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Uphill Running Excessive Training Increases Gastrocnemius Glycogen Content in C57BL/6 Mice

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Summary

The main aim of the present investigation was to verify the effects of three overtraining (OT) protocols performed in downhill (OTR/down), uphill (OTR/up) and without inclination (OTR) on the protein levels of Akt (Ser473), AMPKα (Thr172), PGC-1α, plasma membrane GLUT-1 and GLUT-4 as well as on the glycogen contents in mice gastrocnemius. A trained (TR) protocol was used as positive control. Rodents were divided into naïve (N, sedentary mice), control (CT, sedentary mice submitted to the performance evaluations), TR, OTR/down, OTR/up and OTR groups. At the end of the experimental protocols, gastrocnemius samples were removed and used for immunoblotting analysis as well as for glycogen measurements. There was no significant difference between the experimental groups for the protein levels of pAkt (Ser473), pAMPKα (Thr172), PGC-1α, plasma membrane GLUT-1 and GLUT-4. However, the OTR/up protocol exhibited higher contents of glycogen compared to the CT and TR groups. In summary, the OTR/up group increased the gastrocnemius glycogen content without significant changes of pAkt (Ser473), pAMPKα (Thr172), PGC-1α, plasma membrane GLUT-1 and GLUT-4.

Key words

Akt • AMPKα • Glycogen • Nonfunctional overreaching • PGC-1α

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Introduction

The process of training intensification is named overtraining (OT) and may induce the nonfunctional overreaching state (NFOR), which is defined as a performance drop accompanied or not by psychological and hormonal alterations (Meeusen *et al.* 2013). The carbohydrate hypothesis considers that during long periods of exercise, athletes present a transitory state of hypoglycemia, which can be explained by both the depletion of hepatic and muscular glycogen stores as well as the deficiency of glycolytic metabolic flux in energy generation. After several long-term training sessions, this depletion of glycogen stores may become a chronic problem, especially when there is a deficiency in the intake of this nutrient (Costill *et al.* 1988). To elucidate the molecular mechanisms linked to NFOR etiology, Pereira *et al.* (2015c) proposed three OT protocols for mice based on chronic running sessions in downhill (OTR/down), uphill (OTR/up) and without inclination (OTR) that increased the gastrocnemius and serum contents of interleukin 6 (IL-6) (Pereira *et al.* 2015a).

Weigert *et al.* (2005) verified that IL-6 increases insulin-stimulated glycogen synthesis and phosphorylation of protein kinase B (Akt) at serine473. Also, exercise-induced AMP-activated protein kinase (AMPK) activation enhances the translocation of the glucose transporter type 4 (GLUT-4) to the cell surface and the

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skeletal muscle glycogen content during recovery (Winder and Hardie 1996, Winder and Hardie 1999). GLUT-1 is another cell membrane transporter that regulates glucose uptake in skeletal muscle cells (Manchester *et al.* 1996). On the other hand, IL-6 directly activates the AMPK in skeletal muscle both *in vivo* and *in vitro* (Kelly *et al.* 2009, Kelly *et al.* 2004). Kelly *et al.* (2009) also observed that incubation of skeletal muscle samples with IL-6 increased the glycogenolysis and the protein levels of one of the AMPK genes that acts on mitochondria, the peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α). These authors proposed that IL-6 acts as an autocrine or paracrine factor, enhancing lipolysis and glycogenolysis as well as activating AMPK in the muscle cells during exercise.

Based on these investigations (Kelly *et al.* 2009, Lukaszuk *et al.* 2012, Pereira *et al.* 2015c, Weigert *et al.* 2005, Winder and Hardie 1996, Winder and Hardie 1999), we verified the effects of the OTR/down, OTR/up and OTR protocols on the protein levels of Akt, AMPKα, PGC-1α, plasma membrane GLUT-1 and GLUT-4 as well as on the glycogen contents in mice gastrocnemius. We hypothesize that the OT protocols-induced performance decline is not linked to low levels of muscle glycogen. In fact, we consider that the overtrained rodents will present activation of Akt and AMPKα with enhancement of the translocation of the GLUT-1 and GLUT-4 to the cell surface. As a positive control, we used a trained (TR) protocol that leads to performance increment (Ferreira *et al.* 2007, Pereira *et al.* 2012, Pereira *et al.* 2013, Pereira *et al.* 2014a, Pereira *et al.* 2014b).

