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Developmental glycomics of the *Ixodes ricinus* tick

Ph.D. Thesis

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Annotation

Ticks are increasingly important vectors of human and animal disease; however, the role of their glycans and glycoconjugates in host interaction, pathogen transmission and allergy remains insufficiently understood. This study describes the presence, localisation and origin of sialylated glycans in *Ixodes ricinus* ticks, their organs, and the tick cell line.

Using a combination of microscopic techniques, mass spectrometry, and biochemical analyses, sialylated glycans were found to localise predominantly in salivary glands and the gut, suggesting that most sialylated structures are host-derived and are transported from the blood meal through the gut to the salivary glands. Quantitative analyses of sialic acid, including its metabolically incorporated analogues, further demonstrate that most of the sialylated molecules in adult ticks originate from host blood rather than de novo synthesis, a conclusion supported by similar observations in tick cell cultures.

Despite earlier reports indicating minimal sialic acid presence in arthropods, genomic and biochemical analyses in this study identified active sialyltransferase genes and confirmed the tick's capacity to synthesise its own sialylated glycoproteins, further demonstrated through in vitro feeding experiments and Click chemistry-based confirming tick-origin sialylated glycoproteins in all life stages, from eggs and larvae to adult females.

Declaration

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references.

Jarmila Štěrbová
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15.7.2026

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

- I. Vancova, M., Sterba, J., **Dupejova, J.**, Simonova, Z., Nebesarova, J., Novotny, M.V., Grubhoffer, L. (2012). Uptake and incorporation of sialic acid by the tick *Ixodes ricinus*. J Insect Physiol. 58(9):1277-87. (IF = 2.37)
Jarmila Sterbova did western blot analysis and prepared glycans for MALDI-TOF/TOF analysis. Her contribution was 25 %.
- II. Sterba, J., Vancova, M., **Sterbova, J.**, Bell-Sakyi, L., Grubhoffer, L. (2014) The majority of sialylated glycoproteins in adult *Ixodes ricinus* ticks originate in the host, not the tick. Carbohydr Res. 389:93-9. (IF = 1.9)
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- III. Vechtova, P., **Sterbova, J.**, Sterba, J., Vancova, M., Rego, R.O.M., Selinger, M., Strnad, M., Golovchenko, M., Rudenko, N., Grubhoffer, L. (2018). A bite so sweet: the glycobiology interface of tick-host-pathogen interactions. Parasit Vectors. 11(1):594. (IF = 3.0)
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- IV. **Sterbova, J.**, Kocova, P., Pekarek, L., Selinger, M., Ondrus, J., Grubhoffer, L., Sterba, J. (2021). Click-on-Membrane for Detection of Metabolically Labelled Proteins and RNA. CHEMICKE LISTY, 2021, 115:662-668. (IF = 0.35)
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- V. **Sterbova, J.**, Grubhoffer, L., Sterba, J. (2026). Immunogenic and cross-reactive carbohydrate determinants in ticks and other arthropods. J Appl Biomed, 2026, 24:63-71. (IF = 2.0)
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Jarmila Sterbova prepared the original manuscript, participated in planning, conducting, and evaluation of the experiments, measured the sialyltransferase activity, did metabolic labelling of tick cell line, western blot analyses and fluorescent/confocal visualisation of NeuNAz in tick cells. Her contribution was 60 %.

Co-author agreement

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List of abbreviations

Ab – antibody
Abeta – amyloid-beta peptide
Ac4ManNAz – tetraacetylated ManNAz
ADCC – antibody-dependent cellular cytotoxicity
AGS – α -Gal syndrome
AMP – adenosine monophosphate
APP – amyloid precursor protein
BIF - B-cell inhibitory fraction
BIP - B-cell inhibitory protein
BmAP – *Boophilus microplus* anticoagulant protein
CC – carcasses
CCDs – cross-reactive carbohydrate determinants
CCHFV – Crimean-Congo haemorrhagic fever virus
CNS – central nervous system
CMAH – cytidine monophosphate-N-acetylneuraminic acid hydroxylase
CMAS – CMP-sialic acid-synthetase
CMD – congenital muscular dystrophy
CO₂ – carbon dioxide
Crb – Crumbs
DC-SIGN – dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DG – dystroglycan
DAD1 – defender against cell death 1
EBA – erythrocyte-binding antigen
Eys – Eyes shut
Fuc – Fucose
fV – coagulation factor V
fVIIa – activated factor VII
fXa – activated factor X
fXII – coagulation factor XII
Gal – galactose
GalNAc – N-acetylgalactosamine
GBP – glycan-binding protein
GlcA – glucuronic acid
GlcNAc – N-acetylglucosamine
GPI – glycosylphosphatidylinositol
GPIIb/IIIa – glycoprotein IIb/IIIa
Hex – hexose
HexNAc – N-acetylhexosamine
HA – hemagglutinin
HEF – hemagglutinin esterase fusion protein
HER – host extracellular receptor
HIV – human immunodeficiency virus
HRP – horseradish peroxidase
IgE – immunoglobulin E
IgG – immunoglobulin G
IgM – immunoglobulin M
IAV – influenza A virus
IFN – interferon
IL – interleukin
IP – immunoprecipitation
IpSAP – *Ixodes persulcatus* salivary protein
IRAC – *Ixodes ricinus* anticomplement protein
IRS – *Ixodes ricinus* serpin

IrSPI – *Ixodes ricinus* Serine Protease Inhibitor
ISAC – *Ixodes scapularis* anti-complement
IsC1ql3 – *Ixodes scapularis* complement C1q-like protein 3
IxsS41 – *Ixodes scapularis* salivary serpin 41
IxsS17 – *Ixodes scapularis* salivary serpin 17
Kdn – 2-keto-3-deoxynononic acid
LPS – lipopolysaccharide
LacNAc – lactose N-acetyllactosamine
MALDI – matrix-assisted laser desorption/ionization
Man – mannose
ManNAc – N-acetylmannosamine
ManNAz – N-azido-mannosamine
MERS-CoV – Middle East respiratory syndrome coronavirus
MGAT – mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase
MS – mass spectrometry
N-glycan – nitrogen-linked glycan
NA – neuraminidase
Neu – neuraminic acid
Neu5Ac – N-acetylneuraminic acid
Neu5Gc – N-glycolylneuraminic acid
NeuNAz – N-azidoneuraminic acid
OGA – O-GlcNAcase
OGT – O-GlcNAc transferase
OspA – outer surface protein A
OspC – outer surface protein C
OST – Oligosaccharyltransferase
OV – ovary
PNA – peanut agglutinin
PSA – *Pisum sativum* agglutinin
RGD – Arginine-Glycine-Aspartic acid
ROS – reactive oxygen species
Salp – salivary protein 20
SAT – saliva -assisted transmission
SG – salivary glands
Sia – sialic acid
SiaT – sialyltransferase
Siglecs – sialic acid-binding immunoglobulin-like lectins
ST6Gal1 – beta-galactoside alpha-2,6-sialyltransferase 1
ST6GalNAc1 – alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
STEC – Shiga toxin-producing *Escherichia coli*
TBA – thiobarbituric acid assay
TBEV – tick-borne encephalitis virus
TF – tissue factor
Th – T helper
TSA – Transcriptome Shotgun Assembly
TSLPI – tick salivary lectin pathway inhibitor
TSR – thrombospondin type 1 repeat
UDP – uridine diphosphate
UDP-GlcNAc – uridine diphosphate N-acetylglucosamine
WGA – wheat germ agglutinin
Xyl – xylose

1. Introduction

1.1 General background on *Ixodes ricinus* and its medical importance

1.1.1 Tick systematics

Ticks, together with mites, belong to the subclass Acari, class Arachnida, subclass Chelicerata, phylum Arthropoda. According to Guglielmone and co-authors (2010), the order Ixodida consists of 896 species divided into 3 families: Argasidae, Ixodidae, and Nuttalliellidae. Nuttalliellidae is a monospecific family represented by a single species, *Nuttalliella namaqua*, which is known only from specific areas of South Africa and Tanzania (Mans et al., 2011). The family Argasidae, sometimes called “soft ticks”, includes 193 species, distributed across the genera *Antricola*, *Argas*, *Nothoaspis*, *Ornithodoros*, and *Otobius*. The family Ixodidae, called also “hard ticks”, comprises 702 species in 14 genera: *Amblyomma*, *Anomalohimalaya*, *Bothriocroton*, *Cosmiomma*, *Cornupalpatum*, *Compluriscutula*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Margaropus*, *Nosomma*, *Rhipicentor*, and *Rhipicephalus* (formerly *Boophilus*). Argasidae and Ixodidae differ significantly not only on the molecular level but also morphologically, in their life cycles, feeding strategies, and the pathogens they transmit.

1.1.2 *Ixodes ricinus* and other tick species in the Czech Republic

Ixodes ricinus, the castor bean tick, is the most widespread and abundant tick in the Czech Republic. The distribution of *I. ricinus* extends beyond the Czech Republic into much of Europe, as well as neighbouring areas of North Africa and the Middle East, northward to Iceland and eastward into Russia. Its wide geographic range suggests it can live in diverse environmental conditions. *I. ricinus* occurs mainly in forests, grasslands, parks, and gardens, where suitable hosts are abundant. It prefers high-humidity areas and is largely absent from the Mediterranean, where hot and dry summers prevail. *I. ricinus* ticks don't have eyes, ornamental signs and lack festoons along the back part of the body. They possess a tough scutum that covers part of the female body and the whole male body (Sonenshine & Roe, 2014).

I. ricinus can transmit a variety of pathogens, mainly tick-borne encephalitis virus, *Borrelia burgdorferi* sensu lato (s.l.), *Anaplasma phagocytophilum*, *Babesia* spp., and *Rickettsia* spp. upon their infection while feeding on an infected host to another host in the next life-stage (transstadial transmission) or larvae upon infection of eggs by an infected female (transovarial transmission).

Another Ixodid tick present in the Czech Republic/Central Europe is *Dermacentor reticulatus* (ornate cow tick, meadow tick), associated with wetlands, meadows, and floodplains, currently expanding to the lowlands of the Czech/Slovak Republic, and a vector of *B. canis* and *R. raoultii*

(Kubelová et al., 2011); *D. marginatus* can also be found in Central Europe, but only rarely. Two bird-associated Ixodid species, *I. arboricola* and *H. concinna*, co-occur with the hedgehog tick, *I. hexagonus*, in the Czech Republic (Ghodrati et al., 2025). These animal-associated species can transmit *Rickettsia* spp. or, rarely *B. burgdorferi* s.l. (Spitalská et al., 2011).

1.1.3 Life cycle and feeding of Ixodidae

The family Ixodidae is defined by the presence of a scutum. This sclerotised shield covers part of the female's dorsal surface and the entire male's surface. Ixodidae undergo developmental stages: egg, larva, nymph, and male and female adults. Larva differs from the other active stages in having six legs, whereas both the nymphal and adult stages have eight.

I. ricinus is a three-host tick as each of the active life stages (larvae, nymphs, adult females) feeds on various hosts that range from insectivores, rodents, birds, pets, deer, to horses, and humans. Females after/during mating, ingest a significant volume of blood and increase the size of their bodies to more than 100 times their initial weight (Sauer et al., 1995). *I. ricinus* ticks and the vast majority of Ixodidae have a 3-host cycle (Fig. 1).

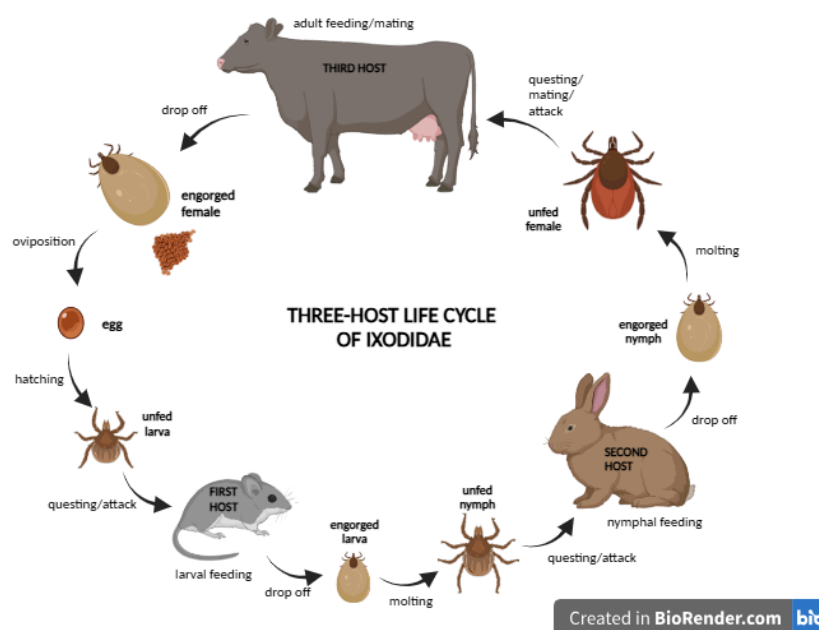


Figure 1. Three-host life cycle of Ixodidae. Larvae feed on a first host, drop off and moult into nymphs. Nymphs then find a second host, feed, detach, and moult into adults. Adult females feed on the third host, mate, and, once fully engorged, drop off the host to lay eggs before dying.

All stages that can suck blood feed on various animals and live freely between feeding intervals. Unfed larvae seek their first host, which is usually a rodent or a lizard. After feeding (2-4 days), larvae drop off from the host and moult to the nymphal stage. Unfed nymphs seek a host, may attach to the

same or a different host, feed (3-5 days), drop off, and moult to the adult stage. Adults find their host; females feed for 7-10 days and mate. After reaching full engorgement, females detach from their host, lay thousands of eggs, and then die. The entire life cycle typically spans 4 to 6 years, but only 2 to 3 years in the southern regions of its territory (Kahl & Gray, 2023). All three blood-sucking stages are parasitic and capable of transmitting tick-borne pathogens.

During evolution, ticks have developed mechanisms to enable prolonged feeding and to overcome the host's immune and defence responses. Ticks suppress host defence mechanisms during feeding through a sophisticated array of salivary molecules that target multiple components of the immune system (Ribeiro, 1995). They produce a range of molecules that suppress the host's natural blood-clotting system, ensure proper blood circulation, and prevent or modulate immune responses (summarised in detail in Brossard & Wikel, 2004; Ribeiro, 1987; Schoeler & Wikel, 2001; Wikel, 2013).

Several Ixodid ticks exhibit a two- or one-host cycle. A tick with a two-host life cycle stays on the host in the larval stage and moults into a nymph, which feeds, engorges, and drops off the host. A fed nymph moults into an adult in the environment. An adult tick attaches to a second host, feeds and mates. Females drop off to lay eggs and die. The representatives are, for example, *H. anatolicum* and *R. bursa*. In the case of one-host life cycle ticks, all parasitic stages (larva, nymph, and adult) feed and moult on the same individual host. The tick remains on that host continuously until the adult female drops off to lay eggs. The representatives include *R. microplus* and *D. nitens* (Apanaskevich & Oliver, 2014).

1.1.4 Feeding of Ixodidae

Generally, Ixodid ticks are considered 'slow feeders,' requiring several days or even weeks to become fully engorged. As a result, they feed on blood in a manner distinct from other blood-feeding parasites in the phylum Arthropoda. To successfully feed for such a long time, ticks must overcome mechanical, physiological and immunological barriers.

Starving ticks use Haller's organ to locate their hosts by detecting carbon dioxide (CO₂), heat, and other chemical cues. Once on the host, the tick's hypostome penetrates the host's skin. This structure is equipped with backwards-facing barbs that act as an anchoring mechanism, securely holding the tick in place while it feeds. The hypostome tears host tissues, creating a feeding lesion. As pool feeders, blood and tissue fluids accumulate around the mouthparts.

Detailed 3D reconstructions of the *I. ricinus* feeding apparatus, provided by Vancová and colleagues (2020), illustrated how the tick cuts and damages the host's skin and dermal vessels and cycles between salivation and blood uptake during engorgement. During feeding, saliva is injected into the lesion, while blood is aspirated into the pharynx without mixing with saliva. This process is enabled by coordinated muscular contractions of the salivarium and the pharyngeal valve (Vancová et al., 2020). Additionally, ticks of the Ixodidae family produce a cement-like substance that enhances their

attachment, allowing them to remain on the host and feed slowly for several days to weeks (Kemp et al., 1982).

In the first 24 hours of feeding, the body mass of the female tick increases only slightly. Then, a phase of slow engorgement occurs, during which a new cuticle is produced to accommodate all incoming blood meals. Over the last 12 to 36 hours, the female organism consumes most of its blood meal (Sonenshine et al., 2014). This allows it to ingest a significant amount of food and increase its body mass by 100-200 times its unfed weight. As a result, ticks must cope with oxidative stress, detoxify host-derived molecules, and restore tissues (summarised in Lou et al., 2026). Transcriptomic studies of *I. persulcatus* reveal significant shifts in metabolic pathways across the unfed, semi-engorged, and fully engorged stages (Lou et al., 2026).

The process of acquiring blood is not simply about consuming blood; it involves a precisely regulated, dynamic exchange of fluid, ions, and molecules between the parasite and the host, which is under the tick's control. The blood is concentrated 2–3 times, while excess water, Na^+ , and Cl^- are returned to the host by the salivary glands, as the main aim is to acquire sufficient nutrients (Bowman & Sauer, 2004). After the successful feeding, the female drops off the host and lays thousands of eggs. Male *I. ricinus* can attach to the host and take small amounts of blood, but their primary goal is to find a female to mate with.

1.1.5 Tick salivary glands, saliva composition, and immunomodulatory effects

Tick salivary glands

Tick salivary glands (SG) and saliva play central roles during tick feeding, host immune modulation, and pathogen transmission. Structurally, SGs consist of multiple cell clusters called acini, categorised into types I–IV depending on tick species and sex. These acini contain specialised cells that produce, secrete, and store saliva during feeding, which contribute to distinct physiological functions.

In Ixodid ticks, females possess acini types I, II, and III; males also have acini type IV (Kaufman, 1989; Kemp et al., 1982; Nuttall, 2019). Acini type I play an important role in water and ion absorption, particularly during periods when ticks are not feeding. They are responsible for maintaining water balance, allowing the ticks to correct fluid imbalance when they are not sucked into the host (Kaufman & Phillips, 1973a, 1973b, 1973c). During the feeding phase, type II and III acini enlarge and regulate the absorption of water and electrolytes from the surrounding haemolymph through their epithelial layer, while simultaneously secreting excess fluid back into the host (Fawcett et al., 1981; Meredith & Kaufman, 1973). Moreover, acini type II express cement proteins with many glycine residues that firmly anchor the tick to the host's epidermis, ensuring stable attachment for prolonged feeding (Kemp et al., 1982). Acini type IV play a role in male tick mating and contribute to the successful transfer of the

spermatophore to the female genitalia (Sonenshine & Roe, 2014).

Tick saliva composition and function

Tick saliva represents a unique liquid in which, after tick feeding, dozens to hundreds of host and tick bioactive molecules are mixed. It is rich in water and ions (Na^+ , Cl^-), derived from the host's blood. It also contains non-peptidic molecules, such as adenosine, prostaglandins, endocannabinoids, and microRNAs (Anatriello et al., 2017; Fezza et al., 2003; Hermance et al., 2019; Sá-Nunes et al., 2007). Additionally, tick saliva includes various tick peptides, such as variegins, hyalomins, and madanins (Iwanaga et al., 2003; Koh et al., 2007; Wu et al., 2010), as well as several tick proteins, including chitinases, mucins, ixostatins, cystatins, defensins, glycine-rich proteins, hyaluronidases, Kunitz-type proteins, lipocalins, and metalloproteases (Beaufays et al., 2008; Francischetti et al., 2002). Furthermore, it encompasses host proteins like immunoglobulins, haptoglobin, transferrin, and exosomes. As a complex biochemical cocktail produced by salivary glands during tick feeding, tick saliva accompanies important processes connected to tick feeding, pathogen transmission and host response.

During feeding, ticks acquire a large volume of host blood; for example, *D. marginatus* females can process 400 times their body weight in blood (Kaufman & Sauer, 1982). For this reason, maintaining homeostasis through the salivary glands and saliva is critical for Ixodid ticks. In females, the salivary glands function as key osmoregulatory organs, regulating internal hydrostatic pressure by eliminating excess water and electrolytes absorbed during blood feeding (Bowman et al., 2008). Moreover, SGs also produce rehydration saliva, which enables unfed ticks to absorb atmospheric water vapour and thereby avoid desiccation.

Soon after the hypostome penetrates the host skin, ticks produce a white substance that rapidly polymerises into a solid cement cone, sealing the feeding site, preventing fluid loss, and anchoring the tick for prolonged attachment (Gregson, 1960; Kemp et al., 1982). Different tick species also differ in the composition of the cement cone, although the glycine-rich cement proteins remain the main common component (summarised in Suppan et al., 2018). Other functions of cement-associated proteins include roles in embryo development (Leal et al., 2018) and in pathogen transmission, which are potentially interesting for anti-tick vaccines and biomedical adhesive applications (summarised in de Souza et al., 2024).

Regulation of host responses is an important part of saliva's functional repertoire. As a cocktail of various bioactive molecules, it can control host pain, blood clotting, inflammation, immune responses and wound healing. All these functions overlap, creating significant complexity. Before the beginning of feeding, ticks irritate the skin of the host using hook-shaped chelicerae and sink the chelicerae into the wound. Tissue damage activates coagulation factor XII (fXII), triggering a cascade that releases

bradykinin, which induces the pain sensation. To suppress painful skin disruption, ticks excrete analgesics, such as endocannabinoids and bradykinin inhibitors (Fezza et al., 2003; Ribeiro & Mather, 1998).

The components of tick saliva also suppress host haemostasis to maintain prolonged and uninterrupted feeding. By producing a bunch of anti-haemostatic molecules secreted into the wound, the whole process, including clot formation, is affected at several levels: inhibition of platelet function, disruption of the coagulation cascade, and promotion of fibrinolysis.

To prevent platelet adhesion, activation, and aggregation at the site of skin disruption, ticks produce molecules that inhibit ADP- (adenosine diphosphate), collagen-, and/or thrombin-induced aggregation. Extracellular ADP is one of the components on which platelet aggregation relies. *I. ricinus*, *I. scapularis*, and ticks from the group *Rhipicephalus spp.* express in their saliva apyrases that hydrolyse ADP and ATP (adenosine triphosphate) into AMP (adenosine monophosphate), thereby inhibiting platelet production (summarised in Stibrániová et al., 2013). Among other platelet aggregation inhibitors are disintegrins and RGD (Arginine-Glycine-Aspartic acid)-containing proteins. Many of these block α IIb β 3 (GPIIb/IIIa), such as Variabilin from *D. variabilis* saliva. Variabilin binds this integrin via its RGD motif and competitively blocks fibrinogen binding (Wang et al., 1996).

In the subsequent phase of haemostasis, both the extrinsic and intrinsic coagulation pathways, as well as clot stabilisation, are inhibited by tick salivary proteins that primarily target thrombin and factor Xa (fXa). These central enzymes are essential for the conversion of fibrinogen into fibrin and are effectively blocked during tick feeding by serine protease inhibitors (serpins), thereby preventing fibrin formation and maintaining blood fluidity at the feeding site (Blisnick et al., 2017; Chmelař et al., 2017).

A variety of inhibitors of factor X and factor Xa were identified in Ixodid ticks. *I. scapularis* protein Penthalaris inhibits the activation of fXa by the complex of tissue factor (TF) and activated factor VII (fVIIa). A new mechanism in thrombin initiation, the factor Xa (fXa)-activated factor V (fV) pathway, was described by the identification of the Tick Inhibitor of fXa towards fV protein (Schuijt et al., 2013). Additionally, *I. scapularis* proteins, including fXa inhibitor Salp14 and Ixolaris, that bind fX and fXa and inhibit the complex TF/fVIIa complex, likely act together to suppress coagulation at multiple points (Francischetti et al., 2002). Regarding thrombin inhibitors, several molecules have been described. *R. microplus* ticks produce Boopihilin, a Kunitz-type protease inhibitor that blocks both active site and exosite I of thrombin (Macedo-Ribeiro et al., 2008), and Microphilin, a 3 kDa inhibitor (Ciprandi et al., 2006), and *Boophilus microplus* anticoagulant protein (BmAP) (Horn et al., 2000). Moreover, Variegin (Koh et al., 2007) and Avathrin (Iyer et al., 2017) were described as components of *A. variegatum* saliva/salivary glands that function as thrombin inhibitors.

Tick modulation of the host immune system during the feeding

Besides enabling prolonged feeding, ticks have evolved mechanisms to overcome the host's immune defences (Ribeiro, 1995). The key players are immunomodulatory molecules secreted from tick saliva into the host during feeding. These bioactive molecules include anticoagulants, vasodilators, and immunomodulators. Vasodilators and anticoagulants maintain blood flow and inhibit clot formation at the feeding site; immunomodulatory proteins suppress host immune responses (summarised in Ribeiro, 1987; Sauer et al., 1995). Furthermore, the salivary glands and saliva serve as pathways for pathogen transmission.

In the early stage of feeding, immediately after the skin has been damaged by the tick's hypostome, the components of innate immunity migrate to the site of injury. In response to the host's early inflammatory immune response, ticks produce numerous immunomodulatory molecules. Sialostatin L from *I. scapularis* inhibits neutrophil infiltration (Kotsyfakis et al., 2006) while *I. ricinus* Serpin-2 (IRS-2) and Iripin-1 suppress neutrophil and monocyte recruitment (Chlastáková et al., 2023; Chmelar et al., 2011). *I. ricinus* Iripin-3 reduces inflammation by lowering macrophage IL-6 production (Chlastáková et al., 2021). Some molecules have more than one function, and some of them also affect adaptive immune responses. For example, Iristatin from *I. ricinus* inhibits adaptive immunity by blocking cathepsins, reducing ROS, and decreasing cytokine levels (Kotál et al., 2019). Ricistatin from *I. ricinus* and Sialostatin L from *I. scapularis* block dendritic cell maturation (Lieskovská et al., 2015; Martins et al., 2023; Sá-Nunes et al., 2009). Moreover, Sialostatin L suppresses interleukin-9 expression in mast cells and cytokine secretion (Klein et al., 2015). Ricistatin has been shown to decrease pro-inflammatory cytokines in macrophages (Martins et al., 2023), and *R. sanguineus* Evasins inhibit the movement of immune cells by targeting both CC and CXC chemokines. The combined action of these molecules results in lowered inflammation and accumulation of immune cells at the sites of tick bites (Déruaz et al., 2008; Frauenschuh et al., 2007).

