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Original research

High-intensity interval training increasing ADP-ribosylation factor 6 and Cytochrome C in visceral adipose tissue of male Wistar rats

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Background: High-intensity interval training (HIIT) can be related to the beneficial adaptations in adipose tissue even though its underlying mechanisms have not yet been clarified. Considering the significant effect of ADP-ribosylation factor 6 (ARF6) and Cytochrome C (CytC, as a marker of mitochondrial content) on adipose tissue plasticity, it was proposed that the positive impacts of high-intensity interval training (HIIT) on visceral adipose tissue (VAT) might be mediated by ADP-ribosylation factor 6 (AEF6) and Cytochrome C (CytC). To examine the research hypothesis, the effect of 6-week high-intensity interval training (HIIT) on ADP-ribosylation factor 6 (ARF6) and Cytochrome C (CytC) protein levels in visceral adipose tissue (VAT) of male Wistar rats was evaluated.

Methods: A total number of 12 male Wistar rats were randomly divided into two groups: control (C) and trained (high-intensity interval training: HIIT) wherein the trained group was exposed to 6 weeks of high-intensity interval running. The ADP-ribosylation factor 6 (ARF6) and Cytochrome C (CytC) protein expressions were also assessed by immunohistochemistry (IHC) analyses. Moreover, independent sample *t*-test was used to compare between-group differences and the significance level was determined by $p \leq 0.05$.

Results: The immunohistochemistry (IHC) analyses showed that protein levels of ADP-ribosylation factor 6 (ARF6) and Cytochrome C (CytC) were significantly higher in the group receiving high-intensity interval training compared with the control group after 6 week ($p = 0.018$ & $p = 0.0001$, respectively).

Conclusions: The increased levels of ADP-ribosylation factor 6 (ARF6) and Cytochrome C proteins due to high-intensity interval training could be related to improved metabolism and glucose homeostasis. It was also suggested to take the physiological consequences of this adaptation into account in future studies.

1. Introduction

Visceral adipose tissue (VAT), considered as the main energy deposits such as triglycerides, consists of adipocytes, connective tissues, nerves, vessels, and immune cells which can be involved in various biological processes such as energy metabolism, neuroendocrine, and immune function. Hence, the VAT is regarded as one of the decisive factors in terms of health status and disease conditions (Shuster et al., 2012). Moreover, it is well known that exercise training (ET) can induce profound physiological adaptations in several tissues including adipose one. Through improvements in glucose tolerance, insulin sensitivity, and lowering of circulating lipid concentrations (Stanford and Goodyear, 2016), as well as increasing adipose tissue lipolysis and free fatty acid mobilization (Cohen et al., 2014); the ET can also have beneficial effects on metabolic health. In this respect, several studies found that high-intensity interval training (HIIT), an exercise training program comprised of brief bouts of intense exercise (90–100% of VO_2 max) followed by periods of recovery, could elicit similar metabolic adaptations compared with classical endurance exercise training but with a much shorter time commitment (Herodek et al., 2014). The HIIT was similarly found to improve exercise capacity and whole-body glucose homeostasis by enhancing liver and adipose tissue insulin sensitivity whose effects were independent of reductions in adiposity/adipose tissue cell size (Marcinko et al., 2015). However, the underlying mechanisms of adipose tissue remodeling effects of the HIIT have not yet been defined despite the given positive metabolic impacts.

The ADP-ribosylation factor 6 (ARF6), as a member of the family of Ras-related proteins, is the trimeric G-proteins (GTP binding proteins) which may have important effects on VAT so that ARF6 depletion in 3T3-L1 adipocytes can result in decreased lipolysis (Liu et al., 2010).

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Also, it has been demonstrated that ARF6 levels can be different between obese or obesity-resistant adipose tissues in mice (Liu et al., 2010). In addition, ARF6 is required for endothelial cell migration (Daher et al., 2008) and it has been associated with insulin resistance (Jayaram et al., 2011), insulin signaling (Hafner et al., 2006), and glucose transporter type 4 (GLUT-4) recycling to the plasma membrane (Li et al., 2012).

