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Short communication

Melatonin and cortisol as components of the cutaneous stress response system in fish: Response to oxidative stress

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ABSTRACT

The skin being a passive biological barrier that defends the organism against harmful external factors is also a site of action of the system responding to stress. It appears that melatonin (Mel) and its biologically active metabolite AFMK (N1-acetyl-N2-formyl-5-methoxykynuramine), both known as effective antioxidants, together with cortisol, set up a local (cutaneous) stress response system (CSRS) of fish, similar to that of mammals. Herein we comment on recent studies on CSRS in fish and show the response of three-spined stickleback skin to oxidative stress induced by potassium dichromate. Our study indicates that exposure of the three-spined stickleback to $K_2Cr_2O_7$ affects Mel and cortisol levels and pigment dispersion in melanophores in the skin. In our opinion, an increased concentration of Mel and cortisol in the skin may be the strategy to cope with oxidative stress, where both components act locally to prevent damage caused by active oxygen molecules. Furthermore, the pigment dispersion may be a valuable, easy-to-observe mark of oxidative stress, useful in the evaluation of fish welfare.

The skin, the largest body organ directly exposed to multiple environmental stressors, is well recognised as a passive biological barrier that protects the organism against harmful external factors, but rarely as a site of production of biologically active compounds that act at the skin level to maintain homeostasis. Indeed, Slominski et al. (1995) proposed that the mammalian skin has a system responding to stress equivalent to the hypothalamic-pituitary-adrenal axis (HPA). They were also the first to provide evidence that hormones related to HPA (corticotropin-releasing hormone, adrenocorticotropic hormone and cortisol) and effective antioxidants such as melatonin (Mel; *N*-acetyl-5methoxy-tryptamine) and its biologically active metabolites, kuramines, respond to local destructive and pathological factors in the skin, partly independent of their systemic action (for a review, see: Slominski et al., 2008, 2017a). It was not a coincidence, but medical reasons for studying the mammalian skin first. Next came the time for fish, in which one might expect to find a similar mechanism. Indeed, fish skin is directly exposed to many compounds in surrounding water with a potential or confirmed detrimental effect on the organism. In our previous articles, we postulated that Mel and N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), both known as effective antioxidants, and cortisol, a glucocorticoid involved in the stress response in vertebrates, establish a local system of response to stress in the skin of fish (cutaneous stress response system; CSRS) (Kulczykowska et al., 2018; Kulczykowska, 2019). The skin, with its active compounds that respond to cortisol administration, mimicking stress conditions, drew our attention as a site of the interplay of many factors to maintain homeostasis in the body. Here, we comment on recent studies on CSRS in fish and show the response of three-spined stickleback skin to oxidative stress induced by potassium dichromate.

It is worth highlighting that the development of our knowledge on Mel originates from observations of the skin made by Carey P. McCord and Floyd P. Allen as early as 1917 (McCord and Allen, 1917). They found lightening of the skin of frogs and tadpoles after administration of bovine pineal gland extract. But the active component that caused skin lightening was isolated by Aaron B. Lerner and his colleagues only in the late 1950s (Lerner et al., 1958) and was named (melatonin) based on its ability to reverse the skin-darkening effects of the melanocyte-stimulating hormone. In 1968, Reed provided indirect evidence that Mel controls a circadian pigment rhythm in the pencil fish *Nannostomus beckfordi anomalus* (Reed, 1968). Unfortunately, this action of Mel in the skin almost disappeared from present knowledge, drowning in a flood of research on countless Mel functions. While Mel is primarily known as the regulator of the circadian clock and annual calendar in vertebrates (Reiter, 1993; Reiter et al., 2010; Sokołowska et al., 2004), it is also an effective antioxidant involved in reactive oxygen species (ROS) trapping and metal neutralization by its chelation as well as activation of antioxidant enzymes and inhibition of pro-oxidative enzymes (Dzięgiel