The host immune response includes complement activation, which is counteracted by tick salivary molecules. CripA from *R. pulchellus* inhibits Properdin, whereas *I. ricinus* anti-complement proteins I and II (IRAC I, IRAC II), *I. scapularis* anti-complement (ISAC), and salivary protein 20 (Salp20), inhibit the alternative complement pathway (Braunger et al., 2022; Daix et al., 2007; Hourcade et al., 2016; Valenzuela et al., 2000). In addition, the *I. ricinus* serpins Iripin-5 and Iripin-8 also function as complement (Kascakova et al., 2021; Kotál et al., 2021).

During the prolonged feeding phase, adaptive immune responses are activated to evade an unwanted ectoparasite. However, tick saliva contains molecules that affect T cell and B cell responses in multiple ways. T cells can be suppressed by Sialostatin L and Iristatin (mentioned earlier), or by IrSPI (*Ixodes ricinus* Serine Protease Inhibitor) and IRS-2 (*Ixodes ricinus* serpin 2), which both suppress Th responses (Blisnick et al., 2019; Páleníková et al., 2015) or by Iripin-3, which reduces CD4⁺ T cell

survival and Th1 response (Chlastáková et al., 2021). The well-characterised *I. scapularis* Salp15 affects T cells on various levels. It blocks calcium signalling and transcription factors (NF-AT, NF- κ B), binds directly to CD4, and promotes the expression of CD73 and adenosine production. It also interacts with DC-SIGN on dendritic cells, thereby attenuating T cell activation, proliferation, and cytokine production (Anguita et al., 2002; Hovius et al., 2008; Tomás-Cortázar et al., 2017).

Salivary gland molecules also affect B cells, which play a significant role in the tick's antimicrobial immune response. During the feeding of Ixodid ticks infected with *B. burgdorferi*, the B-cell inhibitory protein (BIP) was found to interact with B cells. BIP was identified in *I. ricinus* saliva as the factor responsible for saliva-mediated suppression of B-cell proliferation. Hannier and colleagues (2003, 2004) showed that BIP suppresses LPS-induced B-cell proliferation by inhibiting B-cell responses to OspA and OspC. BIP was identified and described in *Hyalomma asiaticum asiaticum*, and its role as an inhibitor of LPS-induced murine B-cell proliferation was shown (Yu et al., 2006).

1.1.6 Ixodidae and their role as a vector of pathogens

Generally, Ixodid ticks are highly effective carriers of pathogens, and their transmission mechanisms are closely linked to their feeding behaviour and saliva composition. They infest every class of terrestrial vertebrates – including mammals, birds, reptiles, and amphibians. Among arthropods, they transmit the greatest variety of disease-causing medically and veterinary important pathogens.

Tick-borne viruses transmitted by Ixodida include several medically important members of different viral families. The pathogen repertoire includes mostly RNA viruses that can cause encephalitis or haemorrhagic disease. Within the family Flaviviridae, ticks transmit tick-borne encephalitis virus (TBEV), mainly transmitted by *I. ricinus* and *I. persulcatus*, which causes encephalitis and represents a major public health problem in Central and Eastern Europe and Russia. Other flaviviruses transmitted by ticks include Powassan virus, vectored by *I. scapularis* in North America (Ebel, 2010; Hermance & Thangamani, 2017), as well as Omsk haemorrhagic fever virus transmitted by genus *Dermacentor* (summarised by Růžek et al., 2010) and Kyasanur Forest disease virus vectored by genus *Haemaphysalis* (Chakraborty et al., 2019), which are responsible for severe febrile and haemorrhagic illnesses in endemic regions.

Within the family Nairoviridae, ticks, particularly species of the genus *Hyalomma*, transmit Crimean-Congo haemorrhagic fever virus (summarised in Gargili et al., 2017), a highly pathogenic virus that causes severe haemorrhagic disease with high fatality rates. The family Phenuiviridae, which includes additional tick-borne viruses such as severe fever with thrombocytopenia syndrome virus, is transmitted by *H. longicornis* and *R. microplus* (Yu et al., 2011), and Heartland virus is transmitted by *A. americanum* (Pastula et al., 2014), both associated with acute febrile illness, thrombocytopenia, and

leukopenia. Finally, Ixodid ticks also transmit viruses from the family Reoviridae, most notably Colorado tick fever virus transmitted by *D. andersoni* (Florio et al., 1950), which causes a biphasic febrile illness and can be associated with neurological complications.

Among the bacterial pathogens, Ixodid ticks serve as vectors for the *Borrelia burgdorferi* sensu lato complex (*B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*), the causative agents of Lyme borreliosis, as well as for Relapsing fever *Borrelia*, such as *B. miyamotoi* or *B. recurrentis*. Another major group comprises bacteria of the order Rickettsiales, such as *Anaplasma phagocytophilum*, responsible for human granulocytic anaplasmosis, and *Ehrlichia chaffeensis* and *E. ewingii*, which cause different forms of ehrlichiosis. In addition, numerous species of the genus *Rickettsia* are transmitted by Ixodid ticks, including *R. rickettsii*, *R. conorii*, and *R. helvetica*, leading to spotted fever and related rickettsioses. Ticks also serve as vectors for bacteria of the family Francisellaceae, most notably *Francisella tularensis*, the etiological agent of tularaemia. Furthermore, they act as natural reservoirs for *Coxiella burnetii*, a bacterium that causes Q fever; although ticks play an important role in maintaining this bacterium in nature, transmission to humans occurs predominantly via inhalation of contaminated aerosols (summarised in Sonenshine & Roe, Chapters 8, 9, 10, 2014).

Hard ticks transmit several protozoan pathogens belonging mainly to the phyla Apicomplexa and Kinetoplastida. Among the Apicomplexa, *Babesia* spp, including *B. microti*, *B. divergens*, and *B. venatorum*, are of major medical importance, as they cause babesiosis, a disease characterised by fever, haemolytic anaemia, and flu-like symptoms. In addition, *Theileria* spp., which are also transmitted by hard ticks, are of major veterinary importance, causing severe and often fatal diseases in domestic and wild ruminants (Pérez de León et al., 2014).

Pathogens can be transmitted transstadially, transovarially, via saliva-assisted and co-feeding transmission. Additionally, Ixodid ticks can carry multiple pathogens simultaneously, leading to co-infection. Pathogens enter the midgut of ticks when ticks feed on infected hosts. Bacterial pathogens multiply in the gut, then they cross the gut epithelium into the hemocoel and further to the salivary glands. Viral pathogens try to escape from the gut as soon as possible to avoid digestion and tick immune responses. This step is critical for the viruses. If viral pathogens evade tick immunity and reach the salivary glands, they replicate there. For all types of pathogenic agents, SGs are the main site where pathogens settle and wait until the subsequent feeding, when they are released into the new host. The extended feeding period allows pathogens to be transferred either from host to tick or from tick to host. The main helper in this process is tick saliva, which contains a mixture of bioactive molecules that help enhance infectivity, suppress host immune responses and create a favourable environment for pathogen survival and transmission. This process is called saliva-assisted transmission (SAT).

Pathogens manipulate both tick biology and host immunity to promote their own survival, transmission, and replication. For instance, Kim and colleagues (2021) demonstrated that *I. scapularis*

salivary protein levels rise following infection with *B. burgdorferi* and Hoxmeier and colleagues (2017) identified 114 metabolites differentially regulated in *B. burgdorferi*-infected versus uninfected *I. scapularis* nymphs. *B. burgdorferi* alters tick metabolism, leading to the production of salivary proteins such as IsC1ql3 (*Ixodes scapularis* complement C1q-like protein 3), TSLPI (tick salivary lectin pathway inhibitor), and IpSAP (*Ixodes persulcatus* salivary protein), which suppress host immune responses by interfering with complement activation and T cell signalling (Jin et al., 2022; Schuijt et al., 2011; Tang et al., 2022).

Moreover, Salp15 and its homologs bind to *Borrelia* surface proteins, enabling the spirochetes to evade antibody-mediated killing (Bierwagen et al., 2021). In addition, the tick serpins IxsS41 (*Ixodes scapularis* salivary serpin 41) and IxsS17 (*Ixodes scapularis* salivary serpin 17) inhibit inflammatory and complement-related proteases, which further protect *Borrelia* and support host colonisation (Bencosme-Cuevas et al., 2023; Nguyen et al., 2024).

Several tick-borne viruses utilise tick saliva to suppress host immunity and enhance transmission. Tick saliva rapidly reduces early skin inflammation and antiviral T cell responses, thereby facilitating viral replication (Maqbool et al., 2022). Tick-borne encephalitis virus (TBEV) and Crimean-Congo haemorrhagic fever virus (CCHFV) interact with dendritic cells to modify adaptive immune response (Lieskovská et al., 2018; Rodriguez et al., 2018). Moreover, TBEV interferes with sphingomyelinase D in *I. scapularis*, leading to lipid accumulation that supports *membrane-associated* viral replication (Regmi et al., 2020).

Non-protein components of tick saliva, such as extracellular vesicles and microRNAs, can alter host immunity during both bacterial and viral infections. For instance, extracellular vesicles from *I. scapularis* can interact with macrophages, thereby enhancing the ability of *A. phagocytophilum* to infect the host. On the other hand, they help reduce *F. tularensis* infection (Oliva Chávez et al., 2021). Furthermore, tick saliva microRNAs (miRNAs) influence host responses during Powassan virus infection, possibly facilitating viral dissemination (Hermance et al., 2019).

Several studies also showed the importance of salivary gland components during the transmission/infection of protozoan pathogens. A study performed by Antunes and colleagues (2018) identified 36 genes in *R. bursa* salivary glands that were differentially regulated during infection with *B. ovis*. Silencing three genes for vitellogenin-3, a cement protein, and lachesin led to higher tick mortality, unsuccessful tick attachment, and reduced *Babesia* levels present in tick SG.

1.2 Glycobiology Basics Relevant to Arthropods and Vertebrates

1.2.1 Glycosylation

The fundamental principle, known as the central dogma, accompanies research in molecular biology. It states that biological information is carried from DNA to RNA to protein. This concept is unique because it is precision-based on the DNA template, enabling us to manipulate one type of molecule by understanding another. Many proteins are, after translation, converted into glycoproteins in the ER by covalent attachment of short oligosaccharide side chains. This process is carried out by glycosylation enzymes (glycosyltransferases and glycosidases), and it is one of the major co- and post-translational processes in proteosynthesis in Eukaryotes.

Glycosylation (the attachment of glycans/saccharide chains) is a common and important protein modification that occurs in more than 50 % of all proteins in eukaryotic organisms, and it also rarely occurs in Eubacteria and Archaea. The biological roles of glycans include functions crucial to the development, growth, functioning, or survival of the organism (Varki et al., 1999). The conformation of monosaccharide rings, their orientation around a glycosidic bond, and the placement of side chains attached to these rings affect molecular shape, distribution of partial charges, intra- and inter-residual hydrogen bonding, solvent accessibility, and the capacity to interact with various biomolecules (Rao, 2019). The variability of glycans is so great and has such potential that it is possible to observe different glycosylation patterns even between evolutionarily related organisms, and these patterns differ even further between tissues within the same organism.

The biological functions of glycans are diverse, ranging from those of little importance to those essential for the development, growth, functioning, or survival of the organism that produces them. The functions of glycans can be divided into two categories: modifications of protein structure and specific interactions of glycans with other molecules. In some cases, glycosylation appears to enhance a protein's primary function rather than, for example, triggering or blocking it. On the other hand, glycosylation enhances the basic properties and functions of proteins (summarised in Varki et al., 1999).

1.2.2 Types of glycosylation

The linkage between the first monosaccharide of a glycan part and the amino acid of a protein part defines the glycosylation class. The linkages could be via the nitrogen atom of the Asn amino group (N-glycans) or the oxygen atom of the Ser or Thr hydroxyl group (O-glycans). N- and O-glycosylation are the most common types and contain most of the glycosylation machinery associated with the pathogenesis and progression of disease.

O-glycosylation

The O-glycosylation process is initiated in the endoplasmic reticulum (ER) or in the Golgi apparatus (GA) with the addition of a glycan to Ser/Thr residues by GalNAc transferases. The first GalNAc can be extended by various monosaccharides, and the formed disaccharide represents the core structure of eight basic mucin-type glycan classes. Depending on the first glycan being attached, the glycans form groups as mucin-type O-GalNAc (N-acetylgalactosamine), O-GlcNAc (N-acetylglucosamine), O-Mannose (O-Man), O-Fucose (O-Fuc), O-Galactose (O-Gal), and O-Glucose (O-Glc) glycans (Patsos & Corfield, 2009). Sialic acid is often added to O-glycans at the final stages of biosynthesis, resulting in the formation of sialylated O-glycans.

O-GalNAc (Mucin type)

Mucins are classified among glycans characterised by the addition of GalNAc to the protein chain. The length of O-GalNAc glycans varies from a single GalNAc residue to more than twenty sugar residues and may include blood group and other glycan epitopes (Patsos & Corfield, 2009). The attachment of GalNAc to Ser/Thr is regulated by O-GalNAc transferase, which creates an O-glycosidic bond. Typically, mucins are involved in normal cell-cell and cell-matrix interactions, moisture retention, and barrier formation in the respiratory, gastrointestinal, and urogenital tracts; however, abnormal glycosylation occurs in various tumours, autoimmune disorders, and neurodegenerative diseases (summarised in Yue et al., 2023; Zhang et al., 2022).

O-GlcNAc

O-GlcNAc glycosylation is a dynamic modification of proteins with nucleolar, mitochondrial, and cytoplasmic localisation in various multicellular organisms. During this process, O-GlcNAc is added/removed rapidly due to the activity of O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) (Hart, 1997; Hart et al., 1989). OGT adds the GlcNAc moiety to proteins using UDP-GlcNAc as the donor sugar, while OGA removes it, rendering this modification reversible and highly dynamic (Varki & Press, 2022). O-GlcNAc-type glycosylation was documented in fungi, worms, insects, plants, and humans. It seems that O-GlcNAc is also present in several prokaryotes, such as *Listeria monocytogenes* (Schirm et al., 2004), but OGT and OGA enzymes are absent in yeast *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* (Kreppel et al., 1997; Lubas et al., 1997).

O-Man

O-mannosylated glycans were reported in various species, from fungi to humans (summarised in Haltiwanger et al., 2022). O-Man can be further decorated with various monosaccharides, such as

GalNAc, GlcNAc, Sia, and Fuc. It is characteristic of certain mammalian tissues (neural tissues), where it represents one-third of all O-glycans. Changes in O-mannosylation in humans are also connected with several diseases. For example, impaired O-mannosylation of α -dystroglycan leads to several types of congenital muscular dystrophy (CMD), and the loss of these glycan structures has been associated with cancer metastasis (summarised in Losasso et al., 2000; Martin, 2007). The structures of O-Man-type glycans vary significantly. Mammalian O-Man glycans can be complex or simple, whereas in yeast, they form poly-mannose chains. Nevertheless, the O-Man glycans in yeasts and prokaryotes are poorly understood to date.

O-Gal

O-galactosylated structures commonly decorate hydroxylysine residues in collagen or proteins with collagen-like structures. The monosaccharide is attached by galactosyltransferase in the Golgi apparatus (GA). The glycan modification often continues by the attachment of Glc upon formation of the disaccharide Glc α 1-2Gal β - (Spiro, 1967). The O-Gal modification is crucial for maintaining collagen stability and ensuring the integrity of the extracellular matrix. O-Gal has been identified in collagens from different organisms, including invertebrates (e.g., Muir & Lee, 1970; Spiro & Bhoyroo, 1980), bacteria (Gerwig et al., 1993), plants (Lamport et al., 1973), and humans (summarised in Haltiwanger et al., 2022).

O-Fuc

L-Fuc is added to the Ser/Thr in the specific domains of the protein backbone. The process takes place in the ER with the help of fucosyltransferases that mainly target Epidermal Growth Factor-like domains in metazoans (Luo & Haltiwanger, 2005), Thrombospondin Type 1 Repeats (TSR) (Hofsteenge et al., 2001), and newly found Elastin Microfibril Interface domains (Houlahan et al., 2024). O-fucosylation impacts the folding and secretion of proteins containing TSR, including Notch receptors, and affects ligand binding and signalling, which, in turn, influence cell fate, proliferation, and differentiation. Defects in O-Fuc lead to the formation of congenital disorders, bone malformations, and cancers.

C-mannosylation

C-mannosylation is a unique type of glycosylation in which mannose is attached to the indole ring of the first Trp in the sequence Trp-Xxx-Xxx-Trp/Cys via a rare C-C bond in the ER (Krieg et al., 1998). The process is catalysed by C-mannosyltransferase, originally discovered in *C. elegans* (Buettner et al., 2013). Later, several homologs were found in mammals and other animals, but not in bacteria and yeast. C-mannosylated proteins belong mainly to the TSR and type I cytokine families (summarised in

Haltiwanger et al., 2022). There is limited knowledge about the biological function of C-mannosylation, but it is known to influence protein folding, stability, and secretion (Shcherbakova et al., 2019). In *Plasmodium*, C-mannosylation is crucial for transmission (Lopaticki et al., 2022), and in humans, it may serve as a receptor in some types of cancer (Kobayashi et al., 2025; Mizuta et al., 2019).

N-glycosylation

N-glycan biosynthesis is a complex process that occurs in the ER and the GA. N-glycans are bound via GlcNAc to the amide nitrogen in the Asp side chain in a specific signal sequence, called a sequon. In eukaryotic organisms, this sequence is Asn-Xxx-Ser/Thr (Marshall, 1974), where Xxx can be any amino acid except Pro. N-glycans may be of the oligomannose, complex, or hybrid type, depending on monosaccharides attached to the first GlcNAc and their bonding.

N-linked glycans biosynthesis

Synthesis of Dolichol-linked oligosaccharide precursor

The process consists of several key steps and starts with the synthesis of the Dolichol-linked oligosaccharide precursor (Dwek, 1996; Lis & Sharon, 1993; Mitra et al., 2006). Two GlcNAc residues are added to Dol-P, followed by the addition of five mannose residues, forming Dol-PP-GlcNAc₂Man₅. The glycolipid is then flipped to the ER lumen, where four more mannoses and three glucoses are added, creating the final Dol-P-P-GlcNAc₂Man₉Glc₃ precursor (Helenius & Aebi, 2004; Kornfeld & Kornfeld, 1985). Oligosaccharide precursor is then transferred to the Asn within the sequon Asn-X-Ser/Thr by Oligosaccharyltransferases (OSTs). In Eukaryotes, OST is a complex of transmembrane subunits that is highly conserved from yeast to humans. The yeast OST complex was among the first to have its individual components identified (Karaoglu et al., 1997; Spirig et al., 1997). It possesses several subunits (Ost1p, Ost2p, Ost3p/Ost6p, Ost4p, Ost5p, Stt3p, Wbp1p, and Swp1p) that have homologs in mammals (ribophorin I, DAD1, N33/IAP, OST4, STT3A/STT3B, Ost48, and ribophorin II) (summarised in Kelleher & Gilmore, 2006). The STT3 subunit possesses catalytic activity, while other components aid in stability, substrate delivery, and regulation of specificity (Roboti & High, 2012). STT3 has STT3A and STT3B isoforms. STT3A attaches N-glycans to the nascent proteins during translation (co-translationally). STT3B can attach glycans to the sequons that STT3A skips during translation, or it can add N-glycans to the sequons located at the C-terminus after translation is complete (post-translationally) (Ruiz-Canada et al., 2009).

Trimming and elongation, oligomannose type N-glycans

After the attachment of the tetradecameric N-glycan precursor to the protein, further reactions are required to trim and consequently elongate the glycan to produce the final structure. Trimming starts

in the lumen of the ER almost immediately after the attachment of the oligosaccharide precursor to the nascent protein, where the α -glucosidases I and II sequentially remove glucose molecules. α -glucosidase I hydrolyses the terminal α 1,2-linked glucose; α -glucosidase II removes the two inner α 1-3 glucoses; and the proteins are controlled to ensure they fold correctly by the calnexin/calreticulin system. If the protein is properly folded, α 1,2-mannosidase cleaves the terminal mannose from the central arm to obtain $\text{Man}_8\text{GlcNAc}_2$, after which the protein is translocated into the Golgi apparatus. Trimming of α 1-2Man residues proceeds in the cis-Golgi by MAN1A1, MAN1A2, and MAN1C1 mannosidases. The resulting $\text{Man}_5\text{GlcNAc}_2$ is called the core structure of N-glycans and is a crucial intermediate in the process of producing hybrid and complex N-glycans. Some $\text{Man}_{5-9}\text{GlcNAc}_2$ glycans are not processed in the cis-Golgi and remain as oligomannose-type N-glycans attached to the mature proteins (Zuber & Roth, 2009).

Complex and hybrid type N-glycans

The biosynthesis of hybrid and complex N-glycans starts in the medial-Golgi with the MGAT1 transferase, which attaches a GlcNAc in an α 1-3 bond to Man of the core. Then, α -mannosidase II enzymes trim N-glycans by removing specific terminal α 1-3Man and α 1-6Man, forming $\text{GlcNAcMan}_3\text{GlcNAc}_2$. This trimming can only occur if MGAT1 has acted first. Next, MGAT2 attaches another GlcNAc in an α 1-6 bond to Man in the other arm of the core to create the precursor for complex N-glycans, $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$. Hybrid N-glycans form if α -mannosidase II does not act on the glycan, resulting in hybrid forms like $\text{GlcNAcMan}_4\text{GlcNAc}_2$. If peripheral GlcNAc is cleaved, paucimannose glycans are formed (Stanley et al., 2022). They are present in plants, in invertebrates, and in vertebrates. In mammals, their levels can be increased in response to cancer, inflammation, and during the development of stem cells.

Di-antennary complex N-glycans are formed by prolonging the two branches of the $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ glycan, while complex glycans with more branches are the result of further branching by the addition of Man in α 1-3 (MGAT4A, MGAT4B, MGAT4C) or α 1-6 bonds (MGAT5) (Abdel Rahman et al., 2015).

Maturation of N-Glycans

During the maturation of N-glycans in the Golgi complex, monosaccharides can be attached to the glycan core and/or the GlcNAc branches can be extended. In most of the complex and hybrid glycans, branch extension occurs through the addition of Gal to the initiating GlcNAc. The resulting $\text{Gal}\beta$ 1-4GlcNAc (LacNAc) may be added sequentially. β -linked GalNAc, instead of Gal, is attached to the initiating GlcNAc, forming the LacdiNAc (Stanley et al., 2022). Elongated branches can be further decorated. The monosaccharides usually present in animal N-glycans are Man, Gal, Glc, GlcNAc,

GalNAc, Fuc, and sialic acids, which are usually formed by α -linkages.

Moreover, further addition of these monosaccharides during glycan maturation expands the limited repertoire and creates a wide range of hybrid and complex mature glycans that affect various biological processes.

Fucosylation is a typical modification. In vertebrates, fucose is typically attached to the terminal or internal Gal/GalNAc residues, or to internal GlcNAc in various types of glycans found in glycoproteins and glycolipids. Vertebrate N-glycans typically undergo fucosylation at the N-glycan core in the α 1-6 position by FUT8. Fuc can also be added as a terminal saccharide, preferentially to the α 1-2, α 1-3, or to the α 1-4 position (Grewal et al., 2021).

1.2.3 Sialic acid

Sialic acids (Sia) are a type of α -keto acid monosaccharide that consists of a 9-carbon backbone. To this date, more than 90 forms of Sia have been documented (summarised in Guin et al., 2022). They are found across a wide range of organisms, primarily in higher Eukaryotes, especially mammals and humans. Sia is widely found on the surfaces of vertebrate cells as a component of glycoproteins, glycolipids, and oligosaccharides. They play key roles in neural transmission, immune response, and cellular recognition, and are also present at lower levels in invertebrates (Ghosh, 2018; Paschinger & Wilson, 2019), microorganisms (Dudek et al., 2022), and microalgae (Wagstaff et al., 2018).

Diversity of sialic acids

The complete repertoire of sialylated glycans on a particular cell is called the sialome. Sia is attached terminally to the N-glycans, O-glycans, glycolipids, and GPI anchors. Even within the same organism, the sialome can differ between cell types, tissues, or regions of the same cell. This high variability is evident at both the structural and functional levels; it is dynamic, and the composition of the sialome can change in response to environmental changes. The complexity of Sia occurs at several levels: Sia structure, linkage, the underlying glycan chain, glycan class, and spatial organisation at the cell surface (Cohen & Varki, 2010; Lewis & Varki, 2009).

Sia synthesis and structure

All Sia have a common core structure comprising a carboxyl group at C1, giving it a negative charge at physiological pH, and a glycerol side chain at C6–C7–C8–C9. The most commonly occurring Sia structures are 2-keto-3-deoxynononic acid (Kdn) and two derivatives of neuraminic acid: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). All of them have a nine-carbon structure with the difference at the C5 position in Kdn and Neu (Fig.2).

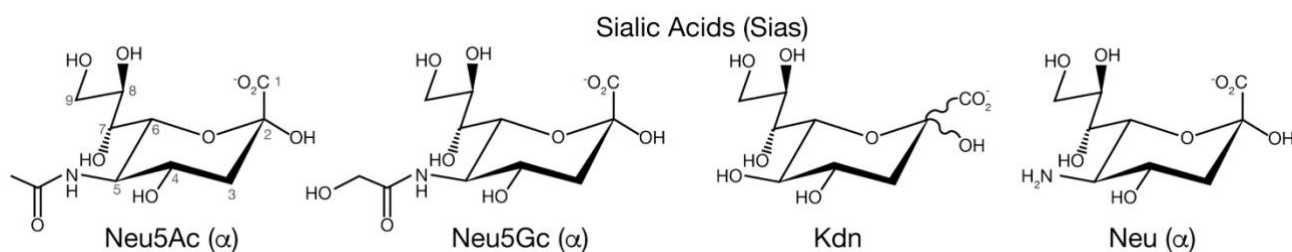


Figure 2. The most common types of neuraminic acid (adapted from Lewis et al., 2022).

Sia synthesis

In vertebrates, NeuAc synthesis begins when the precursor UDP-GlcNAc is converted to ManNAc-6-P by UDP-GlcNAc-2-epimerase/N-acetylmannosamine kinase (Varki & Press, 2022). Then, Neu5Ac-9-P is formed after the reaction of ManNAc-6-P with phosphoenolpyruvate by Neu5Ac-9-P synthase. Finally, the phosphate group is removed from the molecule, and Neu5Ac is released into the cytoplasm. To form Kdn using the same pathway, Man-6-P is used instead of ManNAc-6-P.

