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# The synthesis of UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), α-dystroglycan, and β-galactoside α-2,3-sialyltransferase 6 (ST3Gal6) by skeletal muscle cell as a response to infection with *Trichinella spiralis*

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**Article info**

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**Summary**

The Nurse cell of the parasitic nematode *Trichinella spiralis* is a unique structure established after genetic, morphological and functional modification of a small portion of invaded skeletal muscle fiber. Even if the newly developed cytoplasm of the Nurse cell is no longer contractile, this structure remains well integrated within the surrounding healthy tissue. Our previous reports suggested that this process is accompanied by an increased local biosynthesis of sialylated glycoproteins.

In this work we examined the expressions of three proteins, functionally associated with the process of sialylation. The enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) is a key initiator of the sialic acid biosynthetic pathway. The α-dystroglycan was the only identified sialylated glycoprotein in skeletal muscles by now, bearing sialyl-α-2,3-Gal-β-1,4-GlcNAc-β-1,2-Man-α-1-O-Ser/Thr glycan. The third protein of interest for this study was the enzyme β-galactoside α-2,3-sialyltransferase 6 (ST3Gal6), which transfers sialic acid preferably onto Gal-β-1,4-GlcNAc as an acceptor, and thus it was considered as a suitable candidate for the sialylation of the α-dystroglycan. The expressions of the three proteins were analyzed by real time-PCR and immunohistochemistry on modified methacarn fixed paraffin tissue sections of mouse skeletal muscle samples collected at days 0, 14 and 35 post infection.

According to our findings, the up-regulation of GNE was a characteristic of the early and the late stage of the Nurse cell development. Additional features of this process were the elevated expressions of α-dystroglycan and the enzyme ST3Gal6. We provided strong evidence that an increased local synthesis of sialic acids is a trait of the Nurse cell of *T. spiralis*, and at least in part due to an overexpression of α-dystroglycan. In addition, circumstantially we suggest that the enzyme ST3Gal6 is engaged in the process of sialylation of the major oligosaccharide component of α-dystroglycan.

**Keywords:** α-dystroglycan; GNE; Nurse cell; Sialic acid; Skeletal muscle; *Trichinella spiralis*

## Introduction

Trichinellosis is a food-borne parasitosis caused by nematodes from the *Trichinella* genus (Railliet, 1985). The disease results after consumption of undercooked meat contaminated with infectious *Trichinella* larvae, which reach maturity to adult species of the parasites in the small intestine and reproduce. The newborn larvae travel all over the host body via the blood and the lymphatic system, but can successfully invade only the striated muscle cells. After penetrating the sarcolemma, the larva induces dramatic

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genetic, morphological and functional changes into the occupied portion of muscle fiber that eventually result in a completely new structure called a Nurse cell, capable of supporting the parasite for years. Even if the newly formed cytoplasm had fully lost its contractile capabilities, the Nurse cell remains well integrated within the surrounding unaffected muscle tissue, capable of supporting the parasite for years (Despommier, 1998).

Sialic acids are over than 40 modifications of the neuraminic acid, which derives from N-acetyl mannosamine. The process of sialylation of glycoproteins and glycolipids always occurs into the Golgi and afterwards they are transported to the cell membrane. Because of their terminal position on the carbohydrate chains, the sialic acids participate in almost all types of recognition phenomena and adhesion mechanisms (Varki, 1997). The sialic acids are transferred onto a penultimate sugar residue of a nascent oligosaccharide composition via α-2,3-, α-2,6- or α-2,8-glycosidic bond through enzymes, belonging to different sialyltransferase families (Takashima, 2008). In skeletal muscles, the sialic acids are important for the functional maintenance of glycoproteins involved in muscle excitability (Johnson *et al.,* 2004; Schwetz *et al.,* 2011), cell structure and neuromuscular junctions (McDearmon *et al.,* 2003; Combs & Ervasti, 2005), muscle development and regeneration (Broccolini *et al.,* 2008), and exercise performance (Hanish *et al.,* 2013).

