol was successful in determining the measurement of *BDNF* expression levels in muscle tissue, as well as metabolic stress-delivered and genotype-associated changes in these levels. This protocol appears to be useful for the detection of the Val66Met polymorphism specific *BDNF* expression and can be used in heterogeneous populations.

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Author Contribution

Conceptualization, G.G.A. and E.V.G.; Sample Collection and Analysis, G.G.A. and E.V.G.; Resources, E.V.G.; Data Curation, E.V.G.; Writing, G.G.A., J.R.H. and E.V.G.; Project Administration, G.G.A., Funding, G.G.A. and E.V.G.

Competing Interests

The authors have declared that no competing interest exists.

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