ARF6 activity regulates by diverse guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs); however it's underlying regulatory mechanisms have not been investigated before. In our pervious study, we observed that cerebellar Arf6 gene expression increased in diabetic rats and ET as treadmill running has decreased this elevation (Taherabadi et al., 2019). In addition, a microarray data revealed that ET in mice results in profound altering a vast number of genes in adipose tissue, involved in many cellular functions such as metabolism, mitochondrial biogenesis, oxidant stress and signaling, membrane transport, cell stress, proteolysis, apoptosis, and replication (Stanford et al., 2015). These findings led us to suppose that exercise training in the form of HIIT could effect on several regulatory proteins in VAT such as ARF6. It is well established that ET can induce mitochondrial biogenesis in adipose tissue so that chronic swimming in rodents has been reported to significantly increase the activity of the respiratory chain enzyme cytochrome *c* oxidase and tricarboxylic acid cycle enzyme malate dehydrogenase in VAT (Stallknecht et al., 1991). In addition, the mRNA/protein levels of key transcriptional regulators of mitochondrial biogenesis and mitochondrial DNA content have been found to significantly rise in subcutaneous adipose tissue after a swimming training period (Trevellin et al., 2014). These findings demonstrated that ET could have marked effects on mitochondrial gene expression and activity in adipose tissue. Considering the importance of ARF6 and mitochondrial biogenesis in adipose tissue, it was proposed that the beneficial effects of ET on adipose tissue might be mediated by these proteins. However, the correlation between ARF6 and mitochondrial is not clear, but certain studies have demonstrated that ARF6 is related to mitochondrial through aerobic glycolysis (Liang et al., 2017) and mitochondrial positioning (Onodera et al., 2018). Moreover, mounting evidence indicate that silencing ARF6 expression is associated with decreased mRNA levels of glycolytic genes (glucose transporter 1, (hexokinase 2 and Lactate dehydrogenase A) expression and glucose mitochondrial oxidation in PANC-1 and MiaPaCa-2 cells which suggesting ARF6 is involved in mitochondrial respiration (Liang et al., 2017).In addition Mitochondrial positioning which is controlled by ARF6–AMAP1 pathway is crucial for numerous cellular functions so that blockade of the ARF6–AMAP1 increases the intracellular reactive oxygen species (ROS) levels and induced mitochondrial aggregation (Onodera et al., 2018). Moreover, ARF6-Cyt interaction may play a major role in restructuring lipid bilayers and in preparation for events that require remodeling of biological membranes (Taherabadi et al., 2019). This findings demonstrated that there is strong relation between ARF6, CytC and mitochondria. The aim of the present study was to examine the effect of 6-week HIIT on ARF6 and Cytochrome C (CytC, as a marker of mitochondrial content) protein levels in the VAT of male Wistar rats was evaluated.

2. Methods

2.1. Study design and animals

A total number of 12 adult male Wistar rats were supplied from Razi Institute (Karaj, Iran) and housed four-per-cage in an animal lab under standard conditions (12-h light/dark cycle in a room at a temperature of 20–25 °C) with access to food and water ad libitum. As well, the animals were randomly divided into two groups: (1) healthy control (C, N = 6), 2) and trained (HIIT, N = 6).

2.2. Treadmill training protocol

For further familiarization, the animals of the HIIT group were exercised on the treadmill (Model T510E, Diagnostic and Research, Taoyuan, Taiwan) for 5 sessions per week wherein each session consisted of 10 min of running at the velocity of 10 m/min with 0° incline (Rahmati et al., 2015). Also, the HIIT program included 10 interval running parts for 4 min at the intensity of 85–90% VO2max which was dispersed by 2 min of active rest (5 days per week lasting 6 weeks). It should be noted that the treadmill running speed was gradually increased from 16 to 26 m/min over 6 weeks. In addition, 2–3 min and 3 min were allocated to each training session to warm up and cool down; respectively (Hafstad et al., 2011).

2.3. Tissue extraction

Two days after the last exercise session in the 6th week of the training, the rats (N = 6 for each group) were anesthetized by inhalation of 2% halothane in a mixture of 20% O_2 and 80% CO_2 (Kohler et al., 1999). For the purpose of immunohistochemistry (IHC) analyses, the rats' VAT were also removed immediately and then fixed in the 10 v/v% buffered formalin.