We already reported a positive reaction towards the lectin *Maackia amurensis* II (MAL II) as a permanent characteristic of the cytoplasm of the developing and the mature Nurse cell of *Trichinella spiralis* (Owen, 1835), suggesting a novel biosynthesis of α-2,3-sialylated glycoproteins (Milcheva *et al*., 2020). This suggestion was in accordance to our previous findings of increased levels of free and protein bound sialic acid and elevated total sialyltransferase activity in mice muscles, invaded with *T. spiralis* (Milcheva *et al*., 2015). Therefore, an up-regulation of the enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), which is the key initiator and regulator of the sialic acids biosynthesis (Hinderlich *et al*., 1997), is inevitably expected. On the other hand, the only identified sialylated glycoprotein in skeletal muscles by now is the α-dystroglycan, bearing a sialyl-α-2,3-Gal-β-1,4-GlcNAc-β-1,2-Man-α-1-O-Ser/Thr glycan (Barresi & Campbell, 2006). This glycoprotein is an important member of the dystrophin-associated glycoprotein complex, representing a physical link between the cytoskeleton and basement membrane, and thus providing a structural stability to the sarcolemma (Petrof *et al*., 1993). The role of this glycoprotein for the Nurse cell of *T. spirallis* would be particularly interesting in the light of the fact that after invasion the occupied portion of the muscle fiber loses its contractile properties, but it still remains well integrated within the surrounding non-invaded tissue. A third and very important matter concerns the expression of enzymes from the β-galactoside α-2,3-sialyltransferase family (ST3Gal), involved in the biosynthesis of α-2,3-sialylated oligosaccharide components. One of the members of this family, the enzyme ST3Gal3, transfers sialic acid preferably onto Gal-β-1,4-GlcNAc as an acceptor (Takashima, 2008) – a fact that makes it a suitable candidate in the sialylation of the oligosaccharide of α-dystroglycan.

Based on this hypothesis, the present work was designed to investigate the expressions of GNE, α-dystroglycan and ST3Gal6 sialyltransferse in mouse skeletal muscles, after invasion by *T. spiralis*.

## Material and Methods

*Parasites, invasion, sample collection and tissue preparation*

Infective *Trichinella spiralis* larvae (code ISS03) were isolated from previously invaded laboratory albino mice (*Mus musculus musculus*), between 30$^{th}$ and 40$^{th}$ day post infection (d.p.i.) according to a routine protocol, as already described (Milcheva *et al.,* 2019). Fifteen male mice, 6 – 8 weeks old, were inoculated with 500 infective *T. spiralis* larvae *per os.* The animals (five per group) were humanly euthanized at day 0, 14 and 35 post infection (d.p.i.) and skeletal muscle specimens (front and hind limbs, pectoral and gluteal muscles) were excised and fixed with freshly prepared modified methacarn fixative according to Cox *et al.* (2006). After processing the specimens were embedded in paraffin.

*Immunohistochemistry*

Parallel tissue sections, 5 μm thick, were submitted to an antigen retrieval step with 10 mM Citrate buffer pH 6.2 for 5 min at sub-boiling temperature in microwave oven. The endogenious peroxidase activity was blocked by 0.3 % solution of $H_2O_2$, and then 2.5 % normal goat serum (Vector Laboratories Ltd, Burlingame, CA, USA) was used to prevent non-specific antigen activity. Rabbit polyclonal antibodies against -N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE, dilution 1:100), α-dystroglycan (dilution 1:500, both purchased from Abcam, Cambridge, UK) and β-galactoside α-2,3-sialyltransferase 6 (ST3Gal6, dilution 1:200, Sigma-Aldrich, St. Louis, MO, USA) were applied overnight at 4°C. The sections were then treated with a secondary antibody (ImmPress HRP anti-rabbit IgG polymer detection kit, Vector Laboratories) for 30 min, a color reaction was developed with DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories) and the sections were counterstained with hematoxylin. The immunohistochemical staining was evaluated as negative (-) and positive (+). Additional sections were routinely stained with hematoxylin and eosin (H&E) for basic morphological evaluation.