Modifications of Sia

2-keto-3-deoxynononic acid, Kdn

Kdn is also referred to as deaminated neuraminic acid. It is biosynthesised *de novo* from mannose. Man is converted to Man-6-P, which is then further converted to CMP-Kdn, an activated form. Using Kdn transferases, Kdn is transferred to an acceptor glycan to a terminal position. Kdn is widely present in amphibian eggs (Iwasaki et al., 1990; Maes et al., 1995; Strecker et al., 1992), fish gametes (Nadano et al., 1986; Song et al., 1991), and skin mucus (Kimura et al., 1994; Nakagawa et al., 2001). Moreover, Kdn was found in capsules and cell envelopes of pathogenic bacteria (Gil-Serrano et al., 1998; Knirel et al., 1989), in toxin-producing microalgae (Wagstaff et al., 2018). In mammals, Kdn represents a relatively uncommon type of Sia. It was found in pig submaxillary glands (Inoue et al., 1996) and in rat liver (Angata et al., 1998). In humans, it exists as a free glycan, with elevated levels in foetal red blood cells and cancer cells (Inoue et al., 2011; Inoue et al., 1998; Yabu et al., 2013) compared to maternal and normal cells. Its location indicates that it may play a role in various cancer types, as well as in reproduction and embryonic development.

Neuraminic acid

Neuraminic acid is widely distributed in vertebrates, including humans. In vertebrates, two variants predominantly occur: N-acetylneuraminic acid and its hydroxylated form, N-glycolylneuraminic acid (Fig. 3) (Angata & Varki, 2002).

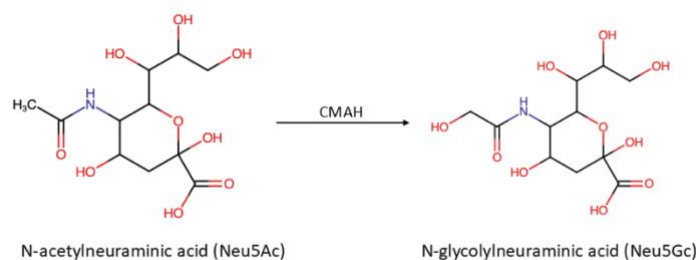


Figure 3. Two predominant forms of sialic acid in vertebrates – N-acetylneuraminic acid and its derivative N-glycolylneuraminic acid, formed by CMP-N-acetylneuraminic acid hydroxylase (CMAH) (adapted from Sterbova et al. 2026a).

N-acetylneuraminic acid, Neu5Ac

N-acetylneuraminic acid is the most prevalent Sia (Lundgren & Boddy, 2007; McNaught, 1997). The backbone is chemically composed of nine carbons with a carboxyl group and an N-acetyl group that plays a crucial role in facilitating hydrophobic interactions. The side chain C7-C9, with a hydroxyl group attached to each carbon, enables hydrogen bonding. Each of these components plays a role in the binding specificity of Sia-binding proteins and the functions of glycans containing Sia. Neu5Ac is found in a wide range of organisms, from bacteria and fungi to protozoa and higher animals, including humans (summarised in Ghosh, 2018).

It is a predominant glycan in higher animals, including humans. As a crucial component of cell surface molecules, secreted glycoproteins, glycolipids, and bacterial surfaces, it mediates various intracellular interactions, including cellular recognition, cell-cell interactions, and cell-pathogen interactions.

N-glycolylneuraminic acid, Neu5Gc

Neu5Gc is a sialic acid whose presence across organisms is largely determined by whether they encode a functional Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) enzyme, which converts CMP-Neu5Ac to CMP-Neu5Gc. Neu5Gc is widely produced by most mammals, but it is also present in many fish, amphibians, reptiles, and birds. CMAH enzyme has been found in Echinoderms, such as starfish and sea cucumbers (Gollub & Shaw, 2003; Peri et al., 2018). Among organisms that do not synthesise Neu5Gc are Old World monkeys and apes, which lost the ability to synthesise it during evolution. Humans possess a nonfunctional CMAH pseudogene with a fixed single-exon deletion (Varki, 2001). As a result, humans do not synthesise Neu5Gc endogenously; however, small amounts are present in human cells and tissues (Bardor et al., 2005; Tangvoranuntakul et al., 2003). These traces come from ingested food, as Neu5Gc levels in mammals are significant. Healthy humans then possess anti-Neu5Gc antibodies that can interact with another Neu5Gc derived from food.

The triggered immune response, called xenosialitis, may contribute to the development of inflammation and atherosclerotic cardiovascular disease (Kawanishi et al., 2019). Moreover, Neu5Gc can contribute to the elevated risk of colorectal cancer progression (Lopes et al., 2025; Samraj et al., 2018; Samraj et al., 2015). Due to these consequences, Neu5Gc is used as a cancer marker.

Sia linkage

The complexity of the sialome partly arises from various α -linkages between Sia C-2 and the underlying structures; Sia can be attached via α 2-3 linkage to Gal or via α 2-6 linkage to Gal/GalNAc, or via α 2-8 to another Sia (Lewis et al., 2022). The attachment is mediated by various sialyltransferases. The differences between linkages reflect the different recognition by glycan-binding proteins (GBPs). Moreover, a combination of Sia attachment and specific modification of underlying glycans, such as the attachment of Fuc or sulphate groups, together with the possibility of attachment of such epitopes to different glycan classes (N-glycans, O-glycans, glycosphingolipids), contributes to increased diversity. The type of glycan class can influence the presentation, localisation, and density of Sia. For example, erythrocyte-binding antigen EBA-175 from malaria parasite *Plasmodium falciparum* binds specifically to Neu5Ac α 2-3Gal on O-linked tri- and tetra-saccharides present in the mucine domain of glycophorin A of human erythrocytes (Tolia et al., 2005).

Additional Sia derivatives, originating from the primary core structures mentioned above, can arise by modification at one or more -OH groups located on C-4, C-7, C-8, and C-9 with O-acetyl, O-methyl, O-sulphate, O-lactyl, or phosphate groups (summarised in Cohen & Varki, 2010). All these modifications can influence how Sias are recognised by proteins, as well as their biological roles and recognition by pathogens or immune cells.

Sia functions in mammals/humans

Sia-containing glycans belong to the most abundant glycans of mammals, predominantly decorating the surface of almost every mammalian cell. Their strategic location, abundance, and chemical properties enable them to perform a wide range of biological functions. Sias at the terminal positions of various molecules functions as an imaginary bridge between cells and their extracellular matrix. Sialoglycans affect biological processes via carbohydrate-protein interactions and/or their negative charge. The negative charge characteristic of Sias creates electrostatic repulsion, giving the cells anti-adhesive properties. For example, it helps red blood cells move freely through blood vessels without interacting with other molecules or with each other (Eylar et al., 1962; Pollack & Reckel, 1977). Sia is also commonly present at high density on mucins as part of O-GalNAc-type glycans in the airways, gastrointestinal and urogenital tract. The hydrophilic properties of O-GalNAc, along with the anionic charge of Sia, allow them to serve as a hydrating and protective barrier at tissue surfaces in

contact with the environment (Lewis et al., 2022).

Sialylation also plays an indispensable role in the vertebrate immune system at multiple levels. Receptors for Sia recognise sialylated molecules as “self,” inhibiting immune system activation and preventing the immune system from attacking its own tissues. Firstly, a well-known mechanism involves Factor H, which binds to “self” cell surfaces and thereby suppresses activation of the alternative complement pathway (Blaum et al., 2015). Notably, the type of glycosidic bond through which Sia is connected to the glycan affects the effectiveness of Factor H binding (Johnston et al., 2008; Ram et al., 2011). Secondly, Sia receptors known as Siglecs (Sialic acid-binding immunoglobulin-like lectins) expressed on immune cells can influence immune response regulation. Siglecs are present on macrophages and leukocytes, and their activation may either suppress or promote inflammation (Angata et al., 2022). Thirdly, the interaction between Sia and selectins (heavily glycosylated membrane proteins) is essential for guiding the leucocytes during the early steps of their movement during the immune response (Sperandio, 2012).

And finally, the increased sialylation of Fc fragments of immunoglobulin G (IgG) negatively affects their functionality. IgGs can mediate Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (Scallon et al., 2007). Moreover, IgG Fc fragments with α 2,6-linked Sia exhibit enhanced anti-inflammatory effects (Anthony & Ravetch, 2010).

Sia is also a promising drug target for the treatment of cancer, viral infections, and neurological disorders at multiple levels. In cancer, Sia-based strategies focus on targeted drug delivery, nanoparticle conjugation, and the development of antimetastatic drugs. In viral infections, Sia-based drugs inhibit viral entry and sialidase activity. Sias are further investigated as markers of inflammation, for example, in Alzheimer’s and autoimmune diseases.

Sia in cancer

Sialylation is a player with multiple roles in the whole cancer process, with an abnormal sialylation as a typical feature of cancer cells. The upregulation of sialyltransferase expression that causes hypersialylation guides cancer progression, metastasis, and immune evasion in a variety of cancer types (breast, lung, pancreatic). For these reasons, Sias are an excellent candidate as biomarkers and therapeutic targets.

The key feature of cancer progression is increased levels of α 2,6-sialylation caused by the overexpression of ST6Gal1 (α 2,6-galactoside sialyltransferase 1) and ST6GalNAc1 (N-acetylgalactosaminide α -2,6-sialyltransferase 1), described in various types of carcinomas (Akamine et al., 2004; Amado et al., 1998; Gc et al., 2022; Liu et al., 2011; Munkley et al., 2015).

Cancer cells with aberrant sialylation can also bind to Siglecs to protect themselves from immune cell-mediated destruction (Ghasempour & Freeman, 2023; Hudak et al., 2014). Various authors have

shown that elevated sialylation enables cancer cells to bind to macrophage or natural killer cell receptors, thereby sending a "don't eat me" signal (Barkal et al., 2019; Huang et al., 2024; Ibarlucea-Benitez et al., 2021), which can inhibit their uptake (Hudak et al., 2014). α 2,6-Sia linked to the terminal Gal on N-glycans can also inhibit the galectin pathway by covering Gal epitopes. Using this strategy, tumour cells from various cancers can progress, evade the immune system, and metastasise (Cagnoni et al., 2016; Peixoto et al., 2019; Zhuo & Bellis, 2011).

Hypersialylation also alters the physical properties of the glycocalyx, which, when Sia levels are elevated, strongly influence membrane dynamics, tension, and signalling. A thick glycocalyx, rich in sialylated and bulky glycans, forms, often accompanied by elevated mucin expression (Woods et al., 2017). Elevated O-glycan sialylation also drives cancer aggressiveness, while its reduction decreases tumour progression at multiple stages (Park et al., 2024).

Neu5Gc was also found to be incorporated into the glycocalyx of rapidly dividing cancer cells, altering their immunogenicity by making them targets for circulating anti-Neu5Gc antibodies (Altman & Gagneux, 2019). Additionally, in a mouse model lacking *CMAH*, anti-Neu5Gc antibodies probably promote the growth of new tumours by increasing tumour-associated inflammation through the induction of “xenosialitis” in the humanised mouse model (summarised in Dhar et al., 2019).

Sia in neurological diseases

Sialylation is highly prevalent in the vertebrate central nervous system (CNS), particularly in gangliosides (65 %) and glycoproteins (35 %) such as NCAM (Brunngraber et al., 1972). Polysialic acid is present in glycans of specific neural cell types, such as neurons, Schwann cells, and stem cells. The dynamic regulation of sialylation affects memory formation, stress responses, and various neurological diseases (summarised in Lünemann et al., 2021).

For example, elevated ST6Gal1 activity leads to over-sialylation of the amyloid precursor protein (APP) and thus increases the extracellular levels of the amyloid-beta peptide (A β), which arises from sequential cleavage of APP (Nakagawa et al., 2006). A β is the crucial component of amyloid plaque characteristic of Alzheimer's disease. Amyloid plaques are suggested to affect neuronal activity and reduce cognitive abilities (Sadigh-Eteghad et al., 2015). The infectious prion protein PrP^{Sc}, which causes prion disease, appears to be highly influenced by sialylation. The infectious form is composed of misfolded and aggregated PrP^C, and the negative charge of Sia on PrP proteins affects the interactions between PrP^{Sc} and PrP^C, particularly the structure and infectivity of PrP^{Sc} (Katorcha et al., 2015; Katorcha et al., 2014). Partially desialylated PrP^{Sc} fails to induce prion disease in animal models, with no prions detected in their brains (Srivastava et al., 2017). Moreover, Bate and colleagues (2016) showed that PrP^C with unsialylated glycosylphosphatidylinositol (GPI) anchor resists conversion to the disease-associated PrP^{Sc}.

Receptors for pathogens

As Sia is a major terminal modification of glycans on the surface of mammalian cells, many pathogens use it as a receptor. Viruses attach to sialylated glycans to enter cells; bacteria synthesise adhesins or toxins that bind to host cell sialic acids; and protozoa utilise them to avoid immune responses.

Viruses

Influenza viruses have several molecules that play key roles during infection of the host. Hemagglutinins (HA), neuraminidases (NA), and the hemagglutinin esterase fusion protein (HEF) are the major viral surface proteins that interact with Sia, which serves as a receptor for all influenza virus subtypes (Gamblin & Skehel, 2010; Shah, 2004). Sia linkage (α 2,3- vs α 2,6-) and the underlying sugar chain structure are critical for viral recognition by hemagglutinins (HA). HA of avian Influenza virus type A (IAV) recognises α -2,3-linked Neu5Ac (Nobusawa et al., 1991; Rogers & Paulson, 1983) while human IAV binds mainly α -2,6-linked Sias (Gambaryan et al., 1997; Matrosovich et al., 2000). Viral neuraminidases cleave Sia residues from host glycoproteins, helping release newly formed viral particles from infected cells and facilitating viral entry into new cells (Air & Laver, 1989; Matrosovich et al., 2004). From the other Sia-binding viruses, the Middle East respiratory syndrome coronavirus (MERS-CoV) binds α -2,3-linked Neu5Ac (Li et al., 2017). In the case of SARS-CoV, sialic acids appear to inhibit infection, whereas in SARS-CoV-2, the spike protein interacts with α 2-6-linked Sia (Saso et al., 2022). SV40 virus attaches to the Sia-containing GM1 ganglioside via its VP1 pentameric capsid proteins and binds more strongly to Neu5Gc-GM1 than to Neu5Ac-GM1 (Campanero-Rhodes et al., 2007).

Bacteria

Bacterial species that use Sia as a receptor use two different strategies. First, they produce molecules, usually toxins with affinity for Sia; second, they produce sialidases that hydrolyse Sia, revealing the rest of the glycan and thus facilitating adhesion to host tissues, biofilm formation, and/or tissue invasion.

Salmonella typhi produces a two-subunit AB₅ six-component toxin type that binds to Neu5Ac (Song et al., 2013). The production of this highly immunogenic toxin is significantly increased upon bacterial invasion of host cells (Spanò et al., 2008). Another member of the AB₅ toxin family is the subtilase cytotoxin, produced by Shiga toxin-producing *Escherichia coli* (STEC). It binds to sialylated glycolipids decorated with terminal α -2,3-linked Neu5Gc. Interestingly, as Neu5Gc is not synthesised in humans, the presence of such a receptor is influenced by the host's metabolism, which incorporates Neu5Gc from dietary sources. This makes the toxin's target dependent on external nutritional factors

(Byres et al., 2008). *Helicobacter pylori* expresses several adhesins specific for sialylated gangliosides, such as the SabA adhesin, which shows a preference for α -2,3-linked Neu5Ac in the sialyl-Lewis x antigen (Mahdavi et al., 2002). *Pseudomonas aeruginosa* uses sialidase (NEU 1) to remove Sia from mucin 1 to interact with human airway epithelial cells and immune cells (Jeffries et al., 2016; Lillehoj et al., 2015). *Clostridium botulinum* produces a toxin complex that includes the botulinum neurotoxin, hemagglutinin components (HA), and some other components. The HA portion can bind to α 2-3- and α 2-6-Neu5Ac on intestinal epithelial cells, facilitating the absorption of the entire toxin complex into the body and enhancing the toxin's oral toxicity (Nakamura et al., 2008; Yamashita et al., 2012). *Vibrio cholerae* uses both strategies mentioned above. It produces cholera toxin (CTx) and a neuraminidase enzyme (VcN). CTx is part of the AB5 toxin group and specifically binds to GM1 gangliosides that are monosialylated with Neu5Ac or Neu5Gc (Lanne et al., 1994; Merritt et al., 1994). However, human epithelial cells predominantly express gangliosides with two or three sialic acid residues. To overcome this, *V. cholerae* uses its sialidase to cleave α -2,3 and α -2,8- Sia, converting complex gangliosides into GM1, which facilitates toxin binding (Moustafa et al., 2004). *Streptococcus pneumoniae* possesses several virulence factors, among them sialidases. They cleave both α -2,3- and α -2,6-Neu5Ac on the T-antigen surface (Coats et al., 2011). This triggers an immunological reaction, resulting in pneumococcal haemolytic uremic syndrome.

Protozoa

Protozoan parasites that cause severe diseases in humans and animals often produce sialidases or proteins that specifically recognise Sia. In contrast to viruses, protozoa seem to use more sophisticated strategies during their interactions with hosts. *Entamoeba histolytica* uses its transmembrane sialidase to hydrolyse α -2,3-Neu5Ac and α -2,8-Neu5Ac (colonic acid) (Nok & Rivera, 2003). The removal of Sia exposes the host receptors that facilitate binding of the *E. histolytica* toxin (Feingold et al., 1985). *Toxoplasma gondii* secretes several microneme proteins that bind to Sia on the host surface – TgMIC1 binds α -2,3 Sias, TgMIC13 binds 4-O-acetylated Sias and α -2,9 Sias (Baba et al., 2015; Friedrich et al., 2010). Moreover, the TgTCP-1 chaperonin and the tachyzoite surface Sia-binding protein 1 interact with Sia. *P. falciparum* uses EBA-175 and EBA-140 proteins to bind Neu5Ac to invade the host erythrocytes (Klotz et al., 1992; Orlandi et al., 1992). Moreover, experiments with human erythrocytes enriched with Neu5Gc showed that *P. falciparum* can bind Neu5Gc as well as Neu5Ac without preference for one or another.

Molecular mimicry

Another pathogen strategy is to use Sia on its own glycans to evade host immunity. Sia can be acquired by the pathogen from the environment or the host (e.g., *P. aeruginosa*, *Haemophilus*

influenzae) or synthesised by the pathogen itself (*E. coli* K1, *Neisseria meningitidis*, *Campylobacter jejuni*, *H. pylori*). Pathogens coated with Sia can hide their antigenic sites, leading to immune cells receiving inhibitory signals and reducing innate immunity. *Trypanosoma cruzi*, for example, expresses a transsialidase TcTS, which cleaves α -2,3-linked Neu5Ac or Neu5Gc from host cells. Then it transfers them to the β -Gal on parasites' mucins, maintaining the same α -2,3 linkage, thus aiding immune response evasion (Agustí et al., 2007; Ruiz Díaz et al., 2015).

The differences between vertebrate and invertebrate glycans

Bacterial species that invade the vertebrate host display Sia, usually on capsules and lipopolysaccharide O-antigens, or as part of flagella, pili, the cell wall, or the S-layer. *E. coli* K1 and *N. meningitidis*, for example, express capsular α 2-8-polySia (Hammerschmidt et al., 1996; Vimr et al., 1995). Group B streptococci display α 2-3-linked Neu5Ac on their surface (Carlin et al., 2007), which is further modified by O-acetylation (Lewis et al., 2004). *H. influenzae* incorporates Neu5Gc to its surface glycans (Taylor et al., 2010). These sialylated bacterial glycans can then interact with Siglecs on host immune cells, thereby suppressing immune signalling. The host immune system also responds to bacterial mimicry by producing ficolins, which recognise Sia and trigger the lectin complement pathway (summarised in Kjaer et al., 2011; Matsushita & Fujita, 2001). The representatives of the group Tripanosomatids (genera *Trypanosoma* and *Leishmania*) evade the host immune system by utilising host Sia with α 2-3 and α 2-6 linkages and incorporating it into their own surface glycans (summarised in Cavalcante et al., 2021).

Vertebrate and invertebrate glycans differ significantly in their structural complexity, terminal sugar residues, and anionic modifications; while vertebrates exhibit complex sialylated glycans, invertebrates often exhibit simpler mannosylated structures, high levels of fucose, and diverse, non-Sia-originating negative charges.

Sialic acids are common terminal residues on vertebrate N- and O-glycans, essential for various cellular processes. In contrast, Sias are rare in most invertebrates, occurring only in specific tissues or at early developmental stages. While Fuc is present in both vertebrates and invertebrates, it is more dominant and diverse in invertebrates. Invertebrate N-glycans frequently contain core α 1,3-Fuc, along with α 1,6-Fuc, which is commonly present in invertebrates (Thomès & Bojar, 2021). Vertebrates generally use Sia to provide a negative charge, whereas invertebrates usually rely on alternative modifications to generate a similar charge, such as sulphation, glucuronic acid (GlcA), or zwitterionic groups like phosphorylcholine, phosphoethanolamine, and aminoethylphosphonate (Paschinger & Wilson, 2019). Finally, vertebrate glycans are generally characterised by complex and hybrid type N-glycans with antennal elongation. Invertebrate N-glycans are often dominated by high mannose and paucimannose types.

1.3 Glycosylation and glycans of arthropods

The investigation of glycomes in arthropods over the last decade has experienced a boom, coinciding with improvements in mass spectrometry (MS) analytical methods. It revealed high glycans variability, especially among insects. As many arthropods are hosts, vectors, or parasites themselves, it is not surprising that they possess a wide range of N- and O-glycans that may be involved in various processes.

1.3.1 N-glycans in Arthropods

Generally, high-mannosylated/paucimannosidic, hybrid/pseudohybrid, and complex glycans constitute most structures identified in arthropod glycomes. Additionally, unique modifications indicating the extensive diversity of N-glycans have also been described. The most in-depth analyses were conducted in mosquitoes and *D. melanogaster*.

Highly mannosylated/paucimannosidic glycans are typical and dominant glycans of invertebrates, found across different arthropod species, especially in insects. A large amount of these glycans bear core-fucose attached in the $\alpha 1,6$ - and/or $\alpha 1,3$ -position. Paucimannosidic glycans were first identified on bee venom glycoproteins (Kubelka et al., 1993), and later also in *A. gambiae* and *A. aegypti* larvae (Kurz et al., 2015), *D. melanogaster* with and without fucosylation (Aoki et al., 2007), and in *T. castaneum* (Walski et al., 2016).

Moreover, in some species, N-glycosylation was shown to be essential for the transition between developmental stages. For example, *T. castaneum* N-glycans participate in metamorphosis, and the N-glycan composition changes during the development from pupa to adult towards more trimmed glycans (Walski et al., 2016). N-glycome featured a high proportion of highly mannosylated glycans, which comprised 70 % in the larval stage, but only 40 % in the adult stage. In comparison, *Tribolium* larvae and adults produce significantly more difucosylated glycans (up to 17%) than *D. melanogaster* (Walski et al., 2016).

In addition to trimmed glycan structures, bi- and tri-antennary complex N-glycans have been reported in *A. gambiae* and *A. aegypti* larvae, further modified with fucose and, unusually, with sulphate or GlcA residues. Anionic modifications were also observed in the glycans of *D. melanogaster* larvae and lepidopteran species. Glycans with GlcA attached terminally were confirmed in *D. melanogaster* (Aoki & Tiemeyer, 2010). Kurz and colleagues (Kurz et al., 2015) showed the presence of sulphate on $\alpha 1,6$ -linked Man and GlcA on non-reducing $\beta 1,3$ -Gal. The authors also speculate that these glycosylated structures function as anionic ligands in the parasite-host relationship. In the *D. melanogaster* genome, Kim and colleagues (2003) identified three glucuronyltransferases and showed that they are catalytically active. Furthermore, in-depth MS analysis of cabbage looper (*Trichoplusia ni*) larvae, cabbage looper High Five cells, and *Lymantria dispar* larvae revealed $\alpha 1,3$ -fucose and up to 5 % of anionic and/or

zwitterionic glycans (Stanton et al., 2017).

Fucosylated glycans are attached to the invertebrate glycans in three different ways: α 1,6-glycosidic linkage to proximal GlcNAc (common in mammals), α 1,3- linkage to proximal GlcNAc (common in plants and insects), and formation of a LacdiNAc structure with α 1,3-bound Fuc. In the phospholipase A2 of the bee *Apis mellifera*, all three types of fucosylated glycans have been found (Kubelka et al., 1993), as well as core-difucosylated N-glycans (Staudacher et al., 1992). Subsequently, difucosylated glycans have been described in other bee (Kubelka et al., 1995), hornet (Kolarich et al., 2005), *D. melanogaster* (Rendić et al., 2010), mosquito (Kurz et al., 2016) and tick (Park et al., 2020) glycoproteins. Core-fucosylated glycans were also found in *C. elegans*, but only in a minority (Natsuka et al., 2002).

1.3.2 O-glycans in Arthropods

Identification of O-glycans is more complicated than that of N-glycans because O-glycans have several different cores. They can be categorised into several types based on the identity of the first monosaccharide that is attached to them. O-glycan profiles have been primarily studied in model organisms such as *D. melanogaster* (Aoki et al., 2008; Kurz et al., 2015), *T. castaneum* (Li et al., 2022), mosquito (Kurz et al., 2016), and silkworm (Soya et al., 2016). The published glycomic data showed that O-glycans are composed mainly of GalNAc, Gal, GlcNAc, Fuc, Man, Glc, GlcA, and Xyl (xylose) monosaccharides (summarised in Li et al., 2020). There is limited knowledge about O-glycosylation during the entire life cycle of arthropods. The O-glycan profile across various developmental stages has been investigated only in *T. castaneum* (Lewis et al., 2022).

The most detailed studies, performed on the *D. melanogaster* embryo and all developmental stages of *T. castaneum*, show that mucin-type O-glycans are dominantly present. Aoki and colleagues (2008) identified predominantly core 1-type O-glycans, and further core 2-type O-glycans, a single O-Fuc glycan, and other glycans with HexNAc-HexNAc cores in *D. melanogaster*. O-linked GalNAc, GlcNAc, Glc, Man monosaccharides, as well as the Xyl-Glc disaccharide, were also observed. Corresponding transferase enzymes in *D. melanogaster* were identified and characterised (summarised in Zhang & Ten Hagen, 2019).

Mucine-type O-glycans are characterised by the attachment of a single GalNAc to the peptide. If a single GalNAc is added to the amino acid, it is called the Tn antigen. O-GalNAc can be further extended with Gal to create core 1-type O-glycan (T antigen) (Lin et al., 2008; Müller et al., 2005; Yoshida et al., 2008).