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et al., 2003; Fischer et al., 2013; Janjetovic et al., 2017; Rodriguez et al., 2004; Romero et al., 2014; Tan et al., 1999), hence it protects the organism from oxidative damage (for a review, see: García et al., 2014; Reiter et al., 2000). The mechanism of Mel action depends on the nature of the oxidative stress inducer (Galano and Reiter, 2018). The main product of the hormone reaction with the hydroxyl radical, one of the most damaging oxidants formed through metal-catalyzed Haber-Weiss-like reactions, is cyclic 3-hydroxymelatonin (Tan et al., 1993) that is transformed to AFMK (Zhang and Zhang, 2014). AFMK itself as a strong antioxidant quickly undergoes free radical reactions, including with the hydroxyl radical (Galano et al., 2013; Manda et al., 2007; Tan et al., 2001). The main product of the reaction, AMK (*N*-acetyl-5-methoxykinuramine), is also a potent scavenger of reactive oxygen and nitrogen species, even stronger than Mel and AFMK (Galano et al., 2013; Galano and Reiter, 2018). Furthermore, AFMK and AMK have been shown to activate antioxidant enzymes and inhibit pro-oxidative and pro-inflammatory enzymes in human melanocytes (Janjetovic et al., 2017). Therefore, in the inactivation of free radicals in mammalian cells, the antioxidant cascade Mel → AFMK → AMK is activated. However, there is an imbalance between research in mammals and fish. In fish, Mel's antioxidant activity is shown only in the regulatory process of oocyte maturation and sperm protection (Maitra and Hasan, 2016; Félix et al., 2021), and AFMK's antioxidant activity has not yet been studied. Therefore, further investigations are highly required in fish, including the skin of the fish.

The question of the synthesis of Mel and AFMK in fish skin cells arises. Mel is synthesized from serotonin by consecutive action of enzymes: serotonin *N*-acetyltransferase (AANAT) and acetylserotonin *O*-methyltransferase (ASMT) mainly at two sites: the pineal organ and the retina, primary in darkness (Benyassi et al., 2000; Falcón et al., 2010, 2011) and transported to various parts of the body with circulation. However, it is also produced locally in different organs/tissues (for review: Acuña-Castroviejo et al., 2014). We have evidence of Mel biosynthesis in the skin of several species of fish: rainbow trout (*Oncorhynchus mykiss*) (Fernández-Durán et al., 2007), three-spined stickleback (*Gasterosteus aculeatus)* (Kulczykowska et al., 2017; Pomianowski et al., 2020) and European flounder (*Platichthys flesus*) (Pomianowski et al., 2021). Indeed, a relative expression of the gene encoding AANAT2 is present in the skin of rainbow trout (*O. mykiss*) (Fernández-Durán et al., 2007), and *aanat*s and *asmt*s and *asmt* alone transcripts are reported in the skin of three-spined stickleback (Kulczykowska et al., 2017; Pomianowski et al., 2020), European flounder (Pomianowski et al., 2021), mudskippers (*Boleophthalmus pectinirosris*, *Periophthalmus magnuspinnatus*) (Zhang et al., 2017) and *Sinocyclochelius* fish (*S. graham, Sinocyclocheilus rhinocerous, S. anshuiensis*) (Zhang et al., 2017). Furthermore, higher levels of Mel in the skin than those in the plasma of round goby (*Neogobius melanostomus*), common carp (*Cyprinus carpio*) and Atlantic cod (*Gadus morhua*) strongly suggest Mel synthesis in the skin (Kulczykowska et al., 2018). Stimulation of Mel synthesis in organs exposed directly to environmental pollutants, such as the skin, has been considered a protective action of the organism by Hardeland (2005) and Tan et al. (2007). It is also an example of how toxic compounds in the diet affect Mel concentration in the gut, the second organ exposed directly to environmental pollutants, in white stork nestlings (*Ciconia ciconia*) living near a copper smelter (Kulczykowska et al., 2007).