*Molecular biology studies*

The experiments described below were designed to evaluate the expression of mRNA of mouse UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (*Gne*), dystroglycan 1 (*Dag 1*) and β-galactoside α-2,3-sialyltransferase 6 (*St3gal6*) by real time RT-PCR in tissue sections from mouse skeletal muscle collected at days 0, 14 and 35 d.p.i. The levels of expressions were estimated via normalization versus the expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as a reference

gene. The infection of the samples was confirmed by end point PCR of the Expansion segment V (ESV) of *T. spiralis* (Zarlenga *et al.*, 2001). The primers used for gene expression analyzes were designed using the NCBI Blast Tool (Ye *et al.*, 2012) in a way to span at least one intron sequence. The full names of investigated genes, the accession numbers of their reference sequences, the primer sequences and the size of the amplified products are shown in Table 1. The oligonucleotides were purchased from HVD Biotech Vertriebs (Vienna, Austria).

*Isolation of gDNA and end point PCR*

Genomic DNA from six paraffin sections from all samples was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden Germany). Genomic DNA from *T. spiralis* infectious larvae was isolated as a positive control, using NZY Tissue gDNA Isolation Kit (NZY-Tech, Lisboa, Portugal). All isolations were performed according to the provided protocols of the producers. The yield and purity of the collected gDNA were measured using S-300 Spectrophotometer (Boeco, Hamburg, Germany). Hot start PCR was designed on approximately 100 ng gDNA as a template by using Veriti thermoblock (Applied Biosystems of Thermo Fisher Scientific), as already described in details (Milcheva *et al.*, 2019). The products of amplification were visualized on 2.5 % agarose gel supplemented with Simply Safe nucleic acid stain (EurX®, Gdansk, Poland) versus 100-1000 bp DNA Ladder (EurX) and the gels were photographed with a gel documentation system Vision (Scie-Plas Ltd, Cambridge, UK).

*Gene expression analyses*

Total RNA from six paraffin sections from all samples was isolated using RNeasy FFPE Kit (Qiagen), according to the provided protocol. The yield and purity of the collected RNA were measured using S-300 Spectrophotometer (Boeco). Approximately 2 µg total RNA from each sample were used for first strand cDNA synthesis, as already described (Milcheva *et al.*, 2019). The generated cDNA was quantified and the samples were stored at -80°C.

Real-time PCR was designed on 1 µl of RT-product, containing approximately 500 ng cDNA as a template, in 20 µl total volume of reaction using RotorGene SYBR Green PCR Kit (Qiagen) following the recommendations of the producer. Three real-time PCR reactions/sample in triplicate were performed for amplification of *Gapdh*, *Gne*, *Dag1* and *St3gal6* using RotorGene™ 6000 Real-time Analyzer (Corbett Life Science-Qiagen). The data were analyzed using Rotor Gene Q Series Software (Qiagen) and the relative quantification of the sialyltransferase expressions was calculated by the ΔΔCt method (Zhang *et al.*, 2014) versus *Gapdh* as reference genes. After each run, a High Resolution Melting Curve Analysis (HRM) was performed to verify the specificity of the amplified products, which were visualized on 2.5 % agarose gel supplemented with Simply Safe nucleic acid stain (EurX) versus 100 – 1000 bp DNA Ladder (EurX) and the gels were photographed with a gel documentation system Vision (Scie-Plas Ltd, Cambridge, UK).

*Statistical analysis of the gene expression quantification*

Statistical analysis of the data was performed using GraphPad Prism 5.03 software (San Diego, CA, USA). Non-parametric one-way analysis of variance (Kruskal-Wallis test) with Dunn`s Multiple Comparison Test (significance level 0.05) was computed to detect statistically significant differences between the Ct values of the qPCR products between the control and infected samples, and the results were interpreted as follows: $P < 0.001$ = highly significant, $P < 0.01$ = very significant, $P < 0.05$ = significant.