The corresponding enzymes are produced in certain tissues during various developmental stages (Tran & Ten Hagen, 2013). O-GlcNAc modification is catalysed by O-GlcNAc transferase, which is present at very low abundance in *D. melanogaster* embryos (Aoki et al., 2008); besides that, O-GlcNAc

is involved in neural processes (Hart et al., 2011), it is important for viability (Sinclair et al., 2009) and embryonal development (Radermacher et al., 2014). In *T. castaneum* O-glycome, mucin-type O-glycans dominate, making up more than 80 % of total glycans. Tetra- and pentasaccharide mucin-type O-glycans were present in high amounts during the pupa stage, indicating a change in the O-glycan profile during the metamorphosis of *T. castaneum*. In total, O-GalNAc and O-GlcNAc glycans covered 16 % of identified glycans (Li et al., 2022). Mucine-type O-glycans with the chains extended with HexNAc-Hex-HexA were also identified in *A. gambiae* and *A. aegypti* larvae with or without terminal sulphate or internal phosphoethanolamine (Kurz et al., 2015).

O-Fuc-type glycans were found in *D. melanogaster* as well as in *T. castaneum* (Aoki et al., 2008; Li et al., 2022). One Fuc extended with GlcNAc and GlcA constitutes 17 % of the *Tribolium* glycome. In *D. melanogaster*, Aoki and colleagues (2008) detected O-Fuc glycan structures, accounting for 11 % of the total glycan content. Furthermore, O-Fuc extended with Glc or GlcNAc was identified (Li et al., 2022). O-Fuc is attached either to EGF repeats or to thrombospondin type 1 repeats (TSR). O-Fuc attached to the EGF repeats of Notch can be further modified by GlcNAc, which is transferred to the target protein thanks to the Fringe transferase (3-beta-N-acetylglucosaminyltransferase) (Moloney et al., 2000); Correia and colleagues (2003) showed that the loss of Fringe is lethal for *Drosophila*.

Although O-Man was identified in *D. melanogaster* at less than 1 % of total O-glycans, it is crucial for *Drosophila* viability. O-mannosylation is carried out by dPOMT1 and dPOMT2 (Ichimiya et al., 2004; Lyalin et al., 2006), homologs of mammalian O-mannosyltransferases. O-mannosylation is essential for muscle development and for the proper function of dystroglycan (DG), a glycoprotein involved in muscle integrity and interactions with the extracellular matrix (Haines et al., 2007; Nakamura et al., 2010).

O-glucosylation, a special type of O-glycosylation, is catalysed by the glycosyltransferase Rumi, which transfers Glc to EGF repeats. In the *D. melanogaster* embryo, O-glucosylated structures belong to a rare class of glycans but play a crucial role in embryonic development by modifying the Notch receptor to regulate signalling (Acar et al., 2008). Rumi has an important role in trafficking Notch out of the ER (Ishio et al., 2015) and targets other proteins with EGF repeats, such as Crumbs (Crb) and Eyes shut (Eys), which are involved in photoreceptor development (Haltom et al., 2014). O-glucosylated glycans can be further extended by the addition of two Xyl residues, with the first one, added by the enzyme Sams transferase, playing a crucial role in regulating the balance between Notch activation and inhibition (Lee et al., 2017). Additionally, this modification may reduce Notch cell-surface expression in certain tissues (Lee et al., 2013).

O-linked glycans were also found in *Bombyx mori* using lectinoblot with PNA lectin. Proteins bearing O-glycan modifications were observed at various developmental stages, with the highest abundance in the pupal stage (Soya et al., 2016). Additionally, the functional enzyme PGANT

N-acetylgalactosaminyltransferase, which initiates O-glycosylation, was discovered in cultured silkworm cells (Xu et al., 2018). Furthermore, GalNAc or GlcNAc and the core 1 structure Gal β 1-3-GalNAc α 1-Ser/Thr were identified as the most abundant glycans present (Morio et al., 2023).

1.3.3 Sialic acid in Arthropods

Sialic acids, typically abundantly present in vertebrates, are generally absent or present at low levels in arthropods. While some studies indicated the presence of Sia in protostomes, including insects (Hollister & Jarvis, 2001; Malykh et al., 1999; Roth et al., 1992), other studies have failed to find evidence of sialylated glycans, the CMP-Neu5Ac donor (a substrate for sialyltransferase), glycoproteins, or glycolipids, and sialyltransferase activity in insect cells (summarised in Koles et al., 2009). From the 1990s to today, only limited information on Sia in arthropods is available.

The controversy was partially resolved when endogenous Sia in *D. melanogaster* was convincingly demonstrated. Mass spectrometry analyses revealed the presence of Sia in the embryonic stages and in the central nervous system of adult *D. melanogaster* (Aoki et al., 2007). Studies addressing this issue suggest that the sialylation pathways of vertebrates and insects share a common evolutionary origin and are therefore very similar (Fig. 3). Findings from studies of *D. melanogaster* support the hypothesis that sialylation in insects is a specialised, developmentally controlled process that likely plays an important role in proper nervous system function (summarised in Koles et al., 2009).

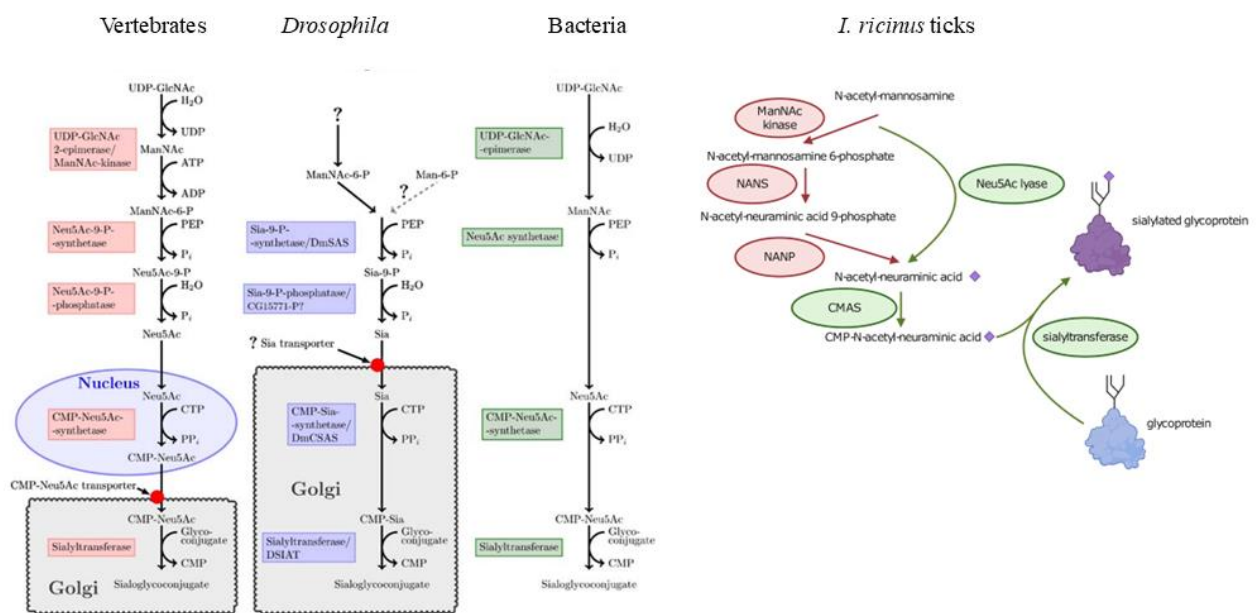


Figure 3. De novo sialylation biosynthetic pathways in vertebrates, *Drosophila*, bacteria and *I. ricinus* ticks (adapted and edited from Koles et al., 2009; Sterbova et al., 2026b). Question marks replace unknown or hypothetical enzymes and pathway steps. In *Drosophila*, the absence of a UDP-GlcNAc 2-epimerase/ManNAc kinase gene makes the synthesis of ManNAc-6-P unclear. The question mark next to Man-6-P suggests a possible alternative pathway, similar to that in lower vertebrates, that may bypass the epimerase/kinase step. CG15771-P denotes the protein encoded by the *Drosophila melanogaster* CG15771 gene. In the *I. ricinus* tick, the enzymes

from the classical pathway via ManNAc kinase, *N*-acetylneuraminate synthase (NANS), and sialic acid phosphatase (NANP) were not found in the published genomes (marked in red circles); the Neu5Ac lyase-like protein was identified together with the CMP-Neu5Ac synthetase and two putative sialyltransferases.

Glycoconjugates containing Sia, specifically α 2,6- and α 2,8-linked Neu5Ac, have been found in Malpighian tubes of cicada *Philaneus spumarius* larvae (Malykh et al., 1999). Histochemical staining of larval tissues with antibodies and lectins specific for Sia was complemented by MS and gas-liquid chromatography analysis. Using microscopic, histochemical, and mass spectrometric methods, lactonized Sia (Neu5Gc7,9Ac₂) was detected in the prothoracic glands of *Galleria mellonella* (Karacali et al., 1997). The first direct evidence of Sia in Arthropods was reported by Aoki et al. (2007), who found complex sialylated N-glycans in *D. melanogaster* embryos. These glycans, carrying α 2-6-linked Sia to terminal Gal β 1-4GlcNAc, are found at very low abundance in both early and late embryos (Aoki et al., 2007). However, quantification of their relative abundance suggests that they increase during embryo development (Aoki et al., 2007). Moreover, Sia was quantified by capillary LC-ESI-MS/MS in the silkworm *B. mori* haemolymph across all developmental and ageing stages, with an increase in its amount seen during the pupal stage and in 3rd instar larvae (Soya et al., 2017). A responsible α 2,6-sialyltransferase was also identified in *B. mori* (Kajiura et al., 2015).

Neu5Ac was also detected in *A. aegypti* tissues, and gene sequences encoding CMP synthase were identified. Authors also hypothesise that Sia in the salivary glands could facilitate dengue virus entry into mammalian cells (Cime-Castillo et al., 2015).

1.3.4 Glycosylation and glycans in Ixodid ticks

The very first information about tick glycans was obtained through indirect analyses. Using Schiff staining, glycosylated structures were found in many tick species, including *D. variabilis* (Coons & Roshdy, 1973), *R. microplus* (Binnington, 1978), or *R. appendiculatus* (Walker et al., 1985). Lectin staining on western blot membranes or microscopical samples revealed the presence of glycan-containing structures in *I. ricinus* (Uhlír et al., 1994; Vancova et al., 2006), *A. polonicus* (Grubhoffer & Dusbábek, 1991) or *R. appendiculatus* (Kamwendo et al., 1993). Later, N-glycans containing Man, Glc, GlcNAc, Gal and O-glycans with Gal, GalNAc were observed in *I. ricinus* larvae and nymphs. Moreover, fucosylated glycans were described in nymphs (Uhlír et al., 1994). In organs of partially fed females, several proteins were stained using different lectins. In the gut, Malpighian tubules, and ovaries, glycosylated proteins were stained with ConA, PSA, PNA, and WGA, indicating the presence of high-mannose and complex glycans containing Man, Glc, Gal, GlcNAc/Sia, and Fuc (Uhlír et al., 1994). The salivary glands of partially fed *I. ricinus* females were also examined by the lectin affinity labelling and visualised using electron microscopy (Vancova et al., 2006). Lectin staining showed differences in glycan composition between secretory granules of b/c2 cells (Man, GlcNAc, Gal, GalNAc, Sia), c1 cells

(Man, GlcNAc, Sia), and f cells (GlcNAc, Man) of Acini type II/III (Vancova et al., 2006). The surfaces of acini types II and III of *R. appendiculatus* also showed the presence of glycans, particularly Gal, Man, and Glc, with galactosylated structures localised only on acini type III (Kamwendo et al., 1993).

Salivary glands and the gut became intensively studied tick organs. The presence of fucosylated glycans was confirmed in the salivary glands and midgut of fed nymphs of the tick *I. scapularis* (Pedra et al., 2010). MALDI-TOF/TOF analysis of SG from fed nymphs revealed N-glycans with core α 1,3-linked Fuc. Moreover, MS analysis showed the presence of less complex oligosaccharides, especially a series of highly mannosylated structures. Hybrid, biantennary, and triantennary complex glycans were detected in small amounts. Protein extracts from SGs and midguts also showed a strong signal after western blot with anti-HRP antibodies that recognise the core α 1,3-Fuc (Pedra et al., 2010). A unique combination of fluorescence and scanning electron microscopy localised α 1,3- and α 1,6-Fuc on the basement membranes of acini types II and III of partially fed *I. ricinus* females, as well as in the small secretory granules within the cytoplasm of “F” cells. Nevertheless, only α 1,6-Fuc was detected in the secretory granules of acini type II, from which the active molecules are released to the host during feeding (Vancova & Nebesarova, 2015).

The presence of various Sia types in N-glycans was investigated in various tissues of partially fed female *I. ricinus* using mass spectrometry (MS), Western blotting, and immunolabeling with anti-Neu5Gc antibodies (Vancova et al., 2012). Western blot analysis confirmed a sialylated (Neu5Gc) 95 kDa protein in tick saliva. Moreover, a protein with the same molecular weight, together with a 72 kDa protein and other proteins stained at lower intensity, was detected in SG homogenates from fed and unfed females. In the SG extract, but not in saliva, MS analysis identified a monosialylated glycan (HexNAc.Hex.Neu5Ac). In the guts of 3- or 6-day fed females, di- and triantennary mono- or desialylated glycans were detected; both Neu5Ac and Neu5Gc were present in the N-glycans. Immunogold labelling localised Neu5Gc within the basement membranes and cytosolic structures of SG acini and in digestive cells and the gut lumen. Based on the glycan structures, their location in tick tissues, and the type of tick samples, the authors speculate on the host origin of the identified glycans (Vancova et al., 2012). To quantify both total and metabolically incorporated Sia in tick tissues, three different methods were employed. Using a commercial Sia quantitation kit, the total amount of Sia was determined in the gut, SG, and OV, expressed as mmol Sia/g protein; in contrast, the enzymatic assay showed the amount of tick Sia to be less than 1000x lower. The authors therefore conclude that the majority of the Sia in tick tissues is of host origin (Sterba et al., 2014).

Based on the experimental data, it is likely that tick glycans play an important role in the tick-pathogen relationship. Hebert and colleagues (2017) assume that *A. marginale* outer membrane protein A targets fucosylated residues on tick ISE6 cells. The hypothesis was supported by Vimonish and colleagues (2022) using the *D. andersoni* primary midgut cell culture system. They showed that the

expression of tick α -1,3-fucosyltransferases A1 and A2 (responsible for core α -1,3-fucosylation) is regulated by *A. marginale* infection of midgut cells. Moreover, the amount of core α -1,3 Fuc, along with the sensitivity of uninfected cells to infection, was lowered after the silencing of α -1,3-fucosyltransferase A2.

The complex analysis of *A. americanum* N-glycome showed distinct patterns in N-glycan structures depending on sex and feeding stage, including in fully fed females and, somewhat surprisingly, in carcasses (CC). In CC, the glycan profile is predominantly composed of paucimannose structures. In female CC, non-fucosylated paucimannoses account for 77 % of their total N-glycan profile. Male CC show a higher degree of fucosylation, with 61 % of their N-glycans being core monofucosylated, and an additional 17 % being di-fucosylated. High-mannose and complex glycans are rare in male CC, comprising only 2 % of the total glycan content. SG samples are generally rich in high-mannose and complex glycans. These include structures containing the α Gal epitope and xylosylation at the core mannose. Additionally, trace amounts of glycans bearing NeuAc and NeuGc have been detected in the SG of male ticks. However, the authors do not discuss the reliability of these results – part of the fed ticks' N-glycome could originate from host glycoproteins, and, in the case of carcasses, degradation could be considered (Park et al., 2020).

Immunologically important tick glycans

The glycan molecules found in various tick species can initiate an immune response, thereby significantly affecting their interactions with the host. The enzymes responsible for glycan maturation in the Golgi apparatus vary among ticks, insects, and their vertebrate hosts, resulting in distinct glycan profiles in ticks. These structural differences in glycans play a crucial role in how ticks interact with both their hosts and pathogens. On one hand, these glycans can engage in molecular mimicry and mechanisms that help reduce the host's immune response and other defence mechanisms. On the other hand, due to their unique structures, they can activate the host's defence systems, contributing to conditions like delayed red meat allergy, or aid in pathogen transmission.

Glycan structures that are present in ticks but absent in humans, the so-called cross-reactive carbohydrate determinants (CCDs), may trigger an immune response, particularly mediated by IgE antibodies (Aalberse et al., 1981; Wilson, 2002). These include α 1-3-linked core Fuc (α 1-3 core Fuc), Gal α 1-3-linked to Gal (α -Gal epitope), sialic acid variant NeuGc, and β 1-2-linked Xyl, which have been identified in several tick species and in some of them, the immunological importance has been proved (summarised in Sterbova et al., 2026a).

The most medically relevant CCD is the α -Gal epitope, which can cause α -Gal syndrome (AGS) in humans after feeding of several tick species (*Amblyomma*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus*), including ticks, for which humans are an accidental host, such as *A. reflexus* (Rolla et

al., 2018). AGS occurs when tick bites introduce α -Gal into the body, which sensitises the human immune system. Following this sensitisation, ingestion of red meat or exposure to products containing α -Gal can trigger an IgE-mediated allergic reaction (Van Nunen et al., 2009). The symptoms of AGS in humans can vary significantly, from no symptoms to mild reactions, and in some cases, it may lead to severe anaphylactic shock (Kleine-Tebbe et al., 2006; Miadonna et al., 1982). Till these days, AGS remains the first allergic disease caused solely by a carbohydrate, triggering IgE-mediated allergic reactions. AGS is primarily associated with American tick species; the lone star tick, *A. americanum*, is responsible for most cases recorded in the U.S. A crucial study by Cabezas-Cruz and colleagues (2018) demonstrates that *I. scapularis* ticks naturally produce α -Gal through the expression of galactosyltransferase genes *b4galt7*, *a4galt-1*, and *a4galt-2*. Their knockdown confirmed these genes are essential for the ticks' endogenous production of α -Gal. Moreover, the elevated α -Gal levels were observed during *A. phagocytophilum* infection, suggesting its role in the pathogen transmission. Antibodies targeting the α -Gal were observed to attach to this epitope in the salivary glands of *A. americanum* and *I. scapularis*. Furthermore, tick protein extracts containing α -Gal prompted basophil activation in individuals with a red meat allergy (Crispell et al., 2019).

The significance of α 1,3-core Fuc was highlighted by Pedra and colleagues (2010), who demonstrated that α 1,3-fucosylated glycans are crucial for *A. marginale* survival in *I. ricinus* ticks. They also found that this bacterium modifies the expression of three α 1,3-fucosyltransferases. Furthermore, silencing the genes encoding these fucosyltransferases significantly reduces *Anaplasma* colonisation of tick cells (Pedra et al., 2010).

Neu5Gc and β 1,2-xylose are other known CCDs. In Ixodid ticks, their presence was shown in *A. americanum*, *A. maculatum*, *I. ricinus*, and *I. scapularis* ticks (Crispell et al., 2019; Park et al., 2020; Vancova et al., 2012). The presence of both structures in the saliva/salivary glands suggests their potential role in tick-host interactions. Nevertheless, the role of these epitopes in tick physiology and tick-host-pathogen interactions needs to be further elucidated.

2. Results

Paper I

**Uptake and incorporation of sialic acid by the
tick *Ixodes ricinus***

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Uptake and incorporation of sialic acid by the tick *Ixodes ricinus*

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ABSTRACT

We describe the detection of sialylated *N*-linked glycans in partially fed *Ixodes ricinus* tick females using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry. Sialylated glycans were detected in salivary glands as well as in tick guts and we propose the host origin of these structures. In addition, we mapped the transport of sialylated structures from the blood meal through the gut to the salivary glands using electron microscopy. Specific localization of sialylated glycans to basement membranes of salivary glands was observed. Finally, the influence of the sample preparation methods for electron microscopy on ultrastructure and immunogold labeling was evaluated.

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1. Introduction

Ticks *Ixodes ricinus* are obligatory blood-feeding parasites, which transmit medically important pathogens such as the tick-borne encephalitis virus or the Lyme disease spirochetes *Borrelia burgdorferi*. When feeding on hosts, some tick tissues, especially the midgut and salivary glands (SGs), undergo substantial changes in their structure, size, and expression of proteins as well. SGs secrete pharmacologically active molecules that allow feeding on hosts for a long time and facilitate the transmission of pathogens while gut produces predominantly the digestive enzymes. Potential *N*-linked glycosylation sites were predicted in medically interesting molecules (such as Isac, Ixolaris, or Salp20 proteins) secreted into the host (Francischetti et al., 2002; Ribeiro et al., 2006; Tyson et al., 2007). However, the glycan structures of such glycoproteins are still completely unknown. The current improvements in analytical techniques have now made it possible to analyze small amounts of glycoproteins obtained from tick organ homogenates and saliva either directly or after preparative chromatography isolations (Dupejova et al., 2011; Man et al., 2008).

The exact structures of tick glycans have been identified only in few cases. Lectin Dorin M from the hemolymph of the soft tick

Ornithodoros moubata was modified solely by the high-mannose-type of *N*-glycans with nine mannose residues and core-fucosylated paucimannosidic glycans with four to five mannoses (Man et al., 2008). Recently, high-mannose and complex *N*-linked glycans without core-fucosylation have been described in a study of Heme-lipoglycoprotein, the protein responsible for binding, transportation, and storage of heme, in *Dermacentor marginatus* (Dupejova et al., 2011). Analysis of glycans from electrophoretically separated proteins from the *I. scapularis* nymph SGs showed high-mannose glycans without core-fucosylation and core-fucosylated paucimannosidic (two to four mannoses) and complex (one or two *N*-acetylglucosamine residues) glycans (Pedra et al., 2010). The complex type glycans are the predominant structures in mammals, whereas the most dominant arthropod glycans are the high-mannose structures as well as truncated (paucimannosidic type) *N*-glycans usually modified by core-fucosylation (Aoki et al., 2007; Wilson et al., 2009). α 1,3-Linked core-fucose was shown to trigger an immune reaction in vertebrates including humans (Altmann, 2007). In ticks, this modification is important for gut colonization by the obligate intracellular tick-borne pathogen *Anaplasma phagocytophilum* (Pedra et al., 2010).

Earlier, we detected the presence of sialic acid (Sia) residues in the secreting granules of SGs isolated from partially fed *I. ricinus* females using indirect methods such as enzymatic deglycosylation and affinity labeling with *Maoackia amurensis* agglutinin (MAA II) and *Sambucus nigra* agglutinin (SNA) (Vancova et al., 2006). SNA binds preferentially to Sia attached to terminal galactose in α 2,

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6-linkage and MAA II is used to detect α 2,3-linked Sia. Total Sia level was measured in homogenates of SGs, dissected from partially fed females, that were treated with neuraminidase, while the presence of Sia was colorimetrically quantified (Vancova et al., 2006).

Sialic acid is a general term for a large group of nine-carbon sugar acids. The most common form is *N*-acetyl-neuraminic acid (Neu5Ac) with *N*-acetyl group at C5 and carboxyl group at C2 sugar ring position. Hydroxylation of 5-*N*-acetyl group produces *N*-glycolylneuraminic acid (Neu5Gc), a carbohydrate present in vertebrates except for humans (Chou et al., 1998). Sia occurs in deuterostomate lineage from echinoderms to humans. In invertebrates, Sia was described only during early neural development of *Drosophila melanogaster* and in cicada *Philaenus spumarius* (Aoki et al., 2007; Malykh et al., 1999; Roth et al., 1992). Moreover, insect cells were shown to synthesize sialylated LacdiNAc structures when transfected with the *Drosophila* α 2,6-sialyltransferase (Koles et al., 2007) or were able to recycle Sia from the medium or host blood meal and incorporate it into their own glycoproteins (Jarvis et al., 2003).

In this study, we verify our previous observations using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) and, for the first time, directly identify the sialylated structures in tick tissues. In addition, we immunolocalized Neu5Gc epitopes at the ultrastructural level using two electron microscopic sample preparative methods. We compare both methods in terms of tick morphology and their potential for labeling of Neu5Gc-containing structures.

2. Material and methods

2.1. Ticks

I. ricinus females were collected from vegetation in South Bohemia, Czech Republic. The ticks were allowed to feed on clean guinea pigs for 3 or 6 days (partially fed females), or until fully engorged (fed females). Tick organs (SGs, gut, ovaria, and Malpighian tubes) were dissected and processed for either transmission electron microscopy (TEM) or mass-spectrometric (MS) analyses.

2.2. Saliva

Saliva was obtained from ticks fed for 6 days after stimulating with 2–4 μ l of methanolic pilocarpin (50 mg/ml) applied on the tick scutum (Valenzuela et al., 2000). The ticks salivated at 35 °C for 3 hr in a humid chamber into glass microcapillaries (Hirschmann laborgeräte, Eberstadt, Germany) that were mounted on chelicerae/over the hypostome. Saliva was immediately sealed in capillaries and stored at –70 °C. The protein concentration was measured using Qubit® Fluorometer (Life Technologies, Carlsbad, CA, USA). Salivation of 25 partially fed females produced the total volume of 45 μ l saliva with protein concentration of 1.8 mg/ml.

2.3. Preparation of extracts from tick tissues

Tick tissues were dissected from unfed females or partially/fully fed females in ice cold water (HPLC grade) containing protease inhibitors (10 μ l/ml, Halt™ Protease Inhibitor Single-Use Cocktail, Thermo Scientific, Waltham, MA, USA). The tissues were carefully removed to prevent contamination from the contents of the gut and separately rinsed thoroughly in water. The gut tissues were rinsed to remove most of the lumen content. The tissues were then sonicated (20 kHz, 30 W, 2 min) and centrifuged at 4 °C to clear the lysates. The protein concentrations in the supernatants were measured using Qubit® Fluorometer. The extracts were stored under liquid nitrogen.

2.4. Preparation of serum from guinea pig

Blood of guinea pigs was collected with the help of a veterinarian under general anesthesia. Blood was allowed to clot and was centrifuged to separate the serum. To decrease the amount of albumin, dealbumination was performed using Blue Sepharose (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions. Protein serum concentration was measured before (5 mg/ml) and after (1.8 mg/ml) the dealbumination and both sera were kept under liquid nitrogen.

2.5. Isolation and permethylation of *N*-linked glycans

N-linked glycans were prepared from tick saliva, and either SGs or guts homogenates obtained from tick females fed for 3 or 6 days. Tissue extracts (2–5 μ g of proteins) were digested using trypsin (37 °C, overnight). Peptides were purified using C18 microcolumns (Harvard Apparatus, Holliston, MA, USA) and dried in vacuum. Peptides were resuspended in HPLC grade water. *N*-linked glycans were cleaved by a combination of PNGase A (0.05 mU, Roche Applied Science, Penzberg, Germany) and PNGase F (50 U, New England Biolabs, Ipswich, MA, USA) treatments. The mixture was incubated overnight at 37 °C. The released glycans were separated using activated carbon microcolumns (Harvard Apparatus). Subsequently, purified *N*-glycans were dried in vacuum.