In different species of fish, various measures of oxidative stress are applied (Birnie-Gauvin et al., 2017). Cortisol plays a role in helping the body respond to stress, and plasma cortisol level is commonly used as an indicator of response to different types of stress in fish (for a review, see: Ellis et al., 2012). Cortisol measurement can provide information on animal well-being in many situations, but some environmental conditions may not lead to a change in cortisol concentration (MacDougall-Shackleton et al., 2019). Furthermore, there are many controversies about the value of applying cortisol or other glucocorticoids to the evaluation of animal well-being (Breuner et al., 2013; Ellis et al., 2012; Romero and Gormally, 2019). It is also critical to study such parameters that may have the most diagnostic value for a given species in a given stress condition. In this study, we examine the reaction of CSRS, where cortisol, Mel and AFMK are important components, to oxidative stress. Due to a relationship between dose and duration of exposure to $K_2Cr_2O_7$ and the level of cortisol in plasma that is apparent in fish (Ko et al., 2019), we have decided to use potassium dichromate as an external factor that induces oxidative stress. Chromium compounds, including potassium dichromate, are highly toxic anthropogenic water pollutants that evoke oxidative stress in aquatic organisms, including fish. Hexavalent chromium compounds readily cross cell membranes and are reduced to a trivalent form in cells. It results in the formation of reactive oxygen species (ROS) and cellular and tissue damage (Saha et al., 2011; Velma et al., 2009). We expected that the levels of antioxidants, Mel and AFMK, and a glucocorticoid, cortisol, in the skin would increase even after a short exposure of fish to such a harmful oxidant. In the study, we have analyzed not only Mel, AFMK, and cortisol, but also melanosome dispersion and direct markers of oxidative stress and oxidative defense, such as reactive substances of thiobarbituric acid (TBARS) and total antioxidant capacity (TAC) in the skin after a short exposure of fish to potassium dichromate ($K_2Cr_2O_7$) in the laboratory.

We raise two questions:

- How do CSRS components react to external oxidative stress?
- What do we learn about fish well-being by studying the skin?

We have carried out the experiments in the three-spined stickleback, which inhabits fresh, brackish, or salt waters in the northern hemisphere and is an indicator species used to monitor, for example, the biological effects of chemical contamination (Von Hippel et al., 2016) and endocrine disruption (Katsiadaki et al., 2012). The three-spined stickleback has also been a model fish in our laboratory for a long time.

Adult three-spined sticklebacks of both sexes ($n = 35$, total weight 0.95–1.95 g and length 54–72 mm) were caught in the Gulf of Gdańsk (southern Baltic Sea) out of the breeding season and transported to the Institute of Oceanology PAS (Sopot, Poland). The fish were acclimatized in 40-L aerated aquaria with brackish water (7 ppt) at a temperature of 10 ± 2 °C and 12 L:12D photoperiod for two weeks and fed frozen food (*Chironomus plumosus*) once a day at 15:00. The fish were randomly assigned to two groups: the control was kept in water without $K_2Cr_2O_7$ (15 individuals) and the experimental group in water with $K_2Cr_2O_7$: 50 mg Cr/1 L for 6 h (20 individuals). The concentration of chromium in water was expressed as a Cr^{6+} ion in milligrams per liter of water. After the experiment, the fish were decapitated and skin samples were taken at midnight under red light and stored at −70 °C for Mel, AFMK, cortisol, TAC and TBARS analyzes. Dorsal, lateral, and ventral skin samples collected for melanosome dispersion analysis (10 mm × 5 mm; $n = 39$) were fixed in 4% buffered formalin.

Mel and AFMK concentrations were measured by HPLC with fluorescence detection preceded by SLE (Simplified Liquid Extraction). Skin samples were homogenized in 1 mL of phosphate buffer (0.05 M; pH 6.8), centrifuged (10,000 *g*, 15 min, 4 °C) and supernatants were loaded onto SLE columns (SLE NOVUM, 100 mg/5 mL; Phenomenex). Mel and AFMK were eluted using 6 mL of dichloromethane: ethyl acetate (1:1, *v*/v). After evaporation of the eluate, a residue was dissolved in 0.1 mL of methanol: PBS buffer (pH 7.4) (1:1, v/v) and analyzed by HPLC according to Kulczykowska et al. (2018).

Cortisol was measured using a commercial Cortisol ELISA kit (Cayman Chemical, USA). Skin samples for cortisol measurement were homogenized in 1 mL of PBS buffer (50 mM, pH 7.4), centrifuged (10,000 *g*, 15 min, 4 °C) and purified using the SLE following the standard protocol (TN-0076, Phenomenex) with minor modifications as follows: skin supernatants were diluted with 50 mM sodium phosphate dibasic (pH unadjusted) before SLE extraction, and dichloromethane was used as an elution solvent.