Methods

Eight-week-old male C57BL/6 rodents from the Central Animal Facility of the Ribeirão Preto campus were maintained in individual cages with controlled temperature (22 ± 2 °C) on a 12:12-h light-dark inverted cycle with food (Purina chow) and water *ad libitum*. The experimental procedures were approved by the Ethics Committee of the University of Sao Paulo (ID 14.1.873.53.0). Mice were randomly divided into naïve (N; sedentary mice), control (CT; sedentary mice submitted to the performance evaluations), trained (TR), overtrained by downhill running (OTR/down), overtrained by uphill running (OTR/up) and overtrained by running without inclination (OTR). The N group was inserted to guarantee that the alterations observed in the CT group did not occur in response to the acute exercise effects (i.e. performance tests).

While the 8-week training protocol was based on the investigation of Ferreira *et al*. (2007), the 8-week overtraining protocols were performed as previously described (da Rocha *et al.* 2015, Pereira *et al.* 2015a, Pereira *et al.* 2015b). Both training and overtraining protocols consisted of 5 days of continuous training interposed by two days of recovery. The performance evaluations were applied on week 0 and 48 h after the last sessions of the TR and OT protocols at the end of weeks 4 and 8 and consisted of the rotarod test, the incremental load test, the exhaustive test and the grip force test (Anderson *et al.* 2004, da Rocha *et al.* 2015, da Rocha *et al.* 2016a, Pereira *et al.* 2015a, Pereira *et al.* 2015b, Turgeman *et al.* 2008). The detailed description of these performance evaluations and the effects of the TR and OT protocols have been previously published in other studies by our research group (da Rocha *et al.* 2015, da Rocha *et al.* 2016a, Pereira *et al.* 2015a, Pereira *et al.* 2012, Pereira *et al.* 2015b, Pereira *et al.* 2013, Pereira *et al.* 2014a, Pereira *et al.* 2014b).

Thirty-six hours after the grip force test performed at the end of the TR and OT protocols, the fasted rodents (i.e. 12 h) were anesthetized with an intraperitoneal (i.p.) injection of 2.5 % (10-20 $\mu l.g^{-1}$) 2,2,2-tribromoethanol. As soon as anesthesia was confirmed by the loss of the pedal reflexes, the abdominal cavity was opened, the portal vein was exposed, and saline with and without human recombinant insulin (10 $U.kg^{-1}$, Eli Lilly, Indianapolis, IN) was injected. At 90 s after saline or saline with human recombinant insulin injection (Pauli *et al.* 2008, Ropelle *et al.* 2006), gastrocnemius samples were removed and used for immunoblotting analysis as previously described (da Rocha *et al.* 2015, da Rocha *et al.* 2016a, da Rocha *et al.* 2017, Pereira *et al.* 2015a, Pereira *et al.* 2012, Pereira *et al.* 2015b, Pereira *et al.* 2014a, Pereira *et al.* 2014b). Herein, we used the following antibodies: beta-actin (SC69879), GLUT-4 (SC53566) and PGC-1α (SC13067) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) at dilution of 1:750; Akt (CELL9272S), phospho-Akt (Ser473; CELL4058S), AMPKα (CELL2532S), phospho(p)-AMPKα (Thr172; CELL2535S) and GLUT-1 (CELL12939S) from Cell Signaling Technology (Beverly, MA, USA) at dilution of 1:1,000.