2.4. Immunohistochemical analysis

The VAT was sliced into 50 μm-thick sections which then underwent the following procedure: dehydration through washing with alcohol, clearing, embedding, deparaffinization, rehydration, and perfusion in water. Then, the tissue was washed with sodium phosphate-buffered saline (PBS at pH 7.4). For the purpose of antigen retrieval, the sections were incubated with hydrochloric acid (HCL) solution (2 N in distilled water, pH 0.6–0.9) for 30 min and borate buffer was added for 5 min to neutralize the acid. After that, the given samples were washed with PBS plus 0.3% Triton X-100 under gentle agitation. The samples were subsequently rewashed with PBS and pre-blocked with normal 10% goat serum added to the background of the sections. Moreover, primary antibodies were added overnight at the dilutions of 1:100 with PBS for anti-CytC and anti-ARF6 (ab13575 and ab108347 respectively; rats polyclonal, Abcam-United states) and the samples were placed in a refrigerator at 2–8 °C for 24 h. Subsequently, the sections were eluted with PBS for four times lasting 5 min. Afterwards, green-conjugated secondary antibodies (goat anti-mouse; Molecular Probes) were added at 1:200 dilution and the samples were incubated at 37 °C for 1.5 h in darkness. Thereafter, the given samples were moved to a dark room and eluted with PBS for four times. Then, the samples were introduced by Propidium Iodide and perfused with PBS once more. Finally; an Olympus CX21 microscope (Olympus Co. Ltd., Tokyo, Japan) was used for the sake of spectroscopy. ImageJ software (1.47v) was also employed for image quantitation.

2.5. Statistical analysis

The SPSS Statistics (Version 22, SPSS Inc., Chicago, IL, USA) was used for statistical analyses. To this end, Shapiro-Wilk test and Levene's test were employed to verify data normality and homogeneity, respectively. Independent-sample *t*-test was also used to compare between-group differences. Moreover, all the data in the Figures and Diagrams were presented as mean ± standard error of the mean (SEM). The significance level was also determined by $p \leq 0.05$.

3. Results

It was observed that all the rats in the trained group could successfully perform the protocols of 6 weeks of the HIIT program. The HIIT consisted of repetitive and interval bouts with maximum or near-maximum intensity which was followed by short rest or low-activity

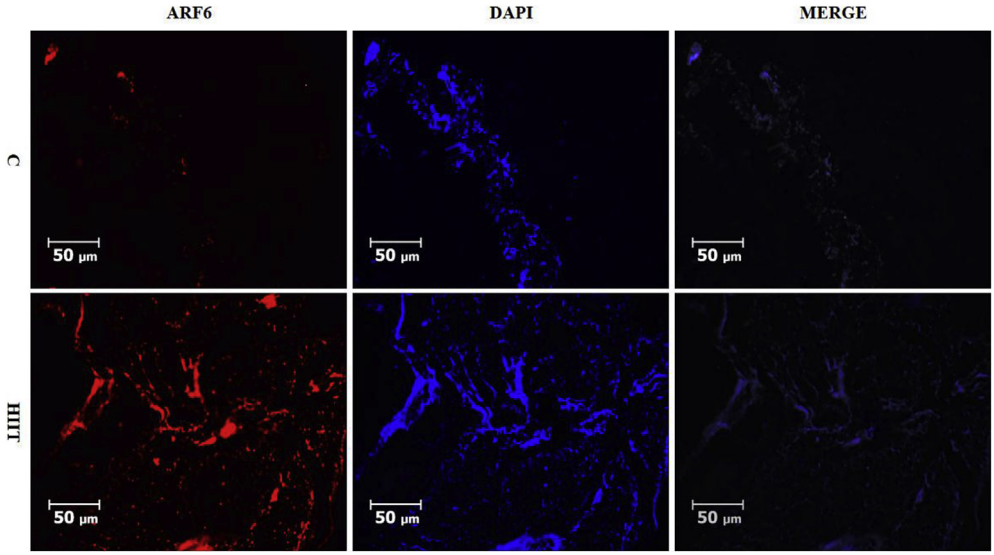

Fig. 1. Fluorescence immunohistochemistry of the ARF6 in the VAT in the C and the HIIT groups. Anti-ARF6; red, with all nuclei which were stained with DAPI (blue), scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

periods. Furthermore, these exercises could be divided into two categories based on the time of exercise bouts in which the HIIT between one to six minutes was considered as long bouts of the HIIT such as the protocol used in the present study.