The dispersion of melanosomes in melanophores was analyzed in dorsal, lateral, and ventral skin samples. Skin samples mounted on

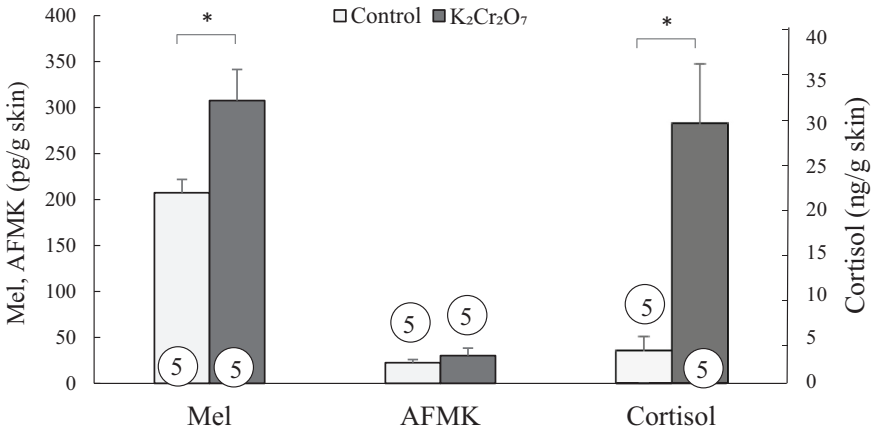

Fig. 1. Mel, AFMK and cortisol levels in the skin of three-spined stickleback (*Gasterosteus aculeatus*): control fish (light bars) and fish after exposure to $K_2Cr_2O_7$: 50 mg Cr/1 L for 6 h (dark bars). Values are means ± SEM. Significant difference $^*P < 0.05$ (Mann-Whitney *U* test). The number of fish is given in the circles.

Eukitt slides (Sigma-Aldrich, USA) were scanned with an Olympus BX60 light microscope (Olympus, Japan) and photographed using trans-illumination with an Olympus XC10 digital camera (Olympus, Japan) coupled with the Olympus microscope. The number of melanophores with different pigment dispersion was quantified using ImageJ 1.53e. The melanophore index (MI) was used to estimate the degree of pigment dispersion, according to Aspengren et al. (2003).

TAC and TBARS were estimated using commercial kits: Antioxidant Assay Kit (Cayman Chemical, USA) and TBARS Assay Kit (Cayman Chemical, TCA Method, USA), respectively. Skin samples for TAC and TBARS analyzes were homogenized in 0.2 mL of TAC assay buffer and 1 mL of RIPA buffer (Sigma), respectively.

Protein concentration was assayed using a commercial Protein Determination Kit (BCA) (Cayman Chemical, USA).

The normality of the data distribution was tested with the W Shapiro-Wilk test. Then the non-parametric Mann-Whitney U test was performed because the data did not follow a normal distribution.

Mel and cortisol levels are significantly higher in the skin of fish exposed to $K_2Cr_2O_7$ ($P < 0.05$, Mann-Whitney U test), but this is not the case with AFMK (Fig. 1). Therefore, Mel and cortisol could be considered indicators of oxidative stress induced by metal redox in fish skin. It should be noted here that the metabolism of Mel in mammalian skin is complex (for a review, see: Slominski et al., 2008; Slominski et al., 2017b); Mel is degraded not only to AFMK but also to 5-methoxytryptamine, 6-hydroxymelatonin, 4-methoxymelatonin, 2-methoxymelatonin and *N*-acetylserotonin (Kim et al., 2013; Fischer et al., 2006). Some of these compounds demonstrate strong antioxidant activity (for a review, see: Galano and Reiter, 2018) and are worth considering in future research in fish.

Exposure of fish to $K_2Cr_2O_7$ causes a significant increase in the number of melanophores with completely dispersed melanosomes (MI 4–5) in the lateral and ventral skin ($P < 0.05$, Mann-Whitney U test) (Fig. 2), but it is not the case with the dorsal. This part of the skin is the most densely covered with bone plates that actually protect the body against external factors. In the skin of several species of fish, administration of cortisol or exposure to stress induces the dispersion of melanosomes, resulting in darkening of the skin (Iger et al., 1995; Ruane et al., 2005). The pigment dispersion analysis could reflect the joint action of cortisol and Mel in the skin because cortisol induces pigment dispersion and skin darkening, and Mel aggregation of melanosomes and skin lightening (Goda and Fujii, 1998; Fujii, 2000). This effect has been discussed earlier in European flounder (*P. flesus*) (Kulczykowska et al., 2018). In the three-spined stickleback, the pigment dispersion related to cortisol appears to outweigh the pigment aggregation related to Mel.