Table 1. The full names of the investigated genes and their primers sequences used in this study.

| Gene | Abbreviation | Species | Accession number | Primers sequences (5`-3`) | Product size (bp) |
|---|---|---|---|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase | *Gapdh* | *Mus musculus* | NM_001289726, transcript variant 1 | TCCTCGTCCCGTAGACAAAATG – F<br>AATCTCCACTTTGCCACTGC – R | 103 |
| Glucosamine (UDP-N-acetyl) – 2 – epimerase/N-acetylmannosamine kinase | *Gne* | *Mus musculus* | NM_015828.3 | AATCCTGCAGATGTGTGTGG – F<br>AATGCAGCACAACTCCTTCC – R | 119 |
| Dystroglycan 1 | *Dag1* | *Mus musculus* | NM_001276485.1, transcript variant 5 | GTTGGCATTCCAGACGGTAC – F<br>AGTGTAGCCAAGACGGTAAGG – R | 136 |
| ST3 beta-galactoside alpha-2,3-sialyltransferase 6 | *St3gal6* | *Mus musculus* | NM_018784.2 | TCCCAGCTGAAGAAATGAGGAC – F<br>TCAGCTCTGCACAGAAATGG – R | 112 |
| Expansion segment V | ESV | *Trichinella spiralis* | * | GTTCCATGTGAACAGCAGT – F<br>CGAAAACATACGACAACTGC – R | 173 |