Glycans were permethylated using sodium hydroxide beads as the solid phase (Alley et al., 2010). The reaction took place in dimethylformamide using methyl iodide. Permethyated glycans were collected and extracted from the mixture with chloroform. The organic phase was washed repeatedly with 0.5 M sodium chloride and HPLC-grade water and vacuum-dried.

2.6. MALDI-TOF/TOF mass spectrometry

The dried permethylated glycans were resuspended in 50% methanol and spotted directly on a MALDI plate. Subsequently, 0.5 μ l of DHB matrix (2,5-dihydroxybenzoic acid, 10 mg/ml) solution in 1 mM sodium acetate was added to each dried spot and vacuum-dried. MS data were acquired using an 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, California, USA) equipped with an Nd:YAG laser with 355-nm wavelength. MALDI spectra were recorded in the positive-ion mode over the *m/z* range from 1000 to 4500. 1000 or 1200 laser shots were acquired per spectrum for MS and MS/MS, respectively. The data files were converted to mzXML files and analyzed by mMass (Strohalm et al., 2008) and Glycoworkbench (Ceroni et al., 2008).

2.7. Western blot analysis

Guinea pig dealbuminated serum, tissue homogenates, and saliva were mixed with SDS loading buffer containing 2 mM DTT (Thermo Fisher, Waltham, MA, USA) and denatured for 5 min at 100 °C. The amount of proteins loaded per line was 2 μ g in the case of SGs and guts from unfed females and ovaria and Malpighian tubes from fed females, 5 μ g for saliva, SGs from females fed for 6 days, and dealbuminated serum, and 10 μ g in the case of gut from females fed for 6 days. Proteins were electrophoretically separated on 10% polyacrylamide gels and transferred to PVDF membranes. The membrane strips were washed in ddH₂O and then blocked in a blocking agent (30 μ l, Gc-free kit, Sialix, Vista, CA, USA) diluted in 1 mL of PBS/0.1% Tween 20 for 2 h at RT. The strips were incubated with anti-Neu5Gc IgY antibodies diluted (0.06 mg/ml, Gc-free kit) in the solution containing 30 μ l of the blocking agent in 1 mL of PBS/0.05% Tween 20 for 2 h at RT. Control membrane strips were incubated with non-immunized control IgY antibody under the same conditions. The membranes were washed in

PBS with 0.05% Tween 20 and incubated with donkey anti-chicken IgY secondary antibody conjugated to HRP (0.1 mg/mL, Thermo Fisher) for 1 h at RT. The bound antibodies were visualized using a DAB substrate kit (Vector Laboratories, Burlingame, CA, USA).

In the case of control Western blots, SGs and gut homogenates from unfed and fed females were treated in 50 mM sodium acetate (pH 5) containing 0.5 U of *Clostridium perfringens* neuraminidase (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C overnight before the anti-Neu5Gc labeling.

2.8. Immunotransmission electron microscopy

Embedding of SGs into resin, Lowicryl K4M, was performed according to the protocol of Vancova et al. (2006). Briefly, SGs were dissected from partially fed female *I. ricinus* and fixed in 4% formaldehyde and 0.5% glutaraldehyde (GA) in 0.1 M phosphate buffer pH 7.2 (PB) for 1 h at RT. After washing, SGs were dehydrated in ethanol (30% at 0 °C, 50–70% at –20 °C; 80–100% at –35 °C for 1 h at each step). After infiltration, the material was embedded into Lowicryl K4M (Polysciences, Warrington, PA, USA) and UV-polymerized for 48 h at –35 °C. Ultrathin sections were contrasted in ethanolic uranyl acetate and lead citrate, and observed in a JEOL 1010 TEM (JEOL Ltd.) at an accelerating voltage of 80 kV. Images were captured using a Mega View III camera (SIS GmH.).

For immunolabeling of thawed cryosections (the Tokuyasu method, Tokuyasu, 1980), the isolated SGs of unfed or partially fed females were fixed in 4% formaldehyde and 0.5% GA in PB for 2 h at RT. The samples were washed in washing buffer (0.1 M PB with 4% glucose) and placed in 2.1 M sucrose in PBS for 48 h at 4 °C. After the sucrose infiltration, the samples were mounted on specimen pins and frozen in liquid nitrogen. Ultrathin sections were cut with a cryoultramicrotome Leica EM FCS equipped with a cryochamber Leica UCT (Leica, Wetzlar, Germany). Sections were collected on droplets of 1% methylcellulose in 1.15 M sucrose (Liou et al., 1996) and transferred to formvar-film-coated and carbon stabilized nickel grids.

Bovine α 1-acid glycoprotein (bAGP, Sigma–Aldrich) and α 1-acid glycoprotein from human plasma (hAGP, Sigma–Aldrich) were diluted in 10% gelatin separately in order to reach concentration of 50 mg/mL at 37 °C. The dissolved solutions were cooled down to 4 °C and fixed in 4% formaldehyde and 0.1% GA in 0.1 M PB for 1 h. Pieces of solidified gelatin containing either bAGP or hAGP were cryoprotected in 2.1 M sucrose for 48 h at 4 °C and finally frozen and sectioned as described above.

The cryosections or ultrathin Lowicryl K4M resin sections were rinsed three times in PBS and then immersed in the blocking solution containing 30 μ L of a blocking agent (Gc-free kit) diluted in 1 mL of PBS with 0.05% Tween 20 and 0.01 M glycine for 2 h at RT. The cryosections were incubated with monospecific polyclonal anti-Neu5Gc IgY antibodies (0.1 mg/mL; Gc-free kit) diluted in the blocking solution for overnight at 4 °C. Cryosections were rinsed six times in a washing solution (10 μ L of the blocking agent in 1 mL PBS with 0.05% Tween 20 and 0.01 M glycine) and incubated with goat anti-chicken IgG conjugated to 10 nm gold particles (dilution 1:40 in the blocking solution, Aurion, Wageningen, the Netherlands) for 2 h at RT. After washing in PBS and dH₂O, cryosections were contrasted with uranyl-methylcellulose mixture (2% methylcellulose and 3% aqueous uranyl acetate mixed in ratio of 9:1) for 10 min and dried.

Specificity of the anti-Neu5Gc labeling was demonstrated by labeling with non-immunized control IgY antibody (Gc-free kit) under the same conditions as described above, and anti-Neu5Gc labeling of thawed cryosections prepared from hAGP (negative control) and bAGP (positive control).

Deglycosylation of cryosections was performed prior to anti-Neu5Gc labeling in a solution containing 2 U of PNGase F in 50 mM PB (pH 7.5) for 24 h at 37 °C.

2.9. Statistical evaluation

Images were taken of random areas of sections at identical magnification. Gold particles were counted over the basement membranes (BMs). Labeling densities were calculated as follows: the total sum of particles divided by the sum of all lengths. The lengths of the BMs were measured using ACC program (SOFO, Brno, Czech Republic).

3. Results

3.1. Identification of Sia in tick salivary glands

We isolated N-linked glycans from tick tissues homogenates, permethylated them, and analyzed using MALDI-TOF/TOF MS. In this paper, we focus exclusively on finding of sialylated glycan structures. Monosialylated glycan HexNAc₄Hex₃Neu5Ac (m/z = 2432.5; Fig. 1, highlighted) was identified in the salivary gland extract (SGE) from tick female fed for 3 days. In saliva alone, we were not able to identify any sialylated structures. The detection of sialylated glycans was confirmed by Western blot analyses.

First, we performed the detection of Neu5Gc-containing structures in saliva from partially fed females. Anti-Neu5Gc antibody stained one approximately 95 kDa protein in saliva (Fig. 2, lane 1). No stain was visible in the saliva sample (Fig. 2, lane 2) labeled using the control antibody.

Further Neu5Gc-positive proteins were present in SGs from unfed and partially fed tick females. In both SG homogenates, the most prominent protein bands were the protein of molecular weight of approximately 72 kDa (Fig. 3, highlighted in rectangle, lanes 1 and 2) and approximately 95 kDa (Fig. 3, lanes 1 and 2, black arrow). In both samples, other proteins in whole range of separated molecular weights were stained with lower intensity. Both major stained proteins (72 kDa and 95 kDa) and the other stained bands corresponded to Sia-positive bands stained in dealbuminated guinea pig serum (Fig. 3, lane 7). Differential staining pattern was observed in SGs homogenates from fed and unfed tick females. Moreover, Neu5Gc labeling of *C. perfringens* neuraminidase treated Western blot strips was negative, confirming the specificity of the staining of sialylated glycoproteins in SGs and gut homogenates from partially fed females (Fig. S1, Supplementary data).

3.2. Identification of Sia in tick midguts

Differential Neu5Gc staining pattern was observed between the homogenates of gut from unfed and partially fed tick females (Fig. 3, lanes 3 and 4, respectively). In both samples, both the approximately 72 kDa and 95 kDa proteins were stained (Fig. 3, lanes 3 and 4, rectangle and black arrow). Moreover, the Sia-positive proteins from tick guts (Fig. 3, lanes 3 and 4) corresponded to proteins observed in the dealbuminated serum (Fig. 3, lane 7).

We performed MS analysis of tick guts as well. Neu5Ac-containing glycans were identified in gut extracts of ticks fed for 3 days (Fig. 4A, highlighted) or 6 days (Fig. 4B, highlighted). In the gut of ticks fed for 6 days, we detected three sialylated glycans HexNAc₄Hex₃Neu5Ac (m/z 2432.2), HexNAc₄Hex₃Neu5Ac₂ (m/z 2793.4), and HexNAc₃Hex₄Neu5Ac (m/z 2881.5) (Fig. 4A). Besides these structures, HexNAc₃Hex₄Neu5Ac₂ (m/z 3241.6) and a weak signal corresponding to HexNAc₄Hex₄Neu5Gc₂ (m/z 2737.5) was present in the gut of females fed for 6 days (Fig. 4B).

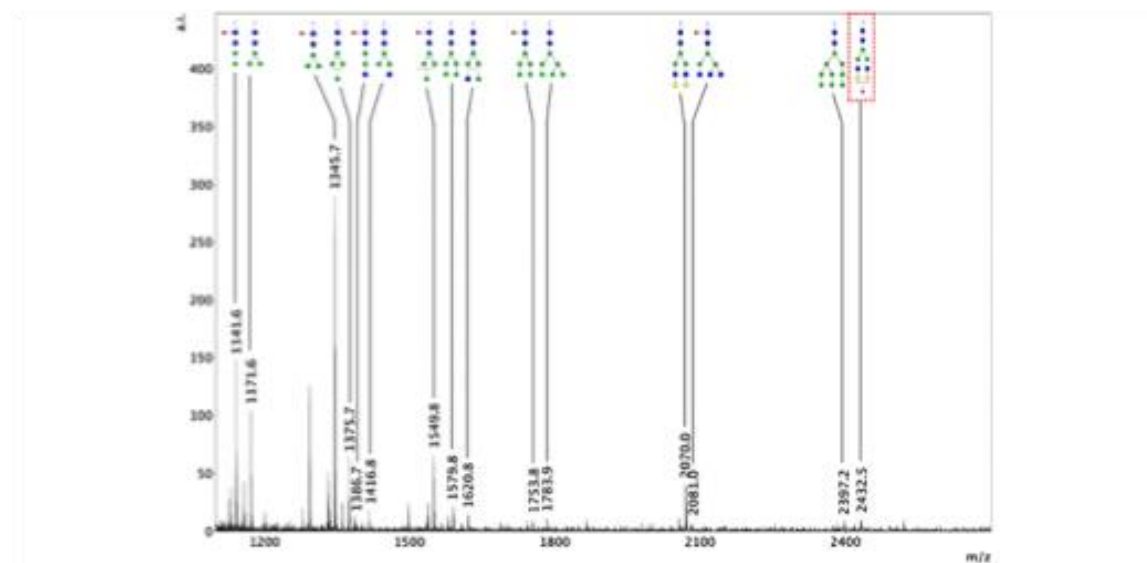


Fig. 1. Mass-spectrometric profiles of permethylated N-glycans. One sialylated glycan structure was observed in salivary glands from *I. ricinus* females fed for 3 days. Symbol key: blue square: N-acetylhexosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose/deoxyhexose; violet diamond: N-acetylneuraminic acid; white diamond: N-glycolylneuraminic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

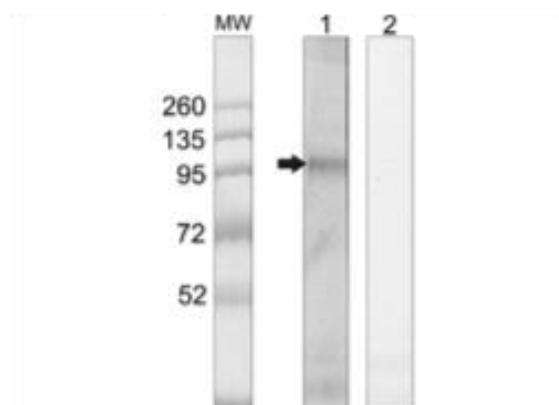


Fig. 2. Immunodetection of Neu5Gc in *I. ricinus* tick saliva. SDS-PAGE separated proteins were transferred to PVDF membranes, and probed with (1) anti-Neu5Gc antibody or (2) control antibody. MW molecular markers in kDa.

The composition of the sialylated N-glycans was confirmed by MS/MS analyses, where the diagnostic ions corresponding to the fragments containing Neu5Ac were present. The MS/MS spectra revealed the presence of monosialylated or disialylated, non-fucosylated, biantennary complex glycans (HexNAc₄Hex₃Neu5Ac, Fig. 5A and HexNAc₄Hex₃Neu5Ac, Fig. 5B) and monosialylated, non-fucosylated triantennary complex glycan (HexNAc₃Hex₆Neu5Ac₂, Fig. 5C). The MS/MS fragments were assigned using GlycoWorkBench. The structure prediction was completed through the common knowledge of the eukaryotic biosynthetic pathway for N-glycans.

3.3. Identification of Sia in ovary and Malpighian tubes of fed tick females

Two additional tissues from fed females were analyzed for the presence of sialylated proteins. The major 72 kDa Sia-positive band

was present in both the ovary and Malpighian tubes (Fig. 3, lanes 5 and 6, respectively, highlighted by rectangle) and other proteins in the whole range of the separated molecular weights showed weak staining as well.

3.4. Localization of Neu5Gc in tick SGs and gut

We used chicken polyclonal antibody against Neu5Gc to localize the sialylated (Neu5Gc-containing) glycans in the tick SGs and the gut isolated from partially fed females. Thawed cryosections (Figs. 6A,B,D and 8A–F), or ultrathin Lowicryl K4M resin sections (UV polymerization at –35 °C; Figs. 6C,E,F) were used. In both cases, we observed strong immune gold labeling of the basement membrane (BM) of the acinus type III (Figs. 6A–C), type II (Fig. 6D), type I, and salivary ducts (data not shown). In Fig. 6A (arrows), the Neu5Gc positive layer was located between two BMs of neighboring acini.

We observed the impact of specimen preparation on the precise localization of gold particles in the BMs. In the cryosections, gold particles were present at the external (hemolymph) side of the layer (Fig. 6B), in contrast to the results of Neu5Gc labeling of the BM in Lowicryl-embedded specimens (Fig. 6C), where the gold particles were more randomly distributed. The structure and thickness of the BMs is shown in greater detail in Fig. S2 (supplementary data); samples were prepared according to the protocol described in Supplementary materials and methods. We determined Neu5Gc labeling densities of the BMs covering acini of types II and III. The results proved higher labeling densities in cryosections (7 ± 1 gold particles/ μm present in acinus type II and 5 ± 1 particles/ μm in acinus type III), than in Lowicryl K4M-embedded specimens, where only 2 gold particles/ μm were observed (same results for both types of acini). In the cryosections the total counts of gold particles and BMs lengths were 504 Au particles per 74.1 μm of BMs (acinus II) and 415 Au particles per 81.2 μm (acinus III); in Lowicryl sections, total counts were 115 Au particles per 70.6 μm (acinus II) and 122 Au particles per 62.1 μm (acinus type III). Using both

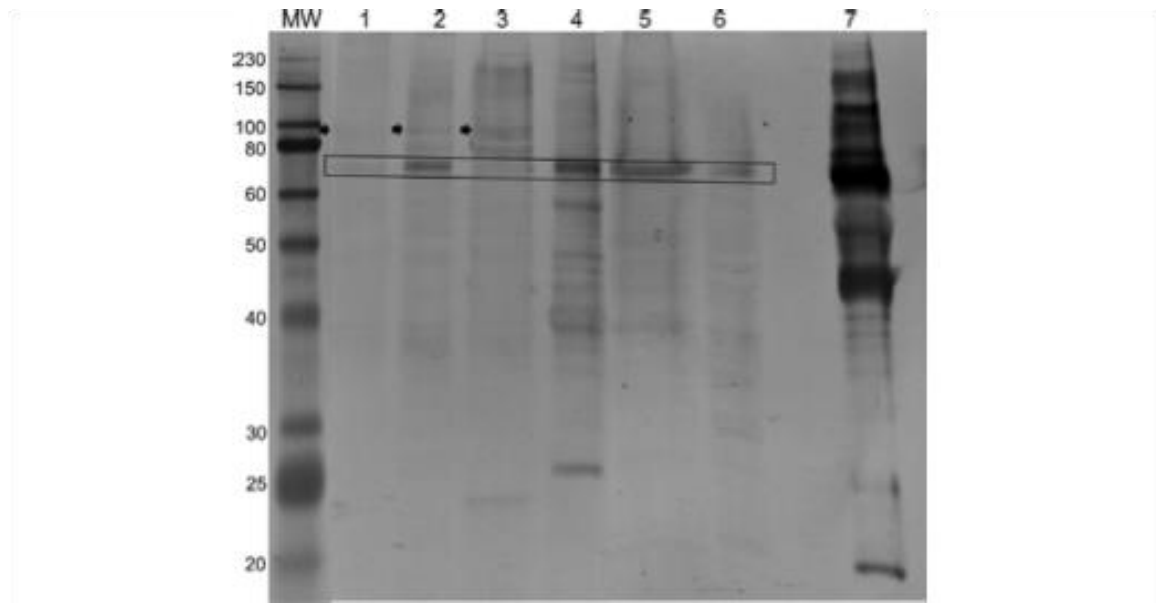


Fig. 3. Immunodetection of Neu5Gc in *I. ricinus* tick tissues and guinea pig serum. SDS-PAGE separated proteins were transferred to PVDF membranes, and probed with anti-Neu5Gc antibody. (1) unfed female SGE, (2) fed female SGE, (3) unfed female gut, (4) fed female gut, (5) fed female ovaria, (6) fed female Malpighian tubes, (7) dealbuminated guinea pig serum. MW molecular markers in kDa.

EM methods of sample preparation, Neu5Gc structures were localized in the acinus type III on membranes of the basal labyrinth, inside the cytosol (Fig. 6E), and on surfaces of microvilli (Fig. 6F) of cells. In the cytosol, Neu5Gc epitopes were found in small vacuoles close to the plasma membrane (Fig. 6E, black arrow). Neu5Gc epitopes were found only rarely associated with the Golgi apparatus (data not shown). *N*-glycosidase F pretreatment of cryosections prior the Neu5Gc labeling led to a negative immunolabeling reaction, confirming that sialylated structures were *N*-glycans. No staining was observed at the BMs or at other sites except for microvilli (results not shown).

Immunohistochemical detection of Neu5Gc structures was performed in tissues isolated from unfed females as well. Very low density of Neu5Gc labeling was observed in the acinar BMs of SGs (Fig. 7A). However, a moderate positive labeling was demonstrated in the basal labyrinth membranes of the acinus type I (Fig. 7B). Neu5Gc was detected also in several parts of gut lumen (Fig. 7C), which can contain residual blood meal. In the gut sections, Neu5Gc structures covered the surface of muscle cells adjacent to the gut (Fig. 7D, arrow).

In the gut isolated from partially fed ticks, the Neu5Gc positive epitopes were found in the lumen containing host blood (Fig. 8A,B). Structures labeled with anti-Neu5Gc antibody were present inside of the clathrin-like coated vesicles (Fig. 8A, white arrows) and accumulated in large granules of the digestive cells (Fig. 8C). Close to the BMs, Neu5Gc epitopes were distributed either inside of the small vacuoles (Fig. 8D) or directly in the cytoplasm of digestive cells (Fig. 8E). Anti-Neu5Gc labeling was absent in the BM of the gut (Fig. 8D,E) except for a few areas (Fig. 8F).

In order to verify the specificity of Neu5Gc labeling, we prepared ultrathin cryosections containing either hAGP or bAGP (Fig. 9). The labeling density achieved in cryosections of Neu5Gc-containing bAGP was high (Fig. 9A, labeling density of 804 ± 170 gold particles per μm^2) and contrasted with the absence of binding in hAGP cryosections (Fig. 9B).

4. Discussion

We report here the structural confirmation of the presence of Sia in the SGs and gut of partially fed females *I. ricinus* for the first time. Sialylated glycans containing both *N*-acetyl- and *N*-glycolneuraminic acid were revealed. Until now, only indirect evidence for the presence of Sia in tick tissues was available (Vancova et al., 2006). Similarly to insects (Koles et al., 2004), sialyltransferase genes were identified in tick *I. scapularis* genome – the putative $\alpha 2,3$ -sialyltransferase (GenBank EEC07371) and $\alpha 2,6$ -sialyltransferase (GenBank ADO17789). A conserved region of the sialyltransferase family (Malissard et al., 2002) is present in another unannotated gene, EEC07911. Other necessary genes coding for the enzymatic machinery required for Sia transfer to glycans or for cytidine monophosphate (CMP)-Neu5Ac conversion to CMP-Neu5Gc were not revealed. It thus appears that ticks have the necessary enzymes enabling the attachment of Sia to glycans.

However, several indirect evidences indicate the host origin of the sialylated glycans. Firstly, all of the sialylated glycans detected in ticks were bi- or triantennary (similar to mammalian sialylated structures) in contrast to the sialylated glycans described in *Drosophila*, where the sialic acid residue was attached to only one antenna (Aoki et al., 2007). Secondly, the sialylated glycans identified in tick samples did not contain core-fucose (Figs. 1 and 4). The result was confirmed by the Neu5Gc-specific staining, which was diminished by PNGase F treatment of cryosections. PNGase F is able to cleave off all the *N*-glycans except for $\alpha 1,3$ -core-fucosylated structures, which are widely present in tick and insect glycans in contrast to mammalian glycans (Fabini et al., 2001; Pedra et al., 2010; Tretter et al., 1993).

Further, Neu5Gc labeling results of ultrathin cryosections prepared from tick guts of partially fed females (Fig. 8) strongly supported this assumption. Neu5Gc epitopes were internalized by the digestive cells (Figs. 8A–C) and were present directly in the cytoplasm of digested cells or inside of small vacuoles, while pass-

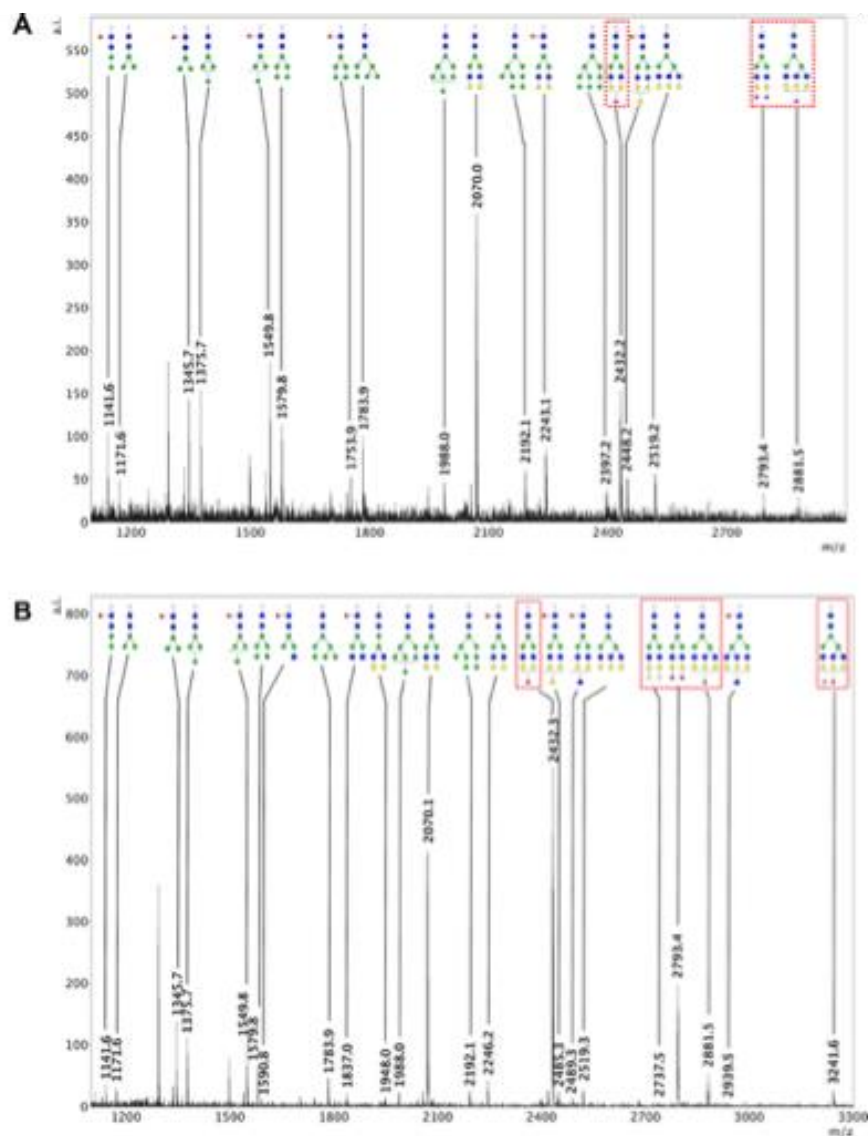


Fig. 4. Mass-spectrometric profiles of permethylated *N*-glycans. (A) Glycans corresponding to sialylated structures were observed in gut isolated from females engorged for 3 days and (B) 6 days. Symbol key: blue square: *N*-acetylhexosamine, green circle: mannose, yellow circle: galactose, red triangle: fucose/deoxyhexose, violet diamond: *N*-acetylneuraminic acid; white diamond: *N*-glycolylneuraminic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ing freely through the gut basement membrane (Figs. 8D–F). It was reported that a small portion of major host proteins (e.g., albumin, transferrin, immunoglobulin G, and hemoglobin) was able to pass through the gut and was found incorporated into other tick tissues (ovary) or even secreted/excreted into tick saliva (Ackerman et al., 1981; Fujisaki et al., 1984; Valenzuela, 2002; Wang and Nuttall, 1994). Similarly, the transcellular transport of intact immunoglobulin fragments, albumin and other proteins across the digestive system was documented in a broad range of non-blood feeding and blood-feeding insect species (Casartelli et al., 2005, 2007; Fian-dra et al., 2009; Jeffers and Roe, 2008).