To investigate the possible effects of ET in the form of the HIIT on CytC and ARF6 protein levels in the rats' VAT, immunohistochemical technique was used (Figs. 1 and 2). After determining the quantification of the images (Table 1), independent-sample *t*-test was employed for comparing the difference of the CytC and ARF6 proteins levels between the C and the HIIT groups. The findings revealed that the animals exposed to the HIIT had elevated protein levels of ARF6 compared with the sedentary rats ($p = 0.018$) (Fig. 3). It should be noted that the ARF6 is considered as a small GTPase in adipocytes that regulates lipolysis and GLUT4 and its elevation on the VAT may be associated with the enhancement of lipolysis and GLUT4.

To evaluate mitochondrial biogenesis (implied by the increase in CytC), the CytC protein levels in the VAT were examined (Fig. 2) and it was observed that the CytC had significantly increased compared with that in the C group after a HIIT program ($p = 0.0001$) (Fig. 3). It should be noted that the CytC is a water-soluble 13 kDa haem-containing protein that normally resides in the spaces within the cristae of the inner mitochondrial membrane (IMM) and its elevation can be associated with increased oxygen consumption and energy expenditure. In addition, the CytC can play a prominent role in apoptosis in other tissues so it can trigger programmed cell death as it is released into the cytosol (Ow et al., 2008). Totally, these data showed that the HIIT as a

Table 1
Quantification results of immunohistochemistry images from the ARF6 and the TMOD2 in the VAT in the C and HIIT groups. C: control, HIIT: trained, SD: standard deviation, SE: standard error.

Groups	*ARF6*		*CytC*	
	C	HIIT	C	HIIT
Mean (μm^2)	653.665	4286.291	301.030	8153.112
SD	273.415	2572.154	156.669	1253.517
SE	11.623	1050.077	63.959	511.746

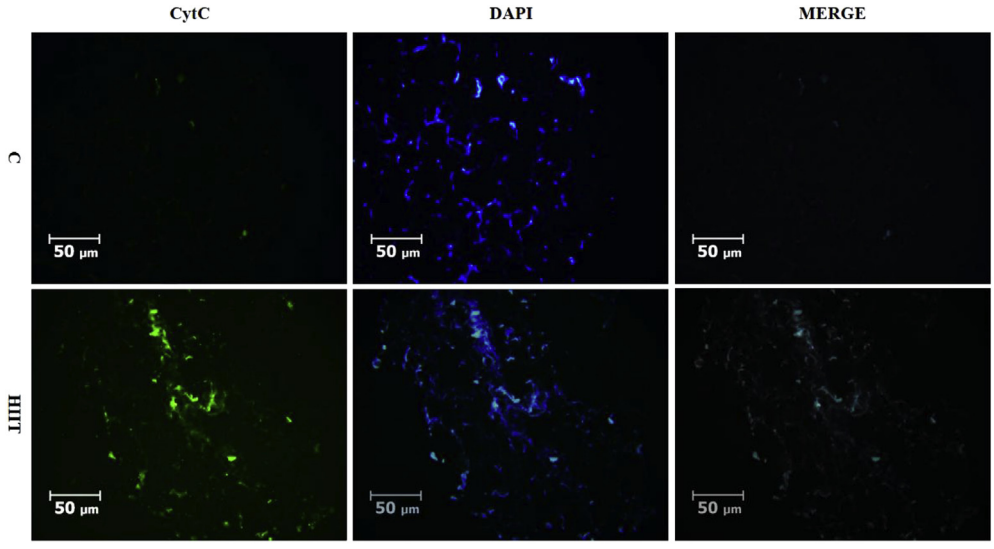

Fig. 2. Fluorescence immunohistochemistry of the CytC in the VAT in the C and HIIT groups. Anti-CytC; green, with all nuclei which were stained with DAPI (blue), scale bar = 50 μm. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