*Zarlenga *et al.*, 2001

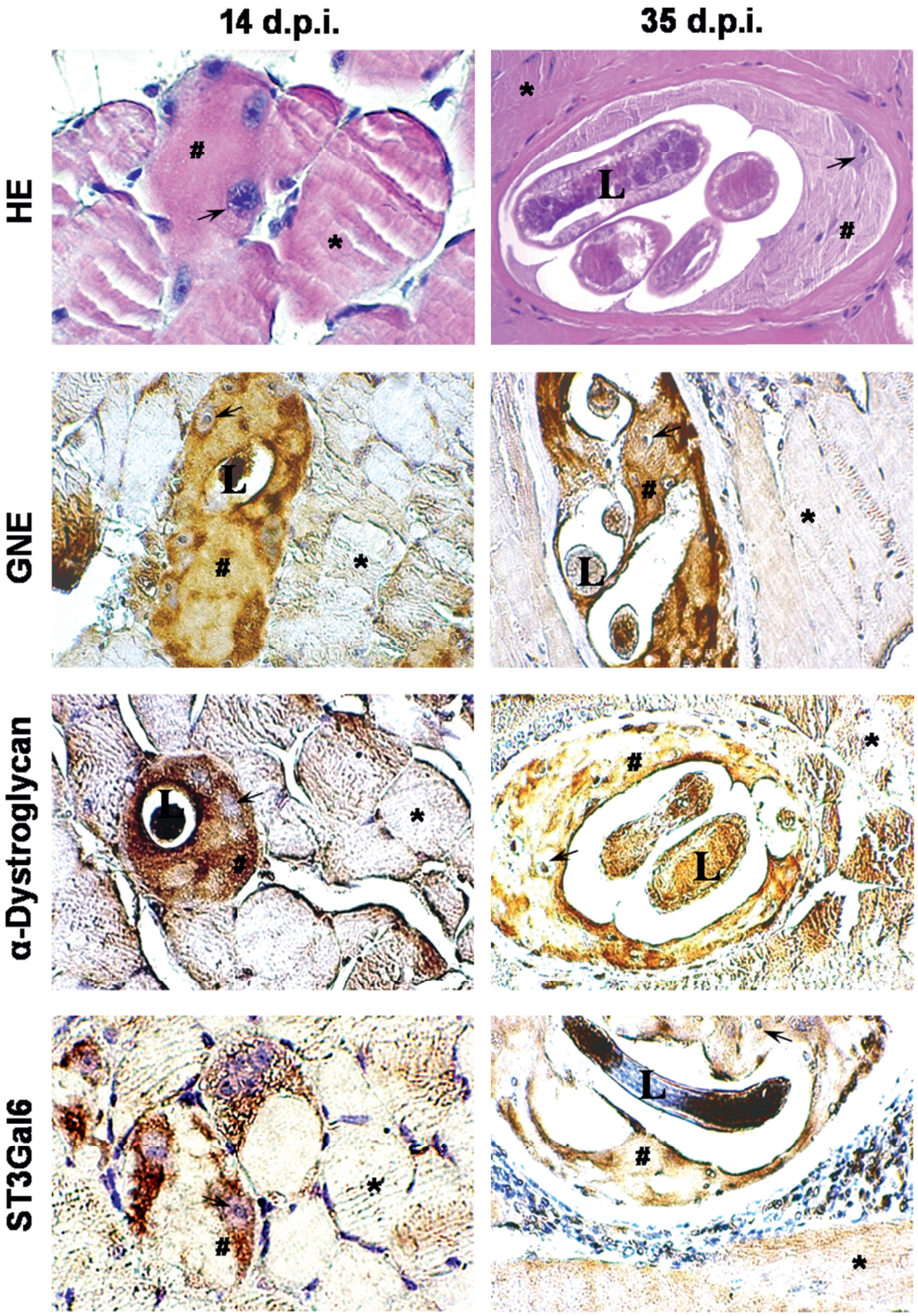


Fig. 1. Immunohistochemistry. Modified methacarn fixed sections from mouse skeletal muscles with *Trichinella spiralis* at days 14 and 35 post invasion (d.p.i.) were stained with rabbit polyclonal antibodies against glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase (GNE), α-dystroglycan and ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (ST3Gal6). Paralel sections were subjected to H&E staining to facilitate the histological orientation. Strong expressions of GNE and α-dystroglycan, and moderate expression of ST3Gal6 were observed on days 14 and 35 after invasion, suggesting these proteins as permanent characteristics of the Nurse cell of *T. spiralis*. The brown colour indicates positive immunohistochemical reaction, hashtag indicates the occupied sarcoplasm, star – non-occupied skeletal muscle cell, arrow – enlarged nucleus, L – larva. H&E, HRP anti-rabbit IgG, DAB. Scale bar 20 μm.

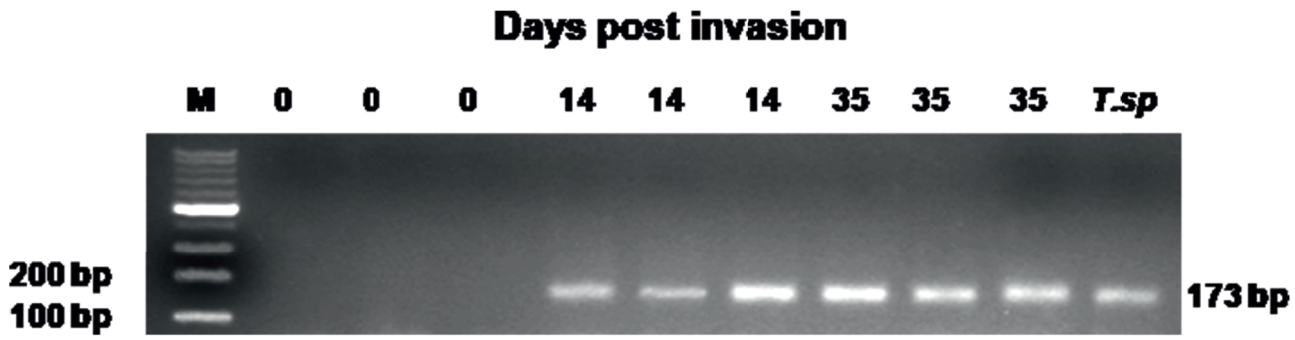


Fig. 2. Agarose gel analysis of *Trichinella spiralis* ESV fragment PCR. Polymerase chain reaction was performed on modified methacarn fixed mouse skeletal muscle tissue sections, selected on days 0, 14 and 35 after *T. spiralis* invasion. Genomic DNA from *T. spiralis* infectious larvae was used as a positive control sample. Presence of 173 bp fragment of expansion segment V of the *T. spiralis* genome was detected only in the mouse samples collected on days 14 and 35 after invasion. The photograph is a representative of three randomly selected samples from each experimental group.