The TAC and TBARS values are as follows: TAC: 0.91 ± 0.17 and 1.84 ± 0.5 (μmol Trolox/mg protein; $n = 5$) and TBARS: 69.51 ± 17.06 and 75.62 ± 11.19 (mmol malondialdehyde (MDA)/mg protein; $n = 7$), in control fish and exposed to $K_2Cr_2O_7$, respectively. For the record, TAC determines the antioxidant activity of all compounds present in the sample (Pellegrini et al., 2020) and TBARS (expressed as the MDA level) is a metric of lipid peroxidation and usually increases after oxidative stress (Kotyzová et al., 2015; Susa et al., 1989). Both TAC and TBARS increase in the skin after exposure of fish to $K_2Cr_2O_7$, but the changes are not statistically different. Regarding TBARS, it may be an effect of a high concentration of Mel after exposure because it is known to decrease lipid peroxidation by scavenging highly reactive hydroxyl and lipid peroxyl radicals that initiate and propagate lipid oxidation reactions (Reiter et al., 2014). Many studies show that Mel reduces the formation of MDA (one of the end products of lipid peroxidation) under oxidative stress. A negative correlation has been observed between the amount of Mel and MDA in isolated hepatocytes of *Labeo rohita* (*Cyprinidae*) exposed to H_2O_2 (Moniruzzaman et al., 2018), the brain of Wistar rats injected with homocysteine (Baydas et al., 2003), the brain, liver and kidney of Buffalo rats injected with benzo(*a*)pyrene (Murawska-Ciałowicz et al., 2011). Therefore, TBARS does not appear to be a good indicator of oxidative stress in fish skin. In the case of TAC, the lack of a significant increase in this parameter may be due to the small number of samples (n = 5) and the large differences in TAC levels between samples.

This study showing the effects of exposure of the three-spined stickleback to $K_2Cr_2O_7$ allows us to assume that an increased concentration of Mel and cortisol in the skin is a strategy to cope with oxidative stress to prevent damage caused by active oxygen molecules in the body organ directly exposed to environmental stressors. Mel, an effective antioxidant, and cortisol, a glucocorticoid that mobilizes various mechanisms to handle stress and restoring balance afterward are important elements of CSRS. There are two ways to increase the pool of Mel content in the skin: activation of local Mel synthesis in skin cells and transport of hormone through circulation from other sites where Mel is produced. There is reason to think that both ways can act (Hardeland, 2005; Kulczykowska et al., 2007; Slominski et al., 2017b), but research dedicated to this issue is required. There is no evidence of local cortisol production in fish skin, but in mammals, skin cells synthesize cortisol (Slominski et al., 2007; Vukelic et al., 2011), so the same may be true in fish; this still needs investigation. This study shows that even a short exposure of fish to $K_2Cr_2O_7$ causes a significant increase in the number of melanophores with completely dispersed melanosomes. The diameter and index of the melanophore in the skin of fish has already appeared to change with the concentration of cortisol (Kulczykowska et al., 2018). Therefore, pigment dispersion may be a valuable and easy-to-observe marker of oxidative stress, useful in assessing fish welfare. Such an approach has considerable merit: a collection of samples (part of the skin or fin) is an easy and quick process when we can avoid killing the fish.