The plasma membrane fractions were obtained

as previously described (Gasparetti *et al.* 2003, Mizukami *et al.* 1997, Pereira *et al.* 2016) and aliquots were subjected to the immunoblotting analysis with the following antibodies: GLUT-4 (SC53566) and GLUT-1 (CELL12939S) for plasma membrane aliquots. The gastrocnemius glycogen contents were measured as described by Dubois *et al.* (1951). Results are expressed as the mean ± standard error of the mean (SE). A one-way ANOVA was used to examine the responses of the analyzed parameters to the experimental groups. When one-way ANOVA indicated statistical significance, a Bonferroni's *post hoc* test was performed. All statistical analyses were two-sided, and the significance level was set at $P<0.05$.

Results

There was no significant difference between the experimental groups for the protein levels of pAkt (Ser473; Fig. 1A), pAMPKα (Thr172; Fig. 1B), PGC-1α (Fig. 1C), plasma membrane GLUT-1 (Fig. 1D) and GLUT-4 (Fig. 1E). On the other hand, Figure 1F shows that the gastrocnemius glycogen content was significantly higher for the OTR/up group (0.28±0.04 mg/g) compared to the CT (0.15±0.01 mg/g) and TR (0.12±0.02 mg/g) groups. The effect sizes of pAkt (with insulin stimulation), pAkt (without insulin stimulation), pAMPKα, PGC-1α, plasma membrane GLUT-1, GLUT-4 and gastrocnemius glycogen are 0.84, 0.74, 0.42, 0.25, 0.49, 0.42 and 0.92, respectively.

Discussion

Recently, we verified that the pAkt (Ser473) was lower in the extensor digitorum longus (EDL) and soleus after the OTR/down and in the soleus after the OTR/up and OTR (Pereira *et al.* 2016). Also, the pAkt (Ser473) was higher after the OTR/up in the EDL. Herein, this protein was not changed after the experimental protocols. These data show that each skeletal muscle presents a different adaptation in response to the same OT protocols. Weigert *et al.* (2005) showed that IL-6 enhances pAkt (Ser473) in mouse muscle; however, they used 50 ng of mouse recombinant IL-6 that was injected intraperitoneally. Our overtrained rodents displayed increased levels of IL-6 in both gastrocnemius and serum samples (Pereira *et al.* 2015a), which were not sufficient to increase the gastrocnemius contents of pAkt (Ser473).

To our knowledge, this is the first study showing the effects of different OT models on the protein levels of pAMPKα (Thr172) and PGC-1α in skeletal muscle samples. Although not significant, the protein levels of pAMPKα (Thr172) for the OTR/up were approximately 84 and 137 % higher compared to the N and TR. Also, the training intensity of the OTR/up was 43.8 % higher compared to the TR. Both AMPK activity and its phosphorylation at threonine 172 are dependent on exercise intensity in humans and rodents (Chen *et al.* 2003, Egan *et al.* 2010, Rasmussen and Winder 1997, Schwalm *et al.* 2015, Tadaishi *et al.* 2011, Wadley *et al.* 2006, Wojtaszewski *et al.* 2000). The relative intensities of the OTR/down and OTR were the same as in the OTR/up; however, the energy cost and mitochondrial adaptation are higher in uphill compared to downhill and running without inclination (Chavanelle *et al.* 2014, Cornachione *et al.* 2011, Schlagowski *et al.* 2016, Vernillo *et al.* 2017).

An important factor that may have influenced the lack of significant alterations in the protein contents of pAMPKα (Thr172) after the TR and OT protocols is the extraction time of the gastrocnemius samples. Regarding the effects of acute and chronic exercise models on the time-course of the intramuscular activation of pAMPKα (Thr172) in humans and rodents, the investigations described a range of significant responses between immediately post-exercise and 6 h of recovery (Camera *et al.* 2010, Gehlert *et al.* 2012, Halling *et al.* 2016, Huh *et al.* 2014, Sriwijitkamol *et al.* 2007). Recently, Wang *et al.* (2016) verified that nine swimming bouts (i.e. 10 min/bout) with 10-min rest periods between each bout did not change pAMPKα (Thr172) levels in rat soleus muscles that were or not stimulated with insulin. In accordance, after six weeks of progressively increasing intensity stationary cycle cycling, Stuart *et al.* (2010) did not observe significant responses of pAMPKα (Thr172) contents in human vastus lateralis muscle biopsies. The extraction times of the skeletal muscle samples previously described were from 3 to 4 h (Wang *et al.* 2016) and 48 h (Stuart *et al.* 2010) after the conclusion of the exercise sessions.