**Ethical Approval and/or Informed Consent**

All animal experiments were performed in a compliance of Regulation № 20/01.11.2012 on the minimum requirements for protection and welfare of experimental animals and the requirements for the sites for their use, breeding and / or delivery, issued by the Ministry of Agriculture and Food of Republic of Bulgaria.

**Results**

Expressions of GNE, α-dystroglycan and ST3Gal6 proteins are increased in skeletal muscle fibers occupied by *T. spiralis*

The routine histology showed the typical features of *Trichinella* infection including centralized enlarged nuclei with disintegrated sarcoplasm at day 14. p.i. and a completed Nurse cell encompassing the grown and developed larva at day 35 p.i. (Fig. 1). Immunohistochemical analysis demonstrated strongly positive immunoreactivity with GNE, α-dystroglycan and ST3Gal6 antibodies with sarcoplasmic localization in the occupied skeletal muscle cells (Fig. 1). The increased expressions of the three proteins were observed in both investigated time points of Nurse cell development. Intensive expressions of the three proteins of interest were absent in the non-invaded areas of skeletal muscle cells.

Up-regulation of *Gne*, *Dag1* and *St3gal6* in skeletal muscles at day 14 p.i.

*T. spiralis* infection was verified in all experimental skeletal muscle samples by the amplification of a fragment of the specific Expansion segment V. Genomic DNA of *T. spiralis* served as a positive control for the ESV fragment. All non-infected skeletal muscle samples were negative (Fig. 2).

The relative expression analysis of the qPCR data showed strong up-regulation of *Gne*, *Dag1* and *St3gal6* in skeletal muscles at day 14 p.i. The levels of expression of *Gne* and *St3gal6* at day 35 p.i. were still significantly higher in comparison with the values of day 0 p.i. (Fig. 3).

**Discussion**

Among all pathological conditions of the skeletal muscle tissue, the establishment of the Nurse cell-parasite complex after invasion by the parasitic nematode *Trichinella* is unique phenomenon. This complex originates from a portion of the skeletal muscle fiber after invasion by a newborn larva. After penetrating the sarcolemma, the larva induces severe genetic, morphological and functional changes within the occupied syncytium area that transforms into a structure called Nurse cell, capable of supporting the parasitic larva for years (Despommier, 1998). During this process of de-differentiation, at least 53 genes associated with apoptosis, satellite cell activation and proliferation, cell differentiation, cell proliferation and cycle regulation, myogenesis and muscle development change in expression (Wu *et al.*, 2008a). The affected areas lose their contractile properties but the membranes of the newly developing Nurse cells remain adherent within the construction of the contractile fiber.

With their outer position on the oligosaccharide chains, the sialic acids are involved in almost all types of recognition phenomena and adhesion mechanisms, either through masking sites of biological recognition or by representing recognition epitopes (Varki, 2007; Schauer, 2009). They also have a crucial role in the process of gene expression and cell differentiation (Weidemann *et al.*, 2010). In skeletal muscles, the sialic acids are important for the functional maintenance of glycoproteins involved in fiber structure and neuromuscular junctions (McDearmon *et al.*, 2003; Combs & Ervasti, 2005), development and regeneration (Broccolini *et al.*, 2008), muscle excitability (Johnson *et al.*, 2004; Schwetz *et al.*, 2011) and exercise performance (Hanish *et al.*, 2013). They obviously play some role in the process of development of the skeletal muscle cell into a nurse cell, as already reported (Milcheva *et al.*, 2015, 2019, 2020).

The results from this work clearly showed that UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), α-dystroglycan and β – galactoside α-2,3-sialyltransferase 6 (ST-