Moreover, comparison of Neu5Gc labeling patterns of guinea pig serum and tick tissue homogenates (Fig. 3) further confirms that the host blood is the source of the detected Neu5Gc-containing glycans. Molecular weights of labeled protein bands did not differ between the unfed and fed tissues homogenates (Fig. 3), but differences in the intensity of staining of particular proteins confirmed specific uptake of sialylated host proteins during the feeding and their persistence after subsequent molting. Differing intensity of staining of gut homogenates from unfed and fed ticks in comparison to guinea pig serum shows also differences in the digestion rate of particular proteins (Fig. 3).

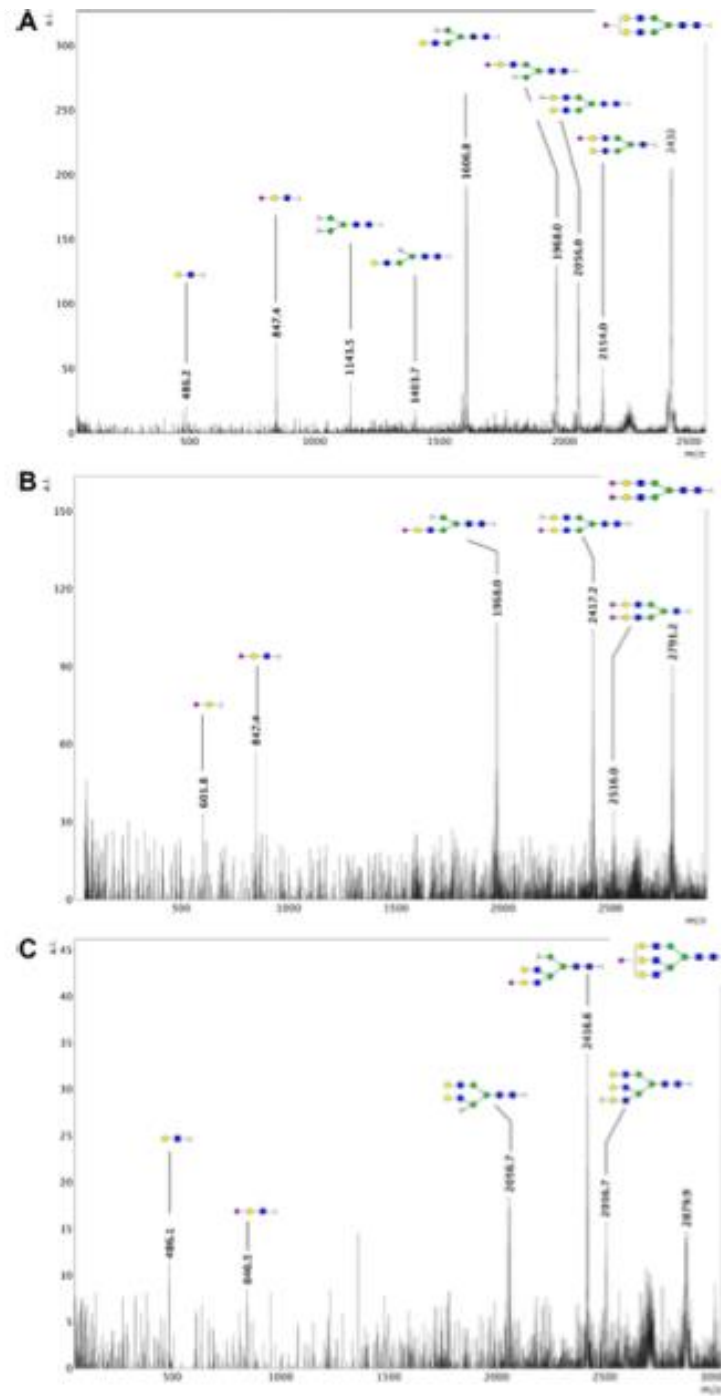


Fig. 5. MALDI-TOF/TOF MS spectra of permethylated *N*-glycan structures with m/z values of (A) 2432, (B) 2791, and 2880 (C) isolated from guts or SGs of partially fed *I. ricinus* females. Symbol key: blue square: *N*-acetylhexosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose/deoxyhexose; violet diamond: *N*-acetylneuraminic acid; white diamond: *N*-glycolylneuraminic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

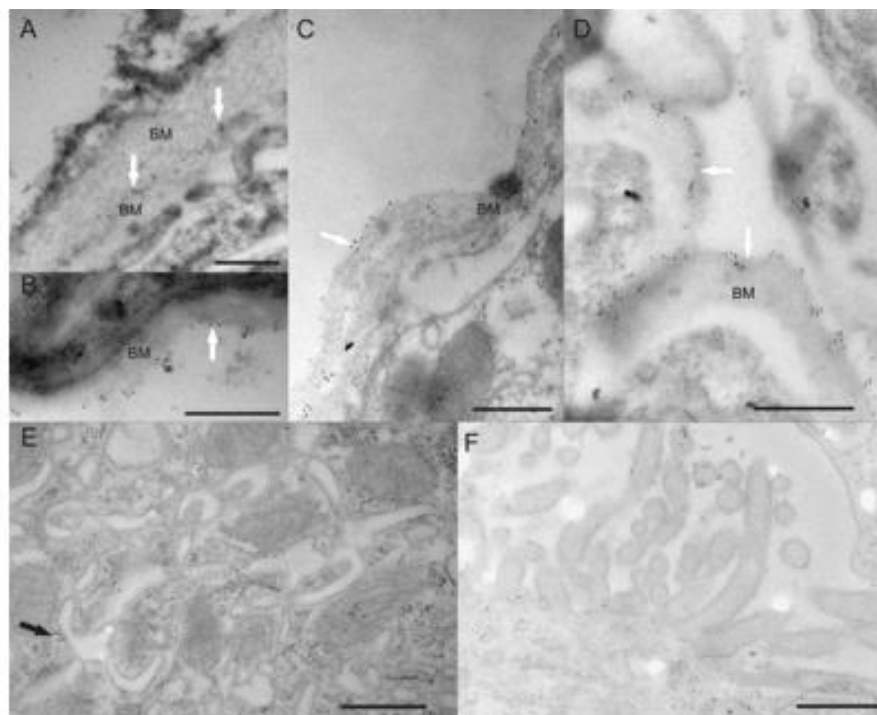


Fig. 6. Electron micrographs of Neu5Gc labeling in salivary glands isolated from partially fed *I. ricinus* females. (A,B,C) Strong Neu5Gc labeling was located on the outer surface layer (white arrow) of the basement membrane of acinus type III and (D) acinus type II. In the acinus type III, Neu5Gc positive structures were located (E) on the highly convoluted membranes of the basal labyrinth (grey arrow), (E) inside of the cytoplasm (black arrow), (F) and on microvillar membranes. (A,B,D) Cryosections, (C,E,F) Lowicryl K4M. Basement membrane (BM). Bar = 500 nm.

In the unfed female tissues, Neu5Gc immunostaining of blotted proteins (Fig. 3, lanes 1 and 3) and of cryosections showed low amounts of Neu5Gc glycans. Trace amount of Neu5Gc structures was identified in gut lumen (Fig. 7C). Other Neu5Gc glycans were present on the hemolymph side of the gut BMs (Fig. 7D), the basal labyrinth of the acinus type I (Fig. 7B), and rarely on the surface of acini BMs (Fig. 7A).

Slight Neu5Gc immunostaining of proteins in the unfed tick females gut (Fig. 3, lane 3) corresponds with the observed incomplete digestion of the blood meal (Wang and Nuttall, 1994, 1995; Wickramasekara et al., 2008). These results are in accordance to well-known fact that intracellular digestion of a blood meal is a gradual process and allows the hard ticks to survive a long period without feeding. Moreover, host proteins have been shown to persist in ticks for long periods (months) after the feeding and the molting events (Wickramasekara et al., 2008).

One of the host serum sialoglycoproteins, which was shown to pass through the gut wall, is the abundant serum transferrin. Transferrin possesses two *N*-linked biantennary glycan chains that differ in the degree of branching and sialylation with up to four Sia residues attached to the molecule (Dijk et al., 1995). Another abundant serum protein, immunoglobulin G contains core-fucosylated biantennary complex glycan molecules with two Sia residues (about 5% of IgG molecules) (Routier et al., 1998). However, the glycan structures identified in the gut or the SGs do not correspond to the oligosaccharides typical for transferrin or IgG molecules. On the other hand, other host blood proteins and lipids are known to be modified by sialylated glycans. In human serum, sialylation of plasma protease C1 inhibitor, orosomucoid, haptoglobin, cerulop-

lasmin, α 1-antitrypsin, and many other glycoproteins has been identified (Alley and Novotny, 2010; Taniuchi et al., 1981).

We suggest that, at least a part of the host sialylated glycoproteins survive the digestion process in the tick gut and are transported into the hemolymph and to the SGs. Neu5Gc structures were localized predominantly on the external surfaces of BMs in all types of SG acini and the salivary duct isolated from partially fed females. In contrast, the BM of the SGs isolated from unfed females was rarely stained with the antibody (Fig. 7A). One can speculate about the function of specific delivery of sialylated glycans to the BM of SG acini and the basal labyrinth of the acinus type I, which have a putative role in uptake of atmospheric moisture by unfed ticks (Needham and Teel, 1986). One of the reasons can be the accumulation of the negative charge on the BM surface (Sias are negatively charged), which helps the permeabilization of water and small solutes but can influence the transport of macromolecules back from the SGs/or to the SGs as well. It was previously proven that sialylation of surface proteins contributes to the function of glomerular filtration barrier in the kidney (Galeano et al., 2007; Quaggin, 2007) and its involvement in the development of SGs in mice has also been suggested (Bernfield and Banerjee, 1972).

Small amount of the Neu5Gc labeling which was found in the cytoplasm of acinus type III, the basal labyrinth, and microvilli in the apical part of *f* cells may suggest excretion of sialylated host molecules from hemolymph into saliva. In ixodid ticks, the *f* cells of the acinus type III are mainly responsible for excretion of excess hyposmotic fluid composed of water and ions. During feeding, these cells substantially enlarge and undergo extensive morpho-

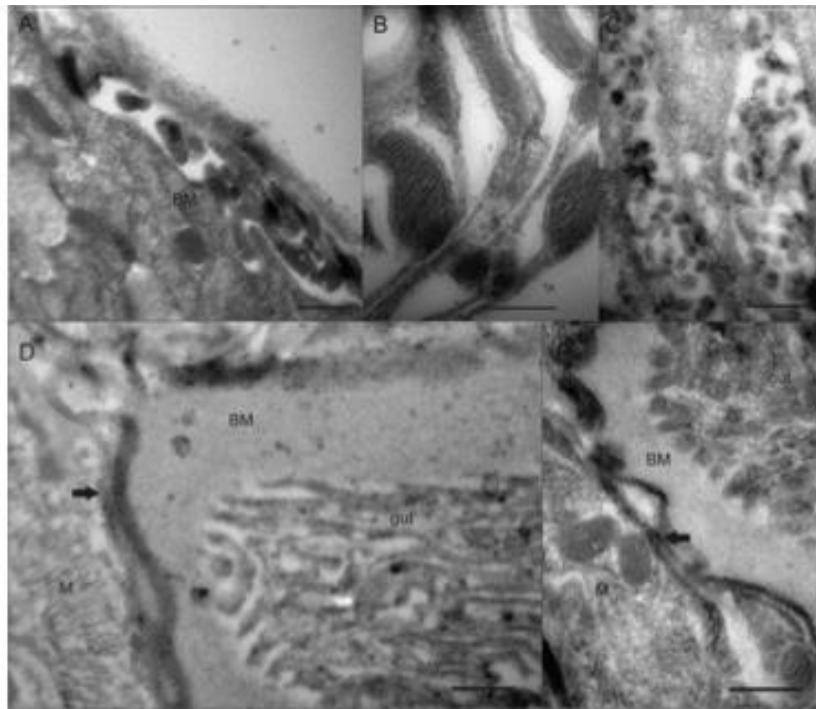


Fig. 7. Neu5Gc labeling of tissue cryosections from unfed *I. ricinus* females. (A) Basement membranes (BM) of the acinus type II showed weak labelling. (B) Moderate labeling was present along the membranes of the basal labyrinth of the acinus type I. (C) Neu5Gc structures were present in gut lumen and microvilli. (D) Neu5Gc positive structures were rarely distributed on the external side of the gut basement membrane (BM) and moderately in the muscle cells (M) membrane (arrow). (E) Control labeling. Bar = 500 nm.

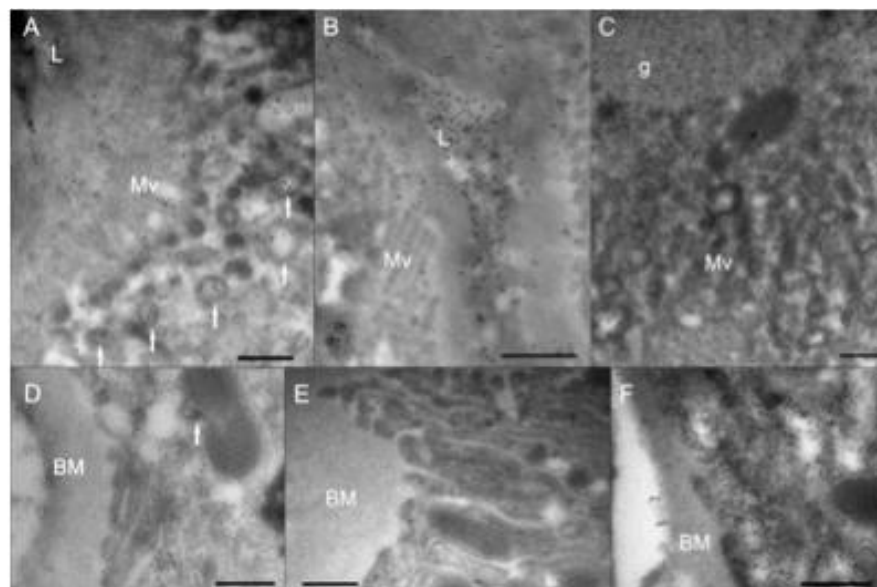


Fig. 8. Electron micrographs of Neu5Gc labeling of ultrathin cryosections of the gut digest cells from partially fed *I. ricinus* females. (A,B) Neu5Gc positive structures were distributed in the lumen (L), along the microvilli (Mv) and inside of endocytic vesicles (arrows). (C) Immunogold labeling showed Neu5Gc modified glycans inside of foaming granules (g). (D,E,F) Close to the gut basement membrane (BM), Neu5Gc epitopes were localized in small vacuoles (arrow) and directly in the cytoplasm. (F) Neu5Gc positive structures found on the basement membrane. Bar = 500 nm.

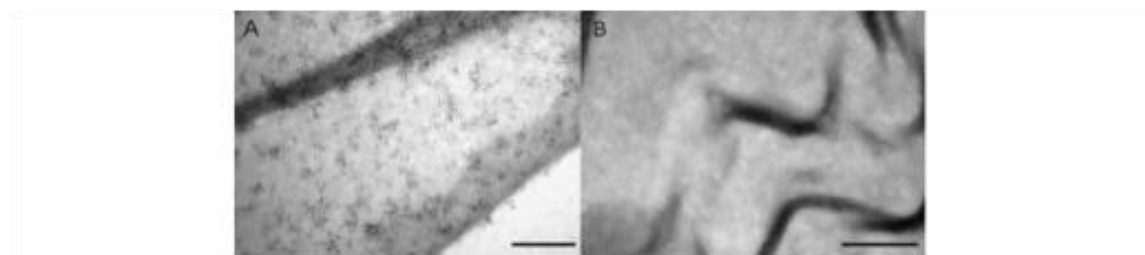


Fig. 9. Electron micrographs of Neu5Gc labeling of (A) bovine and (B) human α 1-acid glycoproteins. Cryosections. Bar = 500 nm.

logical transformation that leads to the development of the basal labyrinth. Extensively and deeply folded plasma membranes in the basal parts of *f* cells form channels and increase the surface area for sufficient uptake of water, ions, and probably other host molecules. Tick SGs were shown to excrete molecules such as intact host immunoglobulin G molecules into saliva in a specific manner (Fujisaki et al., 1984; Wang and Nuttall, 1994). In the hemolymph and SGs of many ixodid tick species, immunoglobulin G binding proteins were revealed (Wang and Nuttall, 1995). Neu5Gc staining was found on the BMs of the granular acini type II, which is (according to ultrastructure) mainly responsible for secretion of many tick salivary components. Plasma membranes of cell types of the acinus type II or secreting granules were not labeled.

Even in the case of the host origin of sialylated glycans, host-derived Sia can be recycled and attached to tick proteins. Glycoconjugates of vertebrates can be desialylated with a lysosomal sialidase. Arthropod sialidase activity has been detected only in the gut and SGs of a blood-feeding bug *Triatoma infestans* to date. This bug excretes sialidase into its saliva during blood feeding (Amino et al., 1995, 1998). Another enzyme, trans-sialidase, is capable of transferring Sia residues to different molecules in the protozoan *Trypanosoma cruzi* and modifying parasite surface molecules (Pereira et al., 1996).

The immunoblotting results confirmed the applicability of the preparative method for tick tissue dissection. Contamination of organs by gut contents (blood meal) is one of the major concerns when analyzing tissue homogenates. Anti-Neu5Gc staining of both unfed and fed tick tissues homogenates showed a different staining pattern in tissue homogenates compared to guinea pig serum which confirms very low contamination of the dissected tissues (Fig. 3).

Finally, localization of Neu5Gc specific structures was demonstrated using two different electron microscope sample preparation techniques, the Tokuyasu method and the embedding into methacrylate based resin. Both methods retained Neu5Gc epitopes, but differed in the labeling density and in the preservation of the fine ultrastructure of the BMs. (Additional information about the BM structure is shown in the Supplementary material). Statistical evaluation confirmed that thawed cryosections are able to retain 2–3 times higher antigenicity for Neu5Gc labeling than Lowicryl K4M resin sections.

5. Conclusions

The previously observed sialic acid-positive lectin staining of tick tissues was confirmed here through Neu5Gc immunostaining and by direct mass-spectrometric identification of sialylated *N*-glycans in tick SGs and guts. Both *N*-acetyl- and *N*-glycolyl-neuraminic acids were detected in the tick samples. Even though ticks confer the sialyltransferase gene, the detected sialylated structures

most probably originate from the host. Trace amounts of Neu5Gc were identified in unfed female tissues. Passage of sialylated structures from the gut lumen containing the blood meal, through digestive cells and the gut basement membrane to SGs, was shown in fed female ticks. In SGs, the sialylated structures specifically localize in the outer surface of basement membrane. Part of the sialylated molecules seems further transferred through the acinus type III of SGs to saliva.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2012.06.016>.

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Paper II

The majority of sialylated glycoproteins in adult *Ixodes ricinus* ticks originate in the host, not the tick

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The majority of sialylated glycoproteins in adult *Ixodes ricinus* ticks originate in the host, not the tick

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ABSTRACT

The presence of sialylated structures in tick organs was observed previously using lectin staining. Recently, we demonstrated the presence of sialylated N-glycans using mass spectrometry in tick salivary glands and the gut. However, we proposed a host (blood) origin for these glycans and mapped the transport of sialylated molecules from the gut to the salivary glands. In this report, we performed quantitation of whole sialic acid and of metabolically incorporated sialic acid (N-azido neuraminic acid) in *Ixodes ricinus* tick samples. We show that the majority of sialylated molecules in the adult tick originate in the host (blood) and are not synthesized by the tick. Similar results were observed for tick cell cultures. The almost complete absence of tick sialylated molecules and the specific transport and localization of host structures into the tick salivary glands and the saliva raises many questions on the role of these molecules in the physiology and, specifically, the blood-feeding of ticks.

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1. Introduction

Sialic acids (Sia) are a group of saccharides providing many functions to glycoproteins in a wide range of organisms, mainly higher Eukaryotes.^{1,2} Moreover, they can be found in some pathogens and parasites where they serve as mimics protecting the microorganisms from the immune system of the host organisms.³ Sialylated structures in the mammalian host cells often serve, on the other hand, as receptors for the pathogens.² While sialylated glycans are widely present in eukaryotes, arthropod glycans are not usually sialylated.^{4,5}

Sia was detected previously in several arthropods including blood-feeding parasites. The best-described species in this regard is the *Drosophila* fly, in which sialylated glycans were described in detail using mass spectrometry.⁶ Here, sialic acid was shown to be present in the embryo, where it occurs in the development of the neuronal system.^{6,7}

The responsible sialyltransferase, a single vertebrate-type sialyltransferase, was identified in *Drosophila melanogaster*.⁸ Putative sialyltransferases were next identified in other *Drosophila* species,

and also in the mosquitoes *Aedes aegypti* and *Anopheles gambiae*, and in other arthropods (*Acyrtosiphon pisum*, *Strongylocentrotus purpuratus*, *Tribolium castaneum* and others).^{9,10}

The $\alpha 2,6$ -sialyltransferase of *Drosophila* was further studied in respect to its specificity and it has been shown to be specific for glycoproteins and oligosaccharides, while no activity has been shown for glycolipids.⁸ Both sialic acid synthesis and sialyltransferase activity are required for the development and function of the nervous system.^{11,12}

Sia-specific lectins (MAA, SNA) have been used for confirmation of the presence of Sia in blood-feeding parasites such as ticks,¹³ and oxidation using sodium metaperiodate suggested the presence of Sia in the gut of mosquitoes.¹⁴ Direct confirmation of Sia in blood-feeding parasites using mass spectrometry was missing until recently, when sialylated N-glycans were described in the ticks *I. ricinus* and *D. marginatus*.¹⁵ However, the analysis of the N-glycans cleaved off the glycoproteins did not prove their tick origin. This is particularly the case for obligatory blood-feeding parasites, in which the ingestion of huge amounts of mammalian blood takes place. The presence of intact host proteins from the tick gut in the haemolymph and other organs including salivary glands was shown previously.^{16–19} Thus it is possible that sialylated host proteins could also be present in the tick haemocoel and internal organs. Accordingly, we proposed a host origin for the observed sialylated glycans and showed the probable route of transport of

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sialylated molecules from the gut through the tick haemocoel into the salivary glands, from where these molecules could be released back into the host.¹⁵

In the present study we performed the quantification of sialylated molecules in tick organs, whole ticks and tick cell cultures and compared the overall amount of Sia to that of bioorthogonally labelled sialylated molecules. Several approaches were used for the quantitation of sialylated molecules, while metabolically labelled Sia (using *N*-azido-mannosamine, ManNAz, which is metabolized to *N*-azido-neuraminic acid, NeuNAz¹⁰) was reacted with Alexa Fluor® 488-alkyne through the Click reaction²⁰ and was subsequently labelled and quantified using anti-Alexa Fluor® 488 antibodies.

Our results show that the majority of sialylated molecules in the adult tick originate in the host (blood) and are not synthesized by the tick. In cultures of a continuous embryo-derived tick cell line, bioorthogonally labelled structures were not observed at all. Fluorescence microscopy (FM) of bioorthogonally labelled tick organs and cultured cells supported these results.

2. Materials and methods

2.1. Ticks and in vitro feeding

I. ricinus females were obtained from the tick rearing facility of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic in České Budějovice. The ticks were allowed to feed using artificial in vitro feeding on ovine blood.²¹ In brief, 3.1 mL aliquots of ovine blood were supplemented with ATP (1 mM), gentamicin (5 µg/mL), Nystatin (100 i.u./mL, all from Sigma–Aldrich, Saint Louis, MO, USA) and 3.1 µL of 25 mM *N*-azido-mannosamine (Life Technologies, Carlsbad, CA, USA) in DMSO; this mixture was placed in wells of a 6-well cell culture microplate (TPP, Trasadingen, Switzerland). In the control experiments, 3.1 µL of DMSO without ManNAz was used. The plate was placed into a water bath at a temperature of 37 °C. Unfed *I. ricinus* females were put into feeding units²¹ (ten ticks in each unit) and the units were placed in the wells of the plate. The blood was replaced with fresh supplemented blood every 12 h during the feeding. To avoid bacterial or fungal contamination, feeding units were washed with sterile phosphate buffered saline (PBS) before each blood change and with Nystatin solution (100 i.u./mL) every two days. After five days of feeding, ticks were collected and dissected if required. Tick organs (salivary glands, gut, Malpighian tubules and ovaries) or whole ticks were homogenized or processed for fluorescence microscopy.

2.2. Tick cell line

The *I. ricinus* embryo-derived cell line IRE/CTVM19²² was grown in L-15 medium supplemented with 20% foetal calf serum, 10% tryptose phosphate broth, 2 mM glutamine and 1% of Antibiotic–Antimycotic Solution (all PAA Laboratories, Pasching, Austria) at 28 °C in flat-sided cell culture tubes (Nunc, Thermo Scientific, Waltham, MA, USA). For metabolic labelling of Sia, the medium was supplemented with 25 µM ManNAz and the cells were grown for at least 4 days. Thereafter, cells were detached by pipetting and the cell suspension was centrifuged at 400g for 10 min at 4 °C to pellet the cells. The pelleted cells were washed 3 × with PBS to remove the remaining medium, sonicated and centrifuged at 14,000g for 10 min at 4 °C to clear the lysates. Lysates of cells from three different culture tubes were analysed by each of the quantitation methods (2.4–2.7); three replicate samples were prepared from each lysate for each method.

2.3. Preparation of homogenates from tick tissues or whole ticks

Tick tissues were dissected from in vitro partially fed females in ice cold distilled water containing 10 µL/mL Halt™ Protease Inhibitor Single-Use Cocktail (Thermo Scientific). The tissues were carefully removed to prevent contamination from the contents of the gut and separately rinsed thoroughly in water. The gut tissues were rinsed to remove most of the lumen contents. Tissues of three to five ticks were pooled, sonicated and centrifuged at 14,000g for 10 min at 4 °C to clear the lysates. The protein concentrations in the supernatants were measured using a Qubit® fluorometer (Life Technologies). The lysates were stored at –20 °C. Three different lysates were analysed by each of the quantitation methods (2.4–2.7); three replicate samples were prepared from each lysate for each method.