In an elegant investigation, Jager *et al.* (2007) showed that AMPK needs PGC-1α for many of its most relevant effects on GLUT-4 translocation in skeletal muscles. Also, Leick *et al.* (2010) suggested that PGC-1α mediates AMPK-induced GLUT-4 regulation in response to chronic exercise. They also verified that the

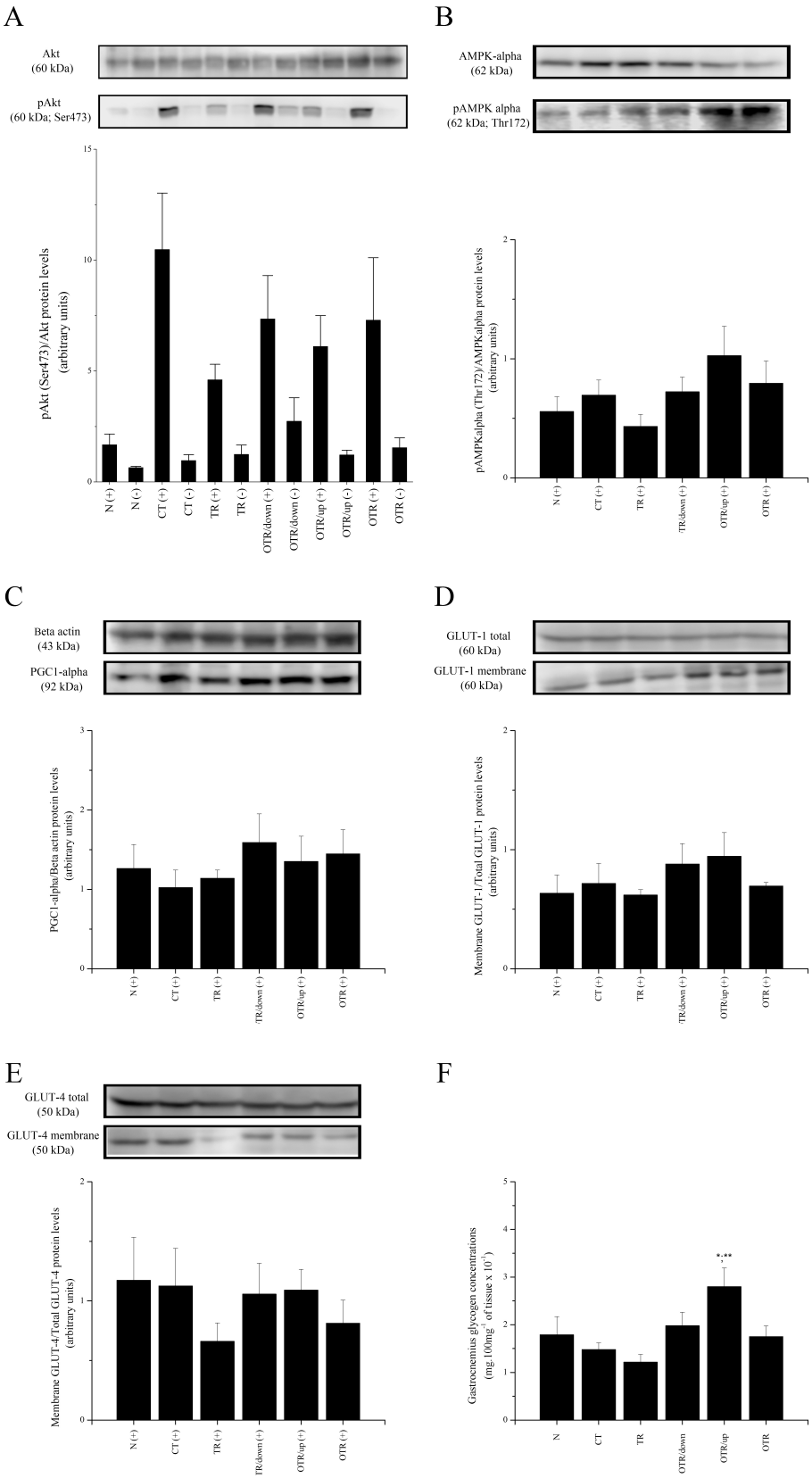

Fig. 1. Protein levels (arbitrary units) of pAkt (Ser473)/Akt (**A**), pAMPKα (Thr172)/AMPKα (**B**), PGC-1α/beta-actin (**C**), membrane GLUT-1/total GLUT-1 (**D**), membrane GLUT-4/total GLUT-4 (**E**) and glycogen concentrations (**F**); mg.100 mg^{-1} of tissue x 10^{-1}) in gastrocnemius for the experimental groups. The original experiments correspond to means ± SE of n=8 mice [i.e. n=8 gastrocnemius with insulin stimulation (+) and n=8 gastrocnemius without insulin stimulation (-)]. N: sedentary mice; TR: trained mice; CT: sedentary mice; OTR/down: overtrained by downhill running; OTR/up: overtrained by uphill running; OTR: overtrained by running without inclination. $^{*}P<0.05$ vs. the CT group; $^{**}P<0.05$ vs. the TR group. The original experiments of Figures 1A-1D are available in the Supplementary Figures 1 and 2. The blots circled in red were used as representative in the respective Figures.

absence of PGC-1α attenuates the skeletal muscle glycogen increase. As the pAMPKα (Thr172) responses, we also did not observe statistical changes of the intramuscular levels of PGC-1α after the TR and OT models. Because the proteins and their targets are phosphorylated at distinct times (Kholodenko 2006), future investigations should measure the time-course of the gastrocnemius levels of pAMPKα (Thr172) and PGC-1α after the TR and OT protocols to clarify whether they were activated before or after the current extraction time. Probably, the current extraction time of the gastrocnemius muscle was the main limitation of the study.