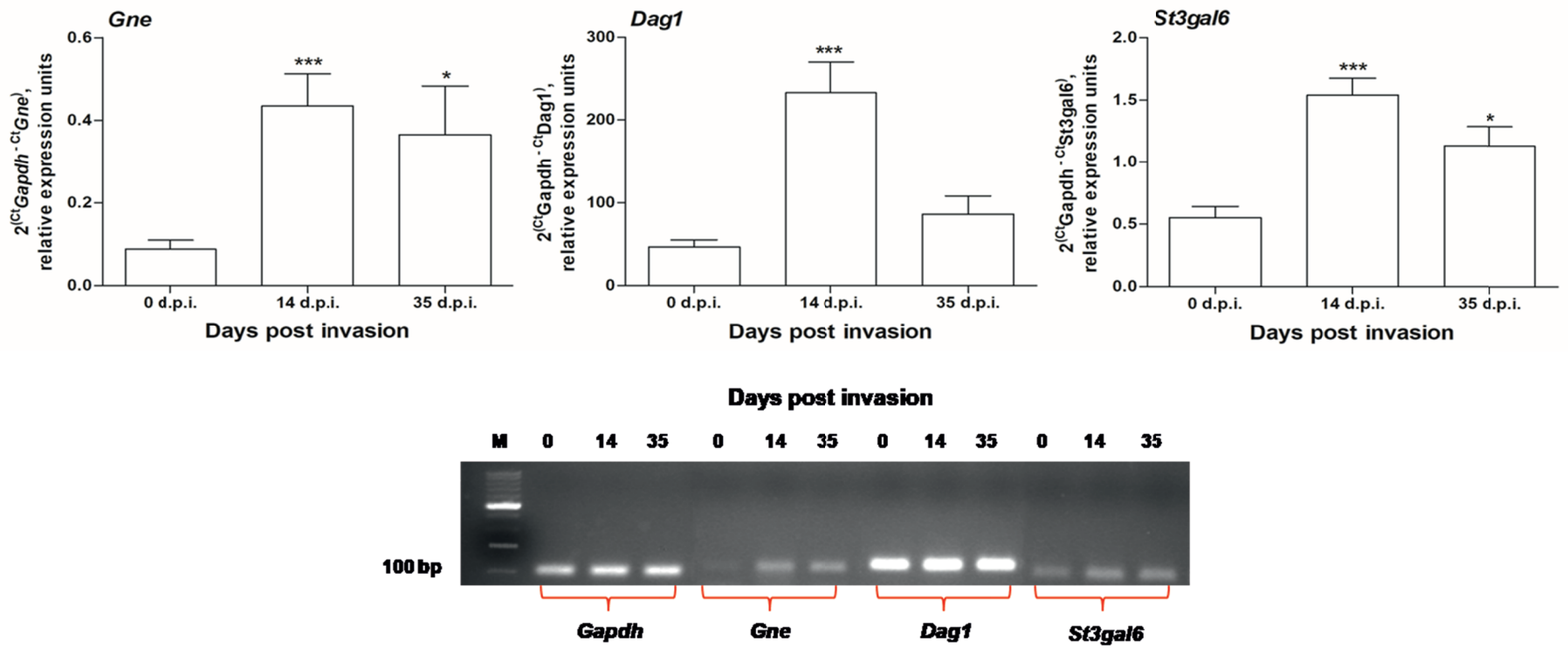


Fig. 3. Expressions of mouse glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase (*Gne*), dystroglycan 1 (*Dag1*) and ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (*St3gal6*) analysed by real time RT-PCR in modified methacarn fixed mouse skeletal muscle tissue sections, selected on days 0, 14 and 35 after *T. spiralis* invasion. The graphs show the relative quantification of the gene expressions calculated by the ΔΔCt method versus glyceraldehyde phosphate dehydrogenase (*Gapdh*) as a reference gene from five individual samples in triplicate. The bars show the standard error of mean. The products of amplification were loaded on 2.5% agarose gel versus Perfect 100-1000 bp DNA Ladder.

3Gal6) take place during the most dynamic period of transformation, and are also a characteristic of the mature Nurse cell.

UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) is a cytosolic bifunctional enzyme that catalyzes the first two key steps in sialic acid synthesis (Stäsche *et al.*, 1997). In skeletal muscles the enzyme has very low expression (Horstkorte *et al.*, 1999) and germinal mutation of the GNE gene is responsible for the pathogenesis of hereditary inclusion body myopathy (HIBM) and distal myopathy with rimmed vacuoles (DMRV). These two skeletal muscle disorders share similar clinical features as a consequence of severe hyposialylation of muscle glycoproteins (Nonaka *et al.*, 2005; Broccolini *et al.*, 2009). On the other hand, GNE is up-regulated after muscle injury in both damaged and regenerating myofibers (Nakamura *et al.*, 2010) – a fact that emphasizes the important role of the sialic acids in the maintenance and recovery of muscles.