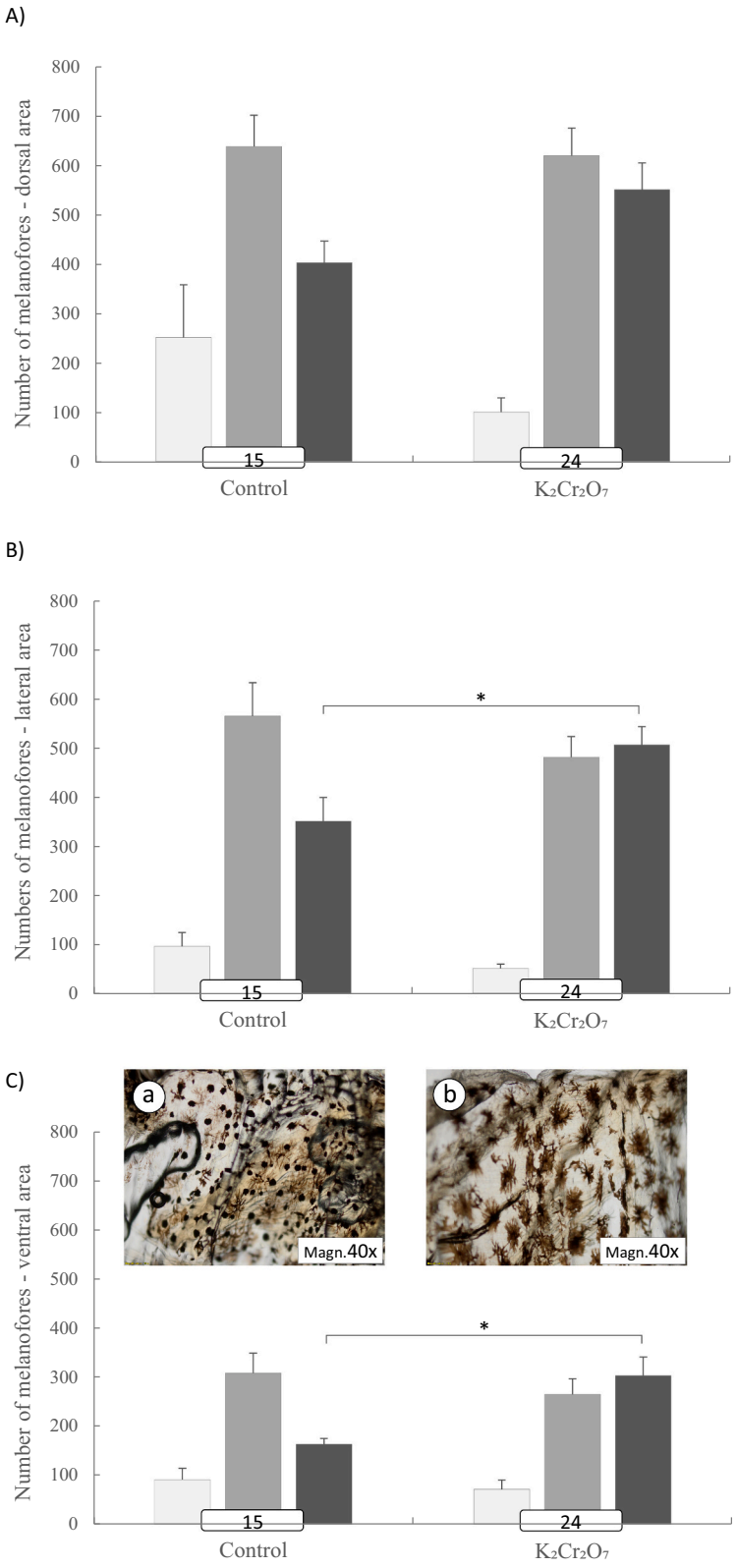

Fig. 2. Dispersion of melanosomes in melanophores in dorsal (A), lateral (B) and ventral (C) skin samples collected from the three-spined stickleback (*Gasterosteus aculeatus*): control group and group after exposure to $K_2Cr_2O_7$. Photos a and b (a 40-fold magnification was applied) show the state of melanophores in the control group and after exposure to $K_2Cr_2O_7$, respectively. Melanophore index: MI 1 (light grey bars): melanophores with completely aggregated melanosomes, MI 2–3 (dark grey bars): melanophores with partially dispersed melanosomes, MI 4–5 (black bars): melanophores with completely dispersed melanosomes. Values are means ± SEM. Significant difference $*P < 0.05$ (Mann-Whitney U test). The number of skin samples is given in rectangles.

Ethics statement

All experiments complied with EC Directive 2010/63/EU for animal experiments and with the guidelines and approval of the Ethics Committee for Animal Experimentation (University of Science and Technology, Bydgoszcz, Poland).

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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