Recently, we showed that the OTR/down downregulated the plasma membrane GLUT-4 contents in both EDL and soleus samples, while the OTR/up and OTR downregulated this protein only in soleus sample (Pereira *et al.* 2016). We argued that the soleus was more recruited than the EDL during the 8 weeks of the OTR/up and OTR. In accordance, Carter *et al.* (1994) concluded that exhaustive concentric exercise might preferentially impair slow twitch fibers. Our research group also observed that these three OT models improved glucose tolerance even with reduced insulin signaling in EDL and soleus (Pereira *et al.* 2016). This finding probably occurred because other tissues such as liver did not present inhibition of this pathway, playing a crucial role in glucose homeostasis (Kotani *et al.* 2004, Zisman *et al.* 2000). In fact, Rocha and coworkers (da Rocha *et al.* 2015) verified that the OTR/down and OTR/up increased the phosphorylation and inhibition of glycogen synthase kinase 3 beta (GSK3beta), enhancing the hepatic glycogen depositions.

Herein, the OTR/up increased the gastrocnemius glycogen content compared to the CT and TR. According to Armstrong and Taylor (Armstrong and Taylor 1993), the higher the exercise intensity, the faster the skeletal muscle glycogen loss. As previously stated, the training intensity of the OTR/up was 43.8 % higher than the TR. Also, Goforth *et al.* (2003) verified that subjects completing a depletion carbohydrate (CHO)-loading protocol achieved higher muscle glycogen concentrations that persisted longer in comparison to those completing the non-depletion CHO-loading protocol. Although GLUT-4 is the predominant glucose transporter isoform expressed in skeletal muscle, and its translocation to the plasma membrane promotes glucose uptake (Klip and Paquet 1990), Fam *et al.* (2012) verified that the deletion of the skeletal muscle GLUT-4 did not impair the whole-body glucose disposal on a pure C57BL6/J background strain. These and other investigations (Charron *et al.* 2005, Fam *et al.* 2012, Ryder *et al.* 1999) suggested that unidentified GLUTs would be increased to compensate the GLUT-4 absence.