2.4. Quantitation of Sia using aminooxy-biotin

In this approach we used the combination of periodate oxidation of Sia with aniline-catalysed oxime ligation (PAL).^{24,25} Quantitation was performed in white 96-well ELISA microplates (Greiner Bio-one, Frickenhausen, Germany). The wells were coated with tick cell lysates, tick organ lysates or control proteins (bovine serum albumin BSA, fetuin) using approximately 20 µg of proteins/well in a coating buffer (0.1 M sodium carbonate buffer, pH 9.6) for 2 h at ambient temperature. After coating, the wells were washed 3 × with Wash buffer (0.05% Tween 20 in phosphate buffered saline PBS, pH 7.4), with the last wash performed on ice. Sia was oxidized with 100 µL of 1 mM sodium periodate/well for 30 min on ice²³ and the reaction was stopped by the addition of 50 µL of 50 mM sodium thiosulphate/well. The wells were washed and subsequently blocked with 3% BSA/PBS overnight at 4 °C. Next, the wells were washed and the aldehyde groups of the Sia were reacted with aminooxy-biotin (100 µL of 100 mM aminooxy-biotin, 10 mM aniline in PBS/well) for 90 min on ice. Each well was then washed with 1% BSA and the biotin molecules were labelled with STREP-HRP (Vector Laboratories, Burlingame, CA, USA, 33.3 pmol conjugate in 1% BSA/well) for 1 h at ambient temperature. After three washing steps, luminescence was measured using the DuoLux substrate (Vector Laboratories). The standard curve was constructed using the signal from wells with serial dilutions of STREP-HRP. Chemiluminescence measurements were performed on an Infinite M200 Pro microplate reader. For each tick sample, the content of Sia was expressed as the mean amount of Sia in µmoles per g of proteins. Measurement of each replicate was performed three times.

2.5. Quantitation of Sia using thiobarbituric acid assay

This method for Sia quantitation uses the coupling reaction of thiobarbituric acid (TBA) with the aldehyde formed during Sia oxidation.²⁶ Sia was released from the control proteins and tick samples (approx. 20–100 µg of total proteins) using 25 U α2-3,6,8-neuraminidase from *Clostridium perfringens* (New England Biolabs, Ipswich, MA, USA) or 0.05 M H₂SO₄.²⁷ A 40 µL aliquot of the neuraminidase-treated sample was oxidized by the addition of 20 µL of 0.2 M sodium metaperiodate in 9 M phosphoric acid for 20 min at ambient temperature. The unreacted metaperiodate was reduced using 200 µL of 10% sodium arsenite in 0.5 M sodium sulfate and 0.1 N sulfuric acid. The aldehyde formed during the oxidation step was coupled to TBA by addition of 600 µL of 0.6% TBA in 0.5 M sodium sulfate and boiling the reaction mixture for 15 min. The mixture was then cooled for 5 min on ice and the coloured product was extracted into 860 µL acidic butanol, the upper organic phase was removed and the absorbance of the product was

measured at 549 nm. For preparation of the standard curve, serial dilutions of sialic acid were used in the procedure. Measurements of absorbance were performed on the Infinite M200 Pro microplate reader. For each tick sample, the content of Sia was expressed as the mean amount of Sia in $\mu\text{moles per g}$ of proteins. Measurement of each replicate was performed three times.

2.6. Quantitation of Sia using enzymatic reactions

Another method of detection used a commercial kit (Sialic Acid Quantitation Kit, Sigma–Aldrich). This kit utilizes a set of enzymes (neuraminidase, *N*-acetylneuraminic acid aldolase and lactate dehydrogenase) which result in NADH oxidation proportional to released Sia concentration. The experiments were performed according to the manufacturer's instructions. For preparation of the standard curve, serial dilutions of sialic acid were used in the procedure. Measurements of absorbance were performed on a Nanophotometer® and the Infinite M200 Pro microplate reader. For each tick sample, the content of Sia was expressed as the mean amount of Sia in $\mu\text{moles per g}$ of proteins. Measurement of each replicate was performed three times.

2.7. Quantitation of metabolically labelled Sia using the Click reaction

Quantitation of metabolically-labelled Sia was performed in 96-well ELISA microplates (Greiner Bio-one, Frickenhausen, Germany). Tick organ lysates were diluted 1:20 and a volume corresponding to approx. 150–250 μg of protein was used for the Click reaction. The Click reaction was performed using the Click-iT® Protein Reaction Buffer Kit with Alexa Fluor® 488 (both Life Technologies) according to the manufacturer's instructions. During the procedure, the non-metabolized ManNAz was removed from the samples using chloroform–methanol precipitation and only NeuNAz metabolically incorporated into proteins was labelled with the Alexa dye. After the Click reaction, protein concentration was measured and approximately 20 μg of sample protein was used per well for coating for 2 h at ambient temperature. After coating, the wells were washed 3 $\times$ with Wash buffer and blocked with 3% BSA/PBS overnight at 4 °C. Next, the wells were washed and incubated with 20 μM rabbit anti-Alexa Fluor® 488 (Life Technologies) in 1% BSA for 1 h at ambient temperature. After another three washing steps, goat anti-rabbit antibodies conjugated with HRP (Vector Laboratories) were added and incubated for 1 h at ambient temperature. Detection was performed as in 2.5. Each measurement was performed three times.

2.8. Detection of metabolically labelled Sia in tick tissues and cell line using fluorescence microscopy

Tissues were dissected from female ticks artificially fed for 5 days on ovine blood supplemented with ManNAz as described above. Each tick organ was placed into a separate well in a 96-well plate, which simplified manipulation of the samples. Organs were washed for 3 $\times$ 10 min with 100 μL of PBS. The tissues were then fixed with 100 μL of 4% formaldehyde for 15 min, permeabilized with 100 μL of 0.25% Triton-X100 in PBS for 15 min, and blocked using 2 washes with 100 μL of 50 mM NH_4Cl in 1% BSA/PBS for 10 min. The azide-labelled molecules were reacted with alkyne–streptavidin in the Click reaction according to the manufacturer's instructions in a volume of 100 μL /well. Tissues were washed with 100 μL of 1% BSA for 1 h after the Click reaction. Biotinylated molecules were labelled with Streptavidin DyLight® 488 or Streptavidin DyLight® 549 (both Vector Laboratories) diluted 1:1000 in 1% BSA for 1 h. Finally, the tissues were washed for 3 $\times$ 20 min in 1% BSA and 3 $\times$ 20 min in PBS and put onto microscopic slides. A drop

of DAPI containing Vectashield® Mounting Medium (Vector Laboratories) was applied and the tissues were covered with a cover slide. Fluorescent images were acquired using an Olympus BX60 fluorescence microscope equipped with an Olympus DP71 camera (both Olympus, Tokyo, Japan).

The tick cell line was grown in complete culture medium as described above. Sterile round cover glasses were put into each well of a 24-well plate. Cells in optimal growth phase were resuspended by pipetting, 200 μL of suspension was transferred into each well of the 24-well plate, and 200 μL of medium with 50 μM ManNAz was added (final ManNAz concentration 25 μM). After 4 days of incubation, the cover glasses covered with tick cells were transferred into a new 24-well plate and each glass was washed 2 $\times$ with 500 μL PBS for 10 min. Next, cells were treated using the Click-iT® Cell Reaction Buffer Kit (Life Technologies) as described above for tick tissues with one difference: the volume of all solutions used was 400 μL except for the volume of Click reaction mixture, which was 100 μL /well. After the labelling, the glasses were mounted and fluorescent images were acquired as described above.

2.9. Statistical analysis

Data were evaluated with the unpaired parametric two-tailed *t*-test using GraphPad Prism version 6.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Values shown are mean $\pm$ standard error of the mean (SEM). Data from at least three independent experiments were used. A *P* value of <0.05 was considered significant.

3. Results

3.1. Quantitation of total Sia by chemical assays

Two different chemical assays were used to quantitate total sialic acid in tick samples. Both methods utilize oxidation of Sia by metaperiodate and detection of the resultant products. In the first method, aminoxy-biotin is conjugated to the aldehyde group formed by Sia oxidation (PAL) and the biotin molecule is detected. In the second method, thiobarbituric acid reacts with the β -formylpyruvic acid (product of Sia oxidation) and the resultant chromophore is quantified.

The amounts of Sia in tick organs obtained using the PAL method ranged from $0.17 \pm 0.054 \mu\text{mol Sia/g}$ of protein in ovaries to $0.573 \pm 0.358 \mu\text{mol Sia/g}$ of protein in the gut (Table 1, Fig. 1A). Malpighian tubules, whole tick samples and cultured tick cells were not tested in this assay.

The TBA assay yielded higher values overall for Sia content compared to the results from the PAL method. The highest Sia content in tick organs was detected in ovaries and gut, while lower levels were detected in salivary glands and Malpighian tubules (Table 1, Fig. 1B). Much higher levels of Sia were detected in whole ticks ($60.45 \pm 15.64 \mu\text{mol Sia/g}$ of protein) and the IRE/CTVM19 cell line ($86.58 \pm 22.52 \mu\text{mol Sia/g}$ of protein) (Table 1, Fig. 1C). In general, high variability of Sia content was observed in the replicate samples (Fig. 1).

3.2. Quantitation of total Sia by enzymatic assay

For comparison, a commercial Sia quantitation kit utilizing enzymatic reactions was also used. The determination efficiency of each replicate of the assay was determined using the commercial standards. The values of the Sia content obtained using the commercial kit were several orders of magnitude higher than those obtained with the chemical methods (Table 1).

Table 1
Content of Sia and NeuNAz in tick samples expressed as mean $\pm$ SEM

Sample [μ mol Sia/g protein]	Ovary	Salivary glands	Gut	Malpighian tubules	Whole tick	IRE/CTVM19
PAL	0.17 $\pm$ 0.054	0.33 $\pm$ 0.184	0.573 $\pm$ 0.358	ND	ND	ND
TBA Assay	8.5 $\pm$ 2.667	4.958 $\pm$ 1.19	8.244 $\pm$ 1.457	1.343 $\pm$ 0.54	60.45 $\pm$ 15.64	86.58 $\pm$ 22.52
Sia quantitation kit	1871 $\pm$ 896.7	1460 $\pm$ 832.9	1220.2 $\pm$ 322.9	ND	ND	175.8 $\pm$ 6.04
NeuNAz quantitation	0.015 $\pm$ 0.003	0.012 $\pm$ 0.003	2.318 $\times 10^{-3}$ $\pm$ 3.148 $\times 10^{-4}$	0.013 $\pm$ 4.903 $\times 10^{-5}$	5.853 $\times 10^{-3}$ $\pm$ 1.424 $\times 10^{-4}$	0

Content is expressed as μ mol of Sia or NeuNAz per gram of protein. PAL—periodate oxidation of Sia with aniline-catalysed oxime ligation, TBA—thiobarbituric acid.

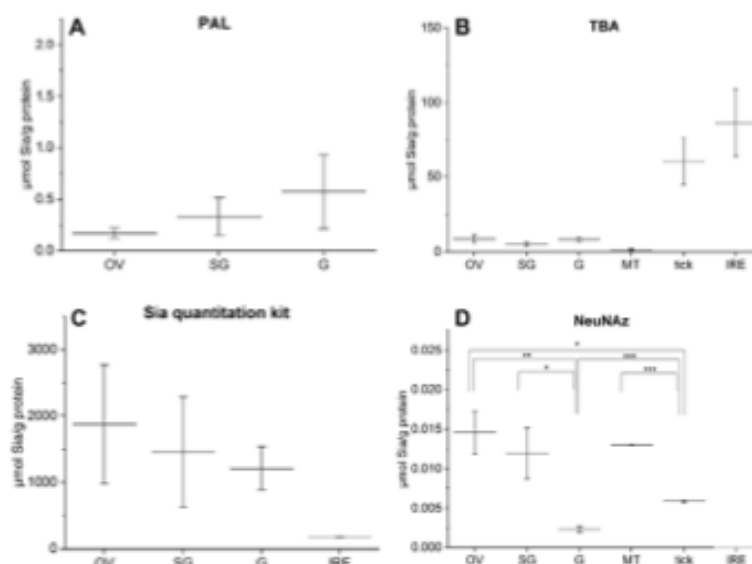


Figure 1. Content of sialic acid (A–C, Sia) and N-azidoneuraminic acid (E, NeuNAz) in 5-day fed female *I. ricinus* tick ovaries (OV), salivary glands (SG), gut (G), Malpighian tubules (MT), in whole tick homogenate (tick) and in tick cell line IRE/CTVM19 lysate (IRE). Sia content was measured using (A) periodate oxidation with aniline-catalysed oxime ligation (PAL), (B) thiobarbituric acid assay (TBA) and (C) Sia quantitation kit. Means are depicted by the horizontal lines; bars represent the standard errors of the means. Content is expressed as μ mol of Sia or NeuNAz per gram of protein. NeuNAz content in the tick cell line was zero. Statistical significance is indicated by asterisks * <0.05 , ** <0.01 , *** <0.001 .

Sia content determined using the commercial kit was again highest in the ovary samples (1871 ± 896.7 μ mol Sia/g of protein); similar values were also obtained for the salivary glands and gut (Table 1, Fig. 1D). Malpighian tubules and whole ticks were not tested in this assay. As with the TBA assay, high variability in Sia content between replicates was observed when measured using the enzymatic kit (Fig. 1).

3.3. Quantitation of metabolically-labelled Sia

Bioorthogonal labelling was used for estimation of the amount of Sia metabolically incorporated in vivo and in vitro into the tick proteins. ManNAz was metabolized by the ticks into NeuNAz, which was incorporated into the proteins. For quantitation, NeuNAz present in the samples was reacted with Alexa Fluor® 488-alkyne (Click reaction) and the dye-containing molecules were quantified using the corresponding anti-dye antibodies conjugated to HRP. As with Sia content, high amounts of NeuNAz were shown to be present in ovaries (0.015 ± 0.003 μ mol NeuNAz/g of protein), salivary glands (0.012 ± 0.003 μ mol NeuNAz/g of protein), and also Malpighian tubules ($0.013 \pm 4.903 \times 10^{-5}$ μ mol NeuNAz/g of protein) (Table 1, Fig. 1E). Much lower amounts of NeuNAz were detected in the gut and the whole tick homogenate (Table 1, Fig. 1E and F). In the IRE/CTVM19 cell line lysate, NeuNAz was not detectable by this method (Table 1, Fig. 1F).

Unpaired parametric two-tailed test (GraphPad Prism) was used for the comparison of the Sia and NeuNAz contents in the organs. While Sia content (mean values) in the tick organs did not differ significantly, NeuNAz content was much more variable, and significant difference was observed between the NeuNAz content in ovaries and the gut ($P = 0.0044$) and salivary glands and the gut ($P = 0.0252$) (Fig. 1). NeuNAz content was also significantly higher in the ovaries ($P = 0.0438$), gut ($P = 0.0003$) and Malpighian tubules ($P = 1.176 \times 10^{-6}$) than in the whole tick (Fig. 1). Furthermore, the Sia content was significantly higher in the ovaries ($P = 0.0084$), salivary glands ($P = 0.0014$) and gut ($P = 0.0002$) than in the whole tick.

The Sia content (determined using the TBA method) was significantly higher than the NeuNAz content in the ovaries ($P = 0.0344$), salivary glands ($P = 0.0167$) and the gut ($P = 0.0065$). Similarly, Sia content of the IRE/CTVM19 cell line was significantly higher than the NeuNAz content ($P = 0.0479$). In order to better represent the ratios of Sia and NeuNAz contents in the different organs, their relative content is presented in Fig. 2, where 100% corresponds to the mean of Sia content in the sample.

3.4. Detection of metabolically-labelled Sia in tick cell line and tick tissues by fluorescence microscopy

Metabolically-labelled Sia was further assayed by fluorescence microscopy in IRE/CTVM19 tick cells grown in culture medium

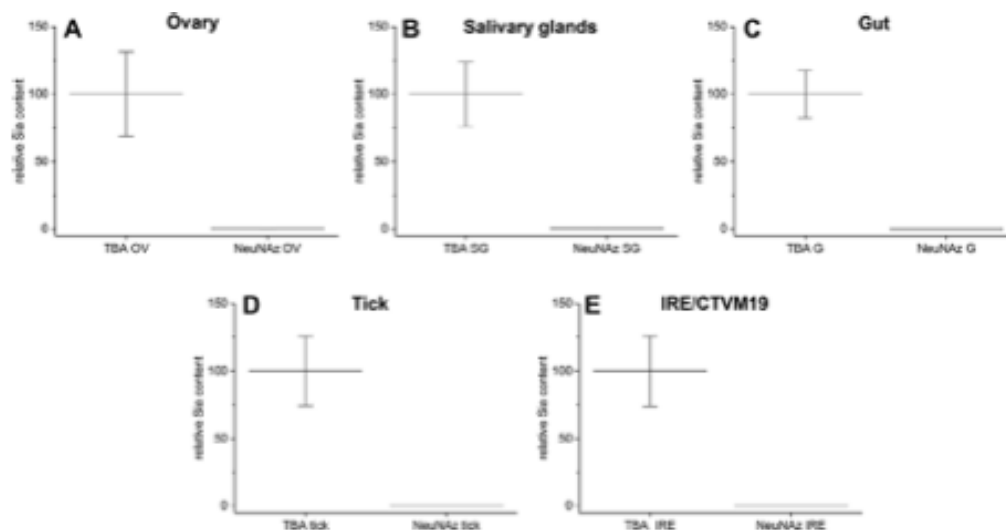


Figure 2. Content of sialic acid measured by thiobarbituric acid assay (TBA) and content of *N*-azidoneuraminic acid (NeuNAz) in (A) fed female *I. ricinus* tick ovary, (B) salivary glands, (C) gut, (D) whole tick homogenate (Tick) and (E) in the tick cell line IRE/CTVM19 lysate. Means are depicted by the horizontal lines; bars represent the standard errors of the means. For better representation of the ratios of Sia and NeuNAz contents in the organs, their relative content is presented, where 100% corresponds to the mean Sia content (in μmol per gram of protein) in the sample.

supplemented with ManNAz and in the tissues of ticks artificially fed on ovine blood with addition of ManNAz. In the tick cell line, we did not detect any signal corresponding to NeuNAz by the fluorescence microscopy (data not shown).

Tick tissues on the other hand showed the presence of low amounts of NeuNAz. In ovaries, some of the oocytes were positively labelled with fluorescently-labelled STREP. The supporting cells surrounding the oocytes, however, did not show any staining (Fig. 3A). Positive staining was also observed in the salivary glands (Fig. 3B). Again, the faint staining was visible only in some granular acini (type II or III), while the majority of the salivary gland was not stained at all (Fig. 2B). The lumen of the gut showed no signal (data not shown), but staining was visible in most of the gut cells, for example in the muscle cells of the gut (Fig. 3C).

4. Discussion

The presence of sialylated structures in tick organs was observed previously using lectin staining.¹³ Heavy sialic acid-specific lectin WGA (wheat germ agglutinin) and MAA II (*Maackia amurensis* agglutinin II) staining was observed in salivary glands in fed and unfed female ticks, but other tissues were not investigated. Recently, we demonstrated the presence of sialylated *N*-glycans using mass spectrometry in tick salivary glands and gut.¹⁵ As with insects,⁸ sialyltransferase genes were identified in the genome of the tick *I. scapularis*—the putative $\alpha 2,3$ -sialyltransferase (GenBank EEC07371) and $\alpha 2,6$ -sialyltransferase (GenBank ADO17789). A conserved region of the sialyltransferase family genes²⁸ is present in another un-annotated gene, EEC07911. However, we proposed a host (blood) origin for these glycans and mapped the transport of sialylated molecules from the gut to the salivary glands. The sialylated molecules have been localized in specific parts of the salivary glands suggesting specialized transport mechanisms and functions in the physiology of the tick.¹⁵

The presence of host proteins/molecules can be expected in all blood-feeding parasites including ticks. Haemolymph and whole

body homogenates of fed ticks contained for example immunoglobulins,^{29,30} and these were also present in the whole body homogenates of unfed ticks.³⁰ Secretion of host immunoglobulins via saliva was assumed to occur during feeding; interestingly, host proteins that had been taken up during feeding of the previous tick life-stage were excreted by the subsequent stage.³⁰ A specific uptake mechanism for immunoglobulins was also suggested and at least parts of the antibody molecules were not degraded by the tick as they retained their antigen-binding ability.³¹ Additionally, host globins, histones and other proteins were detected by mass spectrometry in ticks several months after feeding.³²

To determine the content of Sia in ticks and their organs and the origin of sialylated molecules, we performed the quantitation of total sialic acid and compared the overall amount of sialic acid to biorthogonally labelled sialylated molecules. Several methods were used for quantitation of Sia, including the widely used TBA assay and two other methods, which do not use the highly toxic arsenite.

The three methods used for determination of Sia content provided results which differed greatly for the same samples (Table 1). For each sample tested, the commercial enzymatic kit gave the highest results, most probably due to the presence of pyruvic acid and other α -keto acids in the samples.³³ In contrast, the PAL method resulted in very low values of Sia in the studied organs, even in the gut, which is full of ingested blood. The reason for such low values could be an incomplete reaction with aminoxy-biotin during our 90 min incubation, as reaction times of up to one day were suggested elsewhere.³⁴ Therefore, we used only the results of the commonly used TBA assay for the comparison of Sia and NeuNAz content in the tick samples.

High variability in Sia content per gram of protein was observed between the pooled samples. This could be caused by several factors—inter-individual variability in Sia content in tick organs during feeding, variability of protein content in the tick organs^{35–37} related to variation in proteosynthesis therein,³⁸ or variation in haemolymph volume³⁹ which could affect the concentration of the measured molecules in the haemolymph and consequently

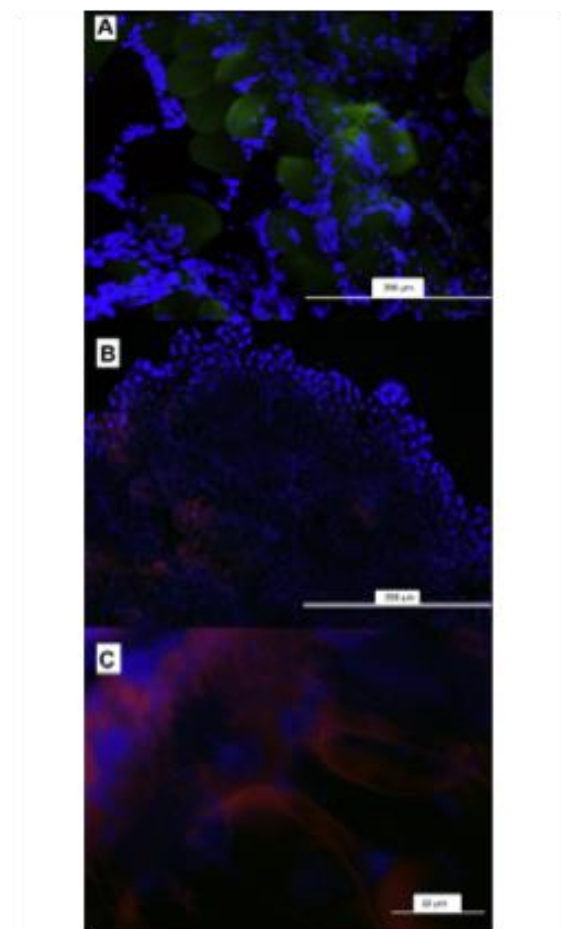


Figure 3. Detection of NeuNAz in (A) ovary, (B) salivary gland and (C) gut of a partially fed female *I. ricinus* tick. Green = Streptavidin DyLight[®] 488, red = Streptavidin DyLight[®] 549, blue = DAPI staining of nuclei. NeuNAz was detected in some of the developing eggs (green), but not in the surrounding supporting cells, in some of the salivary gland acini (red) and in the gut tissue (red).

also in the organs. An important variable is the *in vitro* feeding, as the exact time of the individual tick attachment as well as the feeding process cannot be assessed.

The Sia content measured by TBA assay was between 1.3 and 8.5 $\mu\text{mol Sia/g}$ of protein in the tick organs and approximately 60 $\mu\text{mol Sia/g}$ of proteins in the whole tick homogenates. On the other hand, NeuNAz quantitation showed the content of metabolically incorporated Sia to be three orders of magnitude lower in ovaries and salivary glands and even lower in the gut and whole tick homogenate. The efficiency of ManNAz incorporation into sialylated glycoproteins was shown to be about 3% *in vivo* in mice⁴⁰ and a maximum of 56% *in vitro* in human prostate cancer cells.⁴¹ Even considering that the incorporation efficiency level may be low in ticks, the Sia content was at least one order of magnitude higher compared to the NeuNAz content in ovaries and salivary glands, and three orders of magnitude higher in the gut and whole tick homogenate. Interestingly, NeuNAz content

was significantly lower in the gut than in the other organs and the whole tick (Fig. 1D). These results suggest that the quantified NeuNAz corresponds to the actual metabolically incorporated NeuNAz; the free ManNAz, which is not metabolized and thus not incorporated into glycoproteins, is present in the blood and consequently in the gut at highest concentration.

Overall, the Sia content was significantly higher in individual organs compared to the whole tick homogenate. This suggests the presence of high amounts of host-derived sialylated molecules in the whole tick (and the gut), while the tick-produced sialylated proteins are more prevalent in the salivary glands and ovaries. The presence of host Sia in the gut can be expected, as it contains large amounts of host blood. This factor also explains the presence of host Sia in the whole tick homogenate, which also contains the gut containing host blood.

Relatively high amounts of metabolically labelled NeuNAz-containing molecules that we detected in tick ovaries presented a similar situation to the presence of Sia in another arthropod, the fruit fly *Drosophila*, where it was detected only in the embryo and where it participates in the development of the neural system.^{6,42} It is important to point out that fruit flies do not feed on blood, and thus all the Sia detected in the flies can be attributed to the fly metabolism.

Comparison of Sia and NeuNAz content in the IRE/CTVM19 cell line yielded interesting results. While the Sia content was even higher than that of the whole tick homogenate (86 $\mu\text{mol Sia/g}$ of protein), no NeuNAz was detected in the cell lysate. The presence of Sia can be explained by binding to the cells of sialylated proteins from the bovine serum in the culture medium. The absence of NeuNAz is on the other hand surprising, as the IRE/CTVM19 cell line is embryo-derived. As mentioned above, the presence of Sia was shown in *Drosophila* embryos and we detected NeuNAz in tick ovaries. However, Sia content of the oocyte and the embryo may not correspond and Sia may be present in the oocyte for example in the yolk proteins as suggested for other arthropods.⁴³ Furthermore, not all cell types from embryos are present in continuous tick cell lines²² and thus cells expressing sialyltransferases may not be present or may not normally be subjected *in vitro* to the conditions supporting Sia expression.

5. Conclusions

Our results show that the majority of sialylated molecules in the adult female tick originate in the host (blood) and are not synthesized by the tick. However a low level of sialyltransferase activity seems to be present, based on the quantitation of NeuNAz. These findings, and the specific transport and localization of host molecules into the tick salivary glands and saliva shown previously,¹⁵ raise many questions on the role of these molecules in the physiology and, specifically, the blood-feeding of ticks. Currently, identification of these host proteins and of the associated transport mechanisms is under way to elucidate these mechanisms.