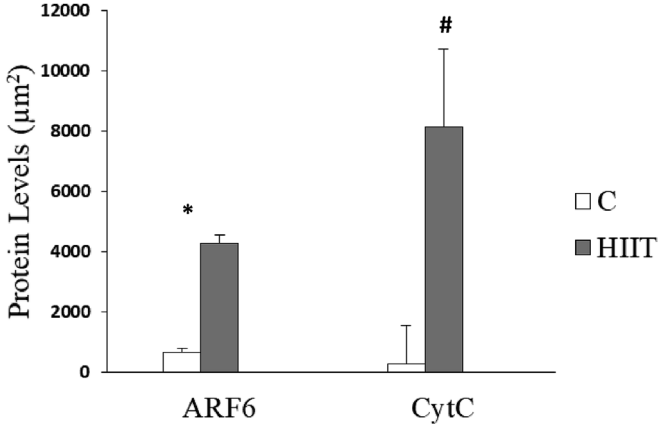

Fig. 3. Independent-sample *t*-test results of the VAT ARF6 and CytC protein levels in the C and the HIIT groups. It was observed that ARF6 and CytC protein levels in the HIIT group had significantly increased in the VAT of the male Wistar rats compared to those in the C group ($p = 0.018^*$ and $p = 0.0001$; respectively). Six rats in each group were used for this analysis.

chronic and intensive ET could have a significant effect on protein expression of the CytC and the ARF6.

4. Discussion

In the present study, the effect of ET as HIIT was investigated on the ARF6 and CytC protein levels in the VAT of male Wistar rats for the first time and it was observed that these protein levels had increased by the HIIT. Although the exact role of the ARF6 in the white adipose tissue (WAT) was not well known, there was some evidence demonstrating that the ARF6 involved glucose homeostasis through regulating GLUT4[6] and also inducing lipolysis via adrenergic stimulation in the adipose tissue (Davies et al., 2014). The WAT also contained 10% of glucose transporter (GLUT4) whose impairment could lead to glucose intolerance (Graham et al., 2006). On the other hand, it was shown that the ET was associated with the enhancement of the GLUT4 levels in the WAT and the reduction of insulin resistance (Stanford et al., 2015). The ET had been also reported to increase the expression of the GLUT4 in the WAT of type-2 diabetic patients (Hussey et al., 2011). However, Hirshman et al. (1993) showed that the GLUT4 levels had not changed in the visceral and subcutaneous WAT after exposure to the ET (Gollisch et al., 2009). In another study, the GLUT4 levels in the plasma membranes in a group of trained mice had significantly increased through insulin stimulation (Hirshman et al., 1993). Besides, Craig et al. (1981) reported that adipocytes of trained rats had higher insulin receptors than non-exercising mice. Therefore, in spite of no changes in the GLUT4 protein levels, the ET could apparently increase the glucose uptake capacity via insulin stimulation (Craig et al., 1981). In line with these observations, it seemed that the increased levels of the ARF6 in the VAT could be a mechanism of increasing glucose uptake by the WAT. The elevated expression of the GLUT4 due to the ET might be also associated with increased triglyceride (TG) synthesis in the WAT. In fact, over-expression of the GLUT4 in the WAT could result in a rise in the TG synthesis capacity through re-esterification and denovo lipogenic pathway, which ultimately led to the accumulation of total fat mass in sedentary animals (Stephenson et al., 2013).