GLUT-1 is also responsible for glucose uptake in skeletal muscle cells (Manchester *et al.* 1996). When incubated with the tumor necrosis factor-α (TNF-α), child myotube cultures up-regulated GLUT-1 expression and glucose transport (Grohmann *et al.* 2005). Also, cultured L6 skeletal muscle cells incubated in the presence of cytokines (i.e. interferon-γ and TNF-α) and lipopolysaccharide (LPS) for 24 h markedly increased both basal glucose transport and GLUT-1 transporter protein (Bedard *et al.* 1997). Although our research group verified high contents of TNF-α in the gastrocnemius samples of the OTR/down and OTR/up (Pereira *et al.* 2015a), the GLUT-1 contents in this particular skeletal muscle sample were not altered after these OT models.

Conclusions

The current data testified our initial hypothesis suggesting that other mechanisms, except the decrease of skeletal muscle glycogen stores, participate in the performance drop linked to the NFOR state. In fact, we verified that uphill running excessive training increased gastrocnemius glycogen content, but did not lead to significant alterations of pAkt (Ser473), pAMPKα (Thr172), PGC-1α, plasma membrane GLUT-1 and GLUT-4. Figure 2 summarizes the current findings regarding the relationship between the OT protocols and the carbohydrate theory.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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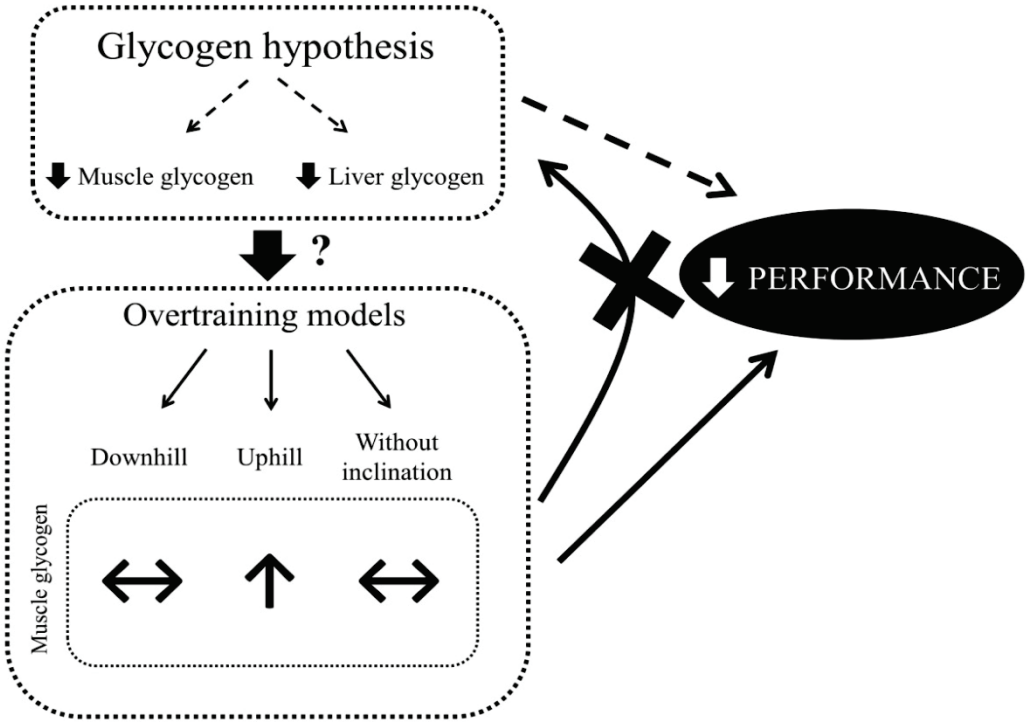

Fig. 2. Schematic model summarizing the current findings regarding the relationship between the OT protocols and the carbohydrate theory.

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