Dystroglycan is an integral membrane component of the dystrophin-glycoprotein complex (DGC) – a large multicomponent structure that mediates the interactions between the cytoskeleton, membrane, and extracellular matrix. The alpha-subunite of dystroglycan is heavily glycosylated, bearing a specific sialyl-α-2,3-Gal-β-1,4-GlcNAc-β-1,2-Man-α-1-O-Ser/Thr glycan as a major oligosaccharide. Aberrant glycosylation of α-dystroglycan is associated with several inherited muscular disorders, including HIBM and DMRV due to GNE mutation (Sasaki *et al.*, 1998; Lapidos *et al.*, 2004; Cohn 2005). Histological expressions of sialylated glycoproteins in muscles were described in details (Marini *et al.*, 2014). The α-dystroglycan, however, is still the only identified sialylated glycoprotein in skeletal muscles. The loss of dystroglycan itself and depletion of proteins that are involved in the post-translational processing of α-dystroglycan, are not compatible with life (Barresi & Campbell, 2006). Except of our discovery of increased expression of dystroglycan in the Nurse cell, we could not find other information concerning up-regulation of this protein in the available literature. Similarly to *Gne* however (Nakamura *et al.*, 2010), it is quite possible that *Dag1* follows the same pattern of expression under conditions of skeletal muscle injury and repair. Indeed, it was already noticed that the Nurse cell formation and the muscle cell regeneration/repair share some events and mechanisms in parallel (Wu *et al.*, 2008b).

The up-regulation of *Gne* described in this work was in a very good agreement with our previous findings concerning the increased sialic acid biosynthesis in the Nurse cell (Milcheva *et al.*, 2015, 2019, 2020). Considering the up-regulation of *Gne* however, we set our attention on the sialyltransferase activity. The finding of increased expression of β-galactoside α-2,3-sialyltransferase 6 (ST3Gal6) was remarkable because this enzyme transfers sialic acid preferably onto Gal-β-1,4-GlcNAc as an acceptor (Takashima, 2008). Therefore, we propose that ST3Gal6 is involved in the sialylation of the major oligosaccharide of α-dystroglycan. Up-regulation of this enzyme has been associated with poor prognosis in patients with multiple myeloma or urinary bladder cancer, and with increased cell proliferation, migration and invasion ability in hepatocellular carcinoma (Glavey *et al.*, 2012; Sun *et al.*, 2017; Dalangood *et al.*, 2020). Considering the skeletal muscle tissue, we could not find information in the available literature whether ST3Gal6 is involved in any pathological or physiological condition.

In summary, we showed that the increased local biosynthesis of α-2,3-sialylated glycoconjugates in the Nurse cell of *Trichinella spiralis* is associated with up-regulation of the gene *Gne*, encoding the enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase, which is the key initiator of the sialic acid metabolic pathway. The elevated sialylation is, at least in part, due to an overexpression of α-dystroglycan - the only identified sialylated glycoprotein in skeletal muscles, bearing a sialyl-α-2,3-Gal-β-1,4-GlcNAc-β-1,2-Man-α-1-O-Ser/Thr glycan. These two events were in a correlation with up-regulation of *St3gal6* sialyltransferase that transfers sialic acid preferably onto Gal-β-1,4-GlcNAc as an acceptor, and thus it was considered as a suitable candidate for the sialylation of the α-dystroglycan. All these interconnected processes could be either part from a self-defense mechanism of the Nurse cell to remain integrated within the surrounding healthy skeletal muscle tissue, or an attempt for healing and regeneration of the distorted tissue.

**Conflict of Interest**

Authors state no conflict of interest.

Authors have no potential conflict of interest pertaining to this submission to Helminthologia

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