The CytC protein levels were also examined as a marker of mitochondrial biogenesis in the VAT and it was observed that these protein levels had significantly increased following the HIIT. Moreover, microarray findings showed that the ET had a significant effect on the WAT genes involved in metabolism, mitochondrial biogenesis, oxidative stress and signaling, membrane trafficking, cellular stress, proteolysis, apoptosis, as well as proliferation and these changes could exhibit a high degree of plasticity in the WAT (Qian et al., 2013). Trevellin et al. (2014) similarly demonstrated that ET had enhanced the expression of mitochondrial biogenesis transcriptional regulators along with mitochondrial DNA content (Trevellin et al., 2014). Sutherland et al. (2009) correspondingly reported that ET had increased the expression of the PGC-1α, Tfam, COX IV, and the citrate synthase activity in the VAT which could further support the claim that ET was an effective stimulus for mitochondrial biogenesis (Sutherland et al., 2009). However, in a human study, Camera et al. (2010) showed that 10-day endurance training in untrained men had no effect on the citrate synthase activity, mitochondrial mass, or the expression of genes predicting enhancement of oxidative capacity in the WAT whose lack of efficacy was probably due to the short training course (Camera et al., 2010). During the ET, the sympathetic stimulation could also cause the activation of the PGC-1α in various tissues including the WAT which ultimately led to mitochondrial biogenesis. It has been suggested that the PGC-1α is only responsible for 40% of this adaptation and the rest is due to hormones (thyroid and glucocorticoid), myokines, as well as other unknown factors. Furthermore, an important part of mitochondrial biogenesis in the WAT can be mediated through the endothelial nitric oxide synthase (Trevellin et al., 2014).

Mitochondrial biogenesis development of the beige phenotype in the WAT whose change can be associated with thermogenesis can also increase energy consumption and insulin sensitivity (Giralt and Villarroya, 2013). Specifically, it has been demonstrated that sedentary mice receiving subcutaneous WAT from the ET donor mice had improved glucose tolerance and enhanced insulin sensitivity compared with those transplanted with subcutaneous WAT from sedentary or sham-treated mice (Stanford et al., 2015). These findings suggested that ET adaptation of the WAT could have beneficial effects on whole-body metabolism and this improvement was independent of the effect of ET on other tissues.

Regarding the relationship between mitochondria and ARF6, it can assumed that the increased levels of these two proteins are related to enhanced aerobic glycolysis (Liang et al., 2017) and quenching reactive oxygen species (ROS) (Onodera et al., 2018). Unfortunately, in the present study the amount of free radicals and aerobic glycolysis has not been measured and also there is no finding to prove this claim. However in this regard, Alkahtani et al. (2013) shown that 12 sessions of HIIT performed in four weeks resulted in significant improvements of fat oxidation, mitochondrial oxidative capacity and reduced lactate accumulation in adipose tissue (Alkahtani et al., 2013). Also Sakurai et al. (2017) reported that one of the unique effects of exercise training is to decrease oxidative stress in adipose tissue (Sakurai et al., 2017). Clarifying the relation between ARF6 with aerobic glycolysis and ROS production in adipose tissue need to be explored in future research.

Although the underlying mechanisms for HIIT inducing elevated ARF6 and mitochondria expression in VAT isn't investigated, it was reported that ET through various ways cause begging of WAT. The increased density of noradrenergic fibers, factors produced by skeletal muscles and adipose tissue such as irisin, Meteorin-like factor, PGC-1α -dependent myokine and UCP1 are the major possible mechanisms of ET-induced WAT plasticity (Tsiloulis and Watt, 2015). The mechanism examination of HIIT increased ARF6 and mitochondria expression in adipose tissue is an interesting topic in future studies.

Overall, ET could induce several adaptations in the VAT such as increased mitochondrial content leading to improved metabolic homeostasis while the decreased mitochondrial activity or mass could be related to cellular dysfunction and cause several diseases (Petersen et al., 2003). For example, mitochondrial dysfunction or decreased number and activity of mitochondria was associated with the pathogenesis of type-2 diabetes, obesity, and insulin resistance (Goodpaster, 2013).

5. Conclusion

In the present study, it was observed that the ET as HIIT was

associated with increased levels of the ARF6 and the CytC proteins in the VAT in male Wistar rats. The augmented levels of these two proteins due to the HIIT might be also related to improved metabolism and glucose homeostasis. However, it was suggested to shed light on the physiological consequences this adaptation in future studies.

Authors' contributions

Mohammad Shariatzadeh 2, Abdolreza Kazemi 3, Seyed Jalal Taherabadi 1.

Masoud Rahmati designed and conducted the study, analyzed the data, and drafted the manuscript; Mohammad Shariatzadeh contributed to the study design, data analysis, and manuscript drafting; Abdolreza Kazemi helped in data analysis and critically reviewed the manuscript; Seyed Jalal Taherabadi contributed in data collection and data analysis. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.obmed.2019.100089.

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