Sia is generally not produced by arthropods,^{4,5} its presence and importance for physiology has only been shown in the *Drosophila* embryo.^{6,7,42} The high content of Sia in the tick body could be simply explained by the presence of host sialylated proteins. We confirmed this assumption by the detection of low amounts of NeuNAz in tick samples. Thus, we propose, that the majority of Sia detected in adult fed female *I. ricinus* ticks originates in host proteins from the blood meal and not in the tick itself. On the other hand, a low level of sialylation was demonstrated in tick organs based on NeuNAz detection, mostly in ovary and salivary glands. Sialylation of tick proteins could therefore be important for the development of eggs and/or embryos.

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Paper III

A bite so sweet: the glycobiology interface of tick-host-pathogen interactions

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REVIEW

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A bite so sweet: the glycobiology interface of tick-host-pathogen interactions

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Abstract

Vector-borne diseases constitute 17% of all infectious diseases in the world; among the blood-feeding arthropods, ticks transmit the highest number of pathogens. Understanding the interactions between the tick vector, the mammalian host and the pathogens circulating between them is the basis for the successful development of vaccines against ticks or the tick-transmitted pathogens as well as for the development of specific treatments against tick-borne infections. A lot of effort has been put into transcriptomic and proteomic analyses; however, the protein-carbohydrate interactions and the overall glycobiology of ticks and tick-borne pathogens has not been given the importance or priority deserved. Novel (bio)analytical techniques and their availability have immensely increased the possibilities in glycobiology research and thus novel information in the glycobiology of ticks and tick-borne pathogens is being generated at a faster pace each year. This review brings a comprehensive summary of the knowledge on both the glycosylated proteins and the glycan-binding proteins of the ticks as well as the tick-transmitted pathogens, with emphasis on the interactions allowing the infection of both the ticks and the hosts by various bacteria and tick-borne encephalitis virus.

Keywords: Tick, Pathogen, Host, Glycan, Lectin, Glycobiology, *Borrelia*, *Anaplasma*, TBEV, Carbohydrate-binding

Background

Vector-borne diseases constitute 17% of all infectious diseases in the world [1]. Pathogenic viruses, bacteria, and protozoa are carried by blood-feeding arthropods on just about all the continents and both livestock and people tend to be affected by these. This becomes a large economic burden on the animal health sector and on the public health system of various countries. Ticks are the first among blood-feeding vectors in terms of the number of pathogens that they can transmit. Unfortunately, there are next to no vaccines against the tick-transmitted bacterial and protozoan diseases and very few against tick-borne viruses [2]. The only successful anti-tick vaccine, based on the glycoprotein Bm86 from the cattle tick *Rhipicephalus microplus*, has been shown to be efficient against ticks of the genus *Rhipicephalus* and Bm86 homologue vaccines have had some efficiency against at

least two species of the genus *Hyalomma*, but this is not the case for other ticks and the pathogens they transmit [3]. The European Centre for Disease Prevention and Control suggests that there will be a rise in tick-borne diseases based on changes in various factors including the environment and socio-economics [4]. Research efforts to combat tick-borne diseases have usually centred, as with most other infectious diseases, on determining the Achilles' heel of the pathogen. Most endeavours have focussed on understanding host-pathogen interactions, primarily at the vertebrate level. Protein-carbohydrate interactions between the pathogen and the host cell are of primary importance, in terms of attachment and/or invasion of the cell, whether in an invertebrate or vertebrate host. The observation that there is conservation in the protein-carbohydrate recognition strategies can be used as part of novel approaches for intervention [5]. It has been shown that many regulatory mechanisms are mediated by post-translational modifications (PTM). One example of a PTM that regulates protein degradation and signaling in eukaryotes is ubiquitination. Pathogens are known to exploit ubiquitination to infect mammalian cells and it

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has been shown that the ubiquitination machinery is present in the tick *Ixodes scapularis*. It was identified that the E3 ubiquitin ligase XIAP restricted bacterial colonization of the vector and *xiap* silencing significantly increased tick colonization by the bacterium *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis [6].

Over the last decade, there has been a slow increase in the knowledge of vector-host-pathogen interactions which start from the time a pathogen invades the vector within a blood meal and attaches to the tick midgut lumen. Later it traverses to the tick salivary glands and completes its life-cycle by transmission to a new mammalian host during the subsequent tick feeding [2].

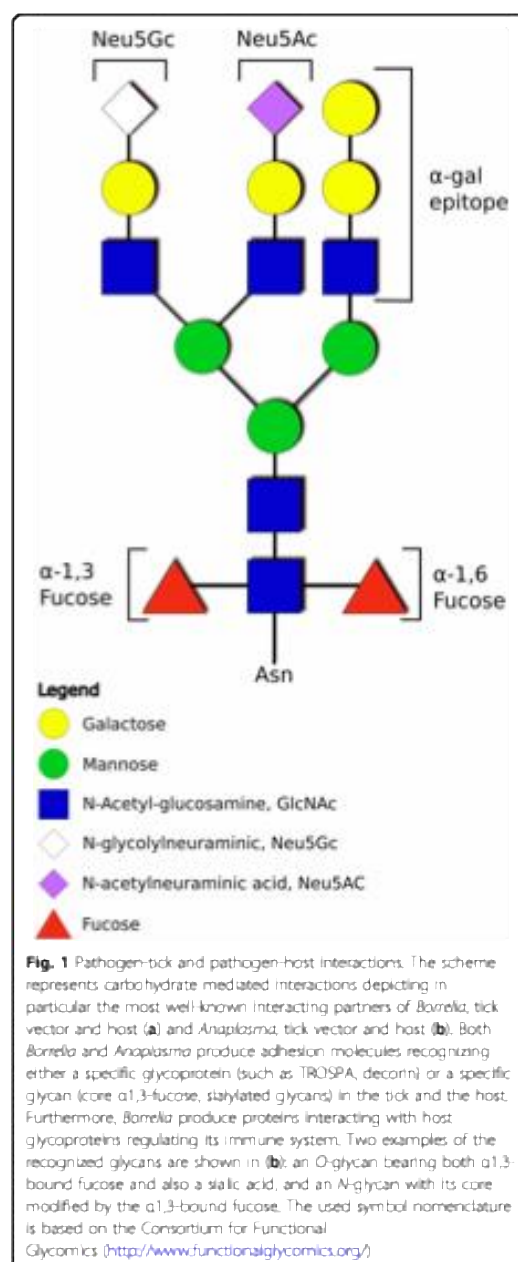
Four possible routes that may facilitate pathogen survival and transmission by most arthropod vectors including ticks have been pointed out. These include: (i) pathogen carbohydrate-binding adhesins that attach to receptors in the tick midgut; (ii) the attachment of carbohydrate-binding proteins of the arthropod to the pathogen as part of its innate immunity; (iii) carbohydrate-binding proteins that are soluble and form a link between the pathogen and midgut surfaces; and (iv) the use of co-receptors to enhance the interactions within the vector [5].

In this review, we would like to highlight the glycobiological aspects of all four of these specific mechanisms that come into play when looking at the vector-pathogen interactions as well as glycobiology-associated processes between the mammalian host and the pathogen (see Fig. 1). Glycobiology of ticks and tick-borne pathogens is developing together with the increased availability and sensitivity of analytical methods; a short overview is listed below, together with some relevant references for readers seeking deeper knowledge.

Importance of glycosylation for protein functions

Post-translational modifications can be found in both prokaryotic and eukaryotic organisms; among them, glycosylation is one of the most abundant and most important. Protein glycosylation affects all the functions of proteins - their structure, activity, interactions with other molecules, half-life in the cell or organism; immune recognition is also dependent on the interaction of immune cells and receptors with glycosylated molecules. A wide variety of possible glycan structures and linkages increases the functionality of proteins [7].

The importance of carbohydrates for the function of proteins can be simply shown on complement proteins. The complement system comprises of more than 30 plasma- or membrane-bound components. Most of them are glycosylated to the various extent and the type of glycosylation generates tissue- or cell-specific population of glycoforms of each complement molecule. The specific



glycoform population then secures functions that are required for a particular cell type or tissue. The repertoire of functions secured by glycans ranges from the control of protein folding, proper assembly within the endoplasmic reticulum, mechanistic shielding of the protein backbone

against protease degradation, preventing inappropriate protein-protein interactions or formation of proper spatial protein conformation or participation in recognition epitope formation [8]. Specific examples show interesting ways in which glycans influence or modulate the complement cascade.

C1q is a recognition molecule of classical complement pathway and mediates initiation of the pathway by binding to the antibody-antigen complexes. The proper function of the C1q is conditioned by the appropriate triple helix formation within C1q monomer and the formation of C1q hexamer whose spatial conformation may be secured by the presence of N-linked glycan of each monomer [9].

Glycosylation was also proven important in complement regulatory factors where factor H (fH) glycosylation mediates its proper folding within the endoplasmic reticulum (ER). The absence of glycosylation or its malfunctioning leads to fH misfolding and retention in ER causing clinical symptoms in children in form of hypocomplementemic renal disease [10].

C1-inhibitor is a plasma glycoprotein and, along with other members of the serpin proteases, its inhibitory activities are enhanced by binding of negatively charged polysaccharides. Most of the polysaccharides binding to the C1-inhibitor induce allosteric changes of the inhibitor molecule causing potentiation of the attachment to the C1 proteases or, as in case of dermatan sulphate, the potentiation is caused by the formation of a negatively charged polysaccharide-mediated linkage between positively charged portions of the C1-inhibitor and C1 protease molecules [11]. Structural characterization of the C1-inhibitor reveals extensive O-glycosylation with a high number of sialylated glycans. The trials for functional characterization of C1-inhibitor glycosylation showed an increased resistance of highly O-glycosylated region against proteolytic degradation [12] and highlighted the importance of sialylation for prolonged serum half-life [13].

Advances in bioanalytical methods for glycobiology

The most frequently used methods in glycobiology are mass spectrometry in combination with chromatography or capillary electrophoresis, glycan/lectin microarrays, or lectin staining. All of these developed greatly recently; for example, the increasing number of mass spectrometers available throughout the world and the development of more sensitive instruments and specifically the introduction of the Orbitrap mass spectrometers, greatly advanced the possibilities for glycan and glycoprotein analysis [14, 15]. The number of commercially available microarrays is also increasing and nowadays allows more or less specific detection of almost any kind of glycan. The availability of lectins together with the possibility to synthesize specific glycan molecules allows also the preparation of in-house glycan-

or lectin-arrays; another possibility is the service provided by the Consortium for Functional Glycomics (<http://www.functionalglycomics.org/>).

Here, we review the current knowledge on how pathogens have evolved "sweet" strategies to overcome the immune responses within the vector and the mammalian host and the use of carbohydrate-binding properties to perpetuate their transmission and dissemination into a vertebrate host (Tables 1 and 2). We also provide a near comprehensive catalogue of all carbohydrate molecules that play a part in the disease cycle that have been characterized to date, be it within the tick or the mammalian host. We would like this to be the start of a renewed interest in the glycobiology of ticks and tick-borne diseases.

Glycosylation in the *Borrelia* infection cycle

Compared to eukaryotes, glycosylation in bacteria produces a much more diverse repertoire of glycoconjugate structures which are often species- or strain-specific. Most of the bacterial glycoconjugates are an integral part of the bacterial cell wall and provide the bacterial cell structural integrity. Additionally, the bacterial glycosylated cell surface structures mediate adhesion and interaction with its environment or host. Although the structural features of bacterial surface glycans have been well described, the function of many of them, including those in pathogenic bacteria, remain unexplored. In principle, pathogenic bacteria use glycosylation for two reasons; they synthesize host-like glycan structures to hide from the host immune system and, conversely, they produce glycosylated proteins that are able to bind more effectively the host immune molecules and thus influence their activity [16].

Since all *Borrelia* species are host-propagated bacteria that shuttle between a vertebrate host and an arthropod vector, these spirochetes have developed strategies to adjust to these diverse environments [17]. This is achieved by regulating the level of gene expression in response to changes in temperature, pH, salts, nutrient content, and other host- and vector-dependent factors. A significant number of *Borrelia* proteins mediate the interactions with host/vector molecules and thus enable *Borrelia* to complete its infectious cycle. Recent findings highlight the importance of carbohydrate moieties in these interactions and in the overall pathogenesis of this infectious spirochete.

Borrelia/tick glycosylated interactions

When entering the vertebrate host during tick-feeding, *Borrelia* must overcome several barriers to successfully invade and disseminate in the host body. The invasion of the host is difficult as it requires the interaction of the existing *Borrelia* surface structures with host tissues without being noticed by the host immune system. *Borrelia* have developed many elaborate strategies to recognize diverse host molecules and cell types to promote

Table 1 Summary of carbohydrate-binding proteins of *Borrelia* spp. recognizing tick or host receptors. The carbohydrate-binding proteins from *Borrelia* spp. are listed together with the recognized molecule from the vector or the host. Glycoproteins or glycans are listed as the recognized molecules depending on the available information. Majority of proteins from Lyme borreliosis spirochetes are listed; in the case of relapsing fever *Borrelia*, the bacterial species is defined

<i>Borrelia</i> spp. protein	Tick binding partner	Reference
<i>Borrelia</i> vs tick		
OspA	TROSPA	[236]
OspC	SALP15	[27]
TSLPVP8	Mannose binding lectin (MBL)	[42]
Vsp33 (<i>B. hermsii</i>)	Unknown receptor in tick SG	[62]
<i>Borrelia</i> vs host		
Bgp (p26)	GAG	[294]
DbpA (p20)	Decorin/dermatansulfate	[48, 295]
DbpB (p19)	Decorin/dermatansulfate/chondroitinsulfate	[48, 295]
Bbk32	Fibronectin/heparansulfate/dermatansulfate	[63]
P66	Integrins	[87]
OspA	Plasminogen	[296]
OspC	Plasminogen	[297, 298]
Enolase	Plasminogen	[299]
Erps (OspE/F related proteins)	Factor H or FHL protein	[105]
CRASPs	Factor H	[105]
PAMPs	Mannose receptor on dendritic cells	[300]
Unknown	Neolacto-(Gal4GlcNAc3Gal4Glc1)-carrying glycoconjugates in human erythrocytes	[301]
VspB (<i>B. burgdorferi</i>)	GAG	[61]

Table 2 Summary of carbohydrate-binding proteins of *Anaplasma* recognizing tick or host receptors. The carbohydrate-binding proteins from *Anaplasma* are listed together with the recognized molecule from the vector or the host. Glycoproteins or glycans are listed as the recognized molecules depending on the available information

<i>Anaplasma</i> protein	Binding partner	Reference
MSP1a (MSP1 complex)	Vector binding partner: Unknown receptor in IDEF8 tick cells	[189, 190]
Unknown molecule	Vector binding partner: Core α 1,3-fucose glycoprotein	[203]
Unknown adhesin-like molecule	Host binding partner: 1,3-Fuc and Sia in sialyl Lewis X, PSGL-1 in human neutrophils	[199, 200]
Unknown adhesin-like molecule	Host binding partner: 1,3-Fuc and Sia in sialyl Lewis X, PSGL-1 in murine neutrophils	[199]
Unknown adhesin-like molecule	Host binding partner: 1,3-Fuc and Sia in sialyl Lewis X, PSGL-1 in human myeloid HL-60 cells	[197, 201]
Unknown molecule of <i>A. phagocytophilum</i> NCH-1 strain	Host binding partner: 1,3-fucose in murine bone marrow-derived mast cells (BMMC), murine peritoneal mast cells	[196]
Unknown molecule of <i>A. phagocytophilum</i> NCH-1 strain	Host binding partner: α 1,3-Fuc in human skin-derived mast cells	[196]
AmOmpA	Host binding partner: α 2,3-sialylated and α 1,3-fucosylated glycan of the sialyl Lewis x in myeloid cells	[182]
AmOmpA	Host binding partner: α 2,3-sialylated and α 1,3-fucosylated glycan of the 6- sulfo-sialyl Lewis x in endothelial cells	[180, 182]
AmOmpA	Host binding partner: α 2,3-sialylated, α 2,6-sialylated, α 1,3-fucosylated glycan receptors in human and murine myeloid HL-60 cells, 6- sulpho-sialyl Lewis x in endothelial cells	[180–182]
Unknown	Host binding partner: α 1,3-fucose	[203]

dissemination and chronic infection [18], and to overcome host immune system surveillance [19]. The concerted action of these structurally and functionally diverse *Borrelia* surface molecules helps the spirochete to successfully adapt and multiply in the host body.

The interacting molecules of *Borrelia* and the tick are often modified by glycosylation producing a diverse pool of structures. Moreover, glycosylation is a dynamic modification and can be readily altered upon environmental cues [20].

The presence of glycoconjugates on the surface of cultured *B. burgdorferi* has been demonstrated by the ability of *Borrelia* to bind a number of lectins [21]. In search of *B. burgdorferi* glycosylation patterns, increased attention has been paid to outer surface proteins that are produced at different stages of the *Borrelia* transmission cycle and represent points of interaction between the spirochetes and their hosts/vectors.

***Borrelia* outer surface proteins**

Borrelia outer surface proteins A and B (*ospA* and *ospB*) are encoded on a bicistronic operon and extensively expressed on the surface of spirochetes in unfed ticks. *OspA* is one of the major and most comprehensively studied *Borrelia* proteins. While *OspA* mediates *Borrelia* attachment to the tick midgut when spirochetes are acquired by ticks during blood-feeding, *OspB* plays a key role in successful colonization of the tick midgut. *OspA* down-regulation is important for *Borrelia* detachment, multiplication, and migration from the tick midgut to salivary glands [22–25]. When ticks are fed to engorgement, *Borrelia* clears *OspA* and *OspB* from the surface expressing instead another outer surface protein C (*OspC*) [22, 26].

OspC, encoded by *bbh19* mapped to the cp26 plasmid, is one of the most divergent genes in *Borrelia* genome, and is crucial for the early stages of mammalian host infection by the spirochete, but not required for acquisition of spirochetes by tick, tick colonization or migration from salivary glands to the gut [27–32].

Erps (*OspE*/*F*-related proteins) are a family of surface integrins with high affinity to factor H and encoded by *erp*-loci localised on each of the cp32 plasmids. Lyme disease spirochetes control Erp synthesis throughout the bacterial infectious tabcycle, producing the proteins during the infection of the host but downregulating their synthesis during tick infection stage. The best-characterized members are *OspE* and *OspF* proteins [33], their paralogues *OspE*/*F*-related proteins [34] and a group of *OspE*/*F*-like leader peptides (Elps) [35].

***OspA*, *OspB* and TROSPA**

Earlier work had indicated that *OspA* and *OspB* are the major *Borrelia* glycosylated proteins [36], yet a later study showed that the suggested *N*-linked glycosylation does not

occur [37]. Colonization of ticks by spirochetes requires the involvement of tick receptor(s). Although a tick receptor for *OspB* has not yet been identified, the tick receptor for *OspA* (TROSPA) is located in the tick gut and is heavily glycosylated. The blockade of TROSPA by TROSPA antisera or by downregulation of TROSPA via RNAi reduced *B. burgdorferi* adherence to the tick gut, hampering the spirochete transmission to the mammalian host. The number of potential posttranslational modification sites in TROSPA is unusually high (> 30), with a predominance of *O*-glycosylation sites [25].

OspC* and *Salp15

When transmitted from the tick vector to the host, *Borrelia* are delivered within the tick saliva. Tick saliva contains a plethora of bioactive molecules, which have been shown to be important for immunosuppression of the host responses [28]. One of the secreted salivary proteins is *Salp15* [29]. This protein specifically interacts with *B. burgdorferi* *OspC* which results in the protection of *Borrelia* from antibody-mediated killing and plays a critical role in establishing *B. burgdorferi* infection [27]. Whereas no data exists about the potential glycosylation of *OspC*, the glycosylation of *Salp15* was demonstrated experimentally [30]. *Salp15* from *L. ricinus* did not deliver the same protection to *B. garinii* and *B. afzelii* against antibody-mediated killing [31], presumably suggesting that the *Salp15* binding for some species is an advantage for surviving in nature [31]. An explanation may lie in a different structural or spatial organization of the *OspC* or *Salp15* molecule causing better access to the binding sites of each of the molecules in *B. burgdorferi*. Another hypothesis claims that *B. burgdorferi* *OspC* holds differently charged areas which interact in a way that favour formation of *OspC* multimers or even a lattice [38]. This structure, along with bound *Salp15*, might form a protective coating on the bacteria preventing an access of anti-*OspC* antibodies or *B. burgdorferi* antiserum [27]. In addition to the direct interplay between *Salp15* and *B. burgdorferi*, *Salp15* indirectly facilitates the host invasion by inhibiting dendritic cell activation by binding to the receptor/lectin DC-SIGN, localized on the surface of macrophages and dendritic cells [32].

***Ixofin3D* and *Ixodes scapularis* dystroglycan-like protein**

Ixofin3D and *I. scapularis* dystroglycan-like protein (ISDLP) are glycoproteins expressed on the surface of midgut cells which were identified as candidate tick midgut binding partners of *B. burgdorferi* using a yeast surface display assay [39]. The expression of both *Ixofin3D* and ISDLP was elevated in *Borrelia*-infected tick midgut during feeding. *Ixofin3D* and ISDLP interact with spirochete cells as was confirmed *in vitro* by immunofluorescence assay and RNA interference. The RNAi-mediated reduction in expression of *Ixofin3D* and ISDLP resulted in decreased

spirochete burdens in the tick salivary glands and in the murine host as well [39, 40]. The full-length Ixofin3D contains four putative fibronectin type III domains. Ixofin3D is glycosylated as shown experimentally by periodic-acid Schiff's staining of a recombinant protein produced in *Drosophila* cells. Even though the importance of Ixofin3D for *Borrelia* infection was shown, the borrelial binding partner for Ixofin3D has not yet been identified [39].

The binding partner for ISDLP is also yet to be discovered. Like Ixofin3D, ISDLP silencing did not reduce the spirochete numbers in the gut but the transmigration process from gut to salivary glands was impaired. The mechanism remains unknown although the collected evidence implies that ISDLP may facilitate gut tissue remodelling or reduced barrier for spirochete transfer to salivary glands [40].

TSLPI

Tick salivary lectin pathway inhibitor (TSLPI) is a secreted salivary protein that protects *Borrelia* from complement-mediated killing. TSLPI facilitates spirochete transmission and acquisition through interference with the host mannose-binding lectin (MBL) and inhibition of the host lectin complement pathway [41]. *N*-linked glycosylation of recombinant *Drosophila*-expressed TSLPI appeared to be vital for its function as a lectin pathway inhibitor, suggesting that TSLPI *N*-glycans are involved in its binding to MBL carbohydrate recognition domains [42, 43].

Borrelia adhesins and extracellular matrix

Adhesion is the first and basic event in establishing an infection. The *Borrelia* cell surface is, at the time of host invasion, covered by adhesion proteins that can recognize and bind to various host cell types and/or extracellular matrix (ECM) components and thus promote *Borrelia* dissemination and settlement in various corners of the host body. Although *Borrelia* adhesins are not glycosylated, the presence of glycosylation has been confirmed in their tick receptors, suggesting a significant role of glycosylation in adhesin-receptor interaction.

Borrelia burgdorferi encodes a variety of adhesins and their characterization and role in the *Borrelia* infection cycle using different approaches was thoroughly described in a review by Coburn et al. [44]. With regard to their overlapping roles in *Borrelia* adhesion to the host tissue, it is important to note that only the concerted action of various adhesins guarantees an effective adhesion and transmigration of spirochetes to different hosts and their tissues [44].

A short overview of host ECM proteins

Glycosaminoglycans

Several ECM-associated molecules are specifically targeted by *Borrelia* adhesins. Exceptionally important seem to be

the glycosaminoglycans (GAGs), large linear polysaccharides constructed of repeating disaccharide units (e.g. hyaluronan, chondroitin, dermatan, heparan, keratan) that decorate the ECM proteins. GAG chains are abundantly modified by sulphurylation, which imparts them a strong negative charge [45]. Numerous studies have shown that binding of GAGs by *B. burgdorferi* enables colonization of the host [46].

Fibronectin

A relevant ECM-associated molecule for *B. burgdorferi* attachment is fibronectin (Fn), a high-molecular weight, dimeric glycoprotein found in body fluids and in the ECM. *Borrelia burgdorferi* fails to bind to the ECM *in vitro* upon exposure to an anti-Fn antibody, implicating the Fn involvement in *Borrelia* attachment [47].

Decorins

Decorins are ubiquitous ECM proteoglycans, which are associated with collagen fibrils in the mammalian connective tissues [48]. Decorins are complex glycoproteins; apart from serine linked GAG chain, they are also modified by up to 3 *N*-glycans [49, 50]. Numerous studies associate decorins with *Borrelia* adhesin attachment and interestingly, the binding is promoted by intact decorin proteoglycan molecule rather than its protein core or GAG chain itself [48].

Laminin

Laminin is a large extracellular matrix multidomain glycoprotein. It is a critical molecule in the basement membrane assembly and, by extension, in tissue formation in the developing organism [51]. Besides its role in basement membrane architecture, it also mediates cellular interactions and provides a dense network for various cellular signalling and attachment events. The existence of different laminin isoforms gives space to developmental regulations mediated by differential responses to cells and newly forming tissues. Laminin, as well as other ECM forming molecules, possesses numerous glycosylation sites and its molecule is modified up to 32 % by *N*-linked glycans [52].

The carbohydrate portion of Laminin was proven to be a mediator of attachment in several bacterial species [53]. Laminin is also a potent target of several borrelial adhesins [54–56] although the direct involvement of the laminin carbohydrate moiety has not been reported yet.

Integrins

Integrins are glycosylated cell surface receptors mediating cell adhesions to the extracellular matrix and some important cell-cell interactions [57]. The presence of *N*-linked glycans in integrin molecule proves integral for the stability of the domain conformation and consequently affects integrin adhesive properties [58]. Integrins possess

a typical heterodimeric structure combining different α and β polypeptide chains and their combination determines the specificity of integrins [59]. Integrins are expressed on all mammalian cells except erythrocytes. The expression of different integrin subtypes produces unique cell surface signature of each cell type [60].

***Borrelia* adhesins**

Vsp

Relapsing fever *Borrelia*, unlike Lyme disease-causing *Borrelia*, are vectored by soft ticks of the genus *Ornithodoros*. They are present in the blood of the mammalian host in high numbers which lead to high fevers followed by bouts of relapses. They recognize glycosaminoglycans (GAG), which mediates the attachment of *Borrelia* to mammalian cells. GAG recognition is partly dependent on the presence of some of the variable small proteins (Vsps). *Borrelia turicatae*, a relapsing fever borrelia that is vectored by *O. turicata*, recognizes GAGs via VspB which allows binding of *B. turicatae* to cultured mammalian cells as well as increased spread and replication in the mammalian host. *Borrelia hermsii* also attaches to cultured mammalian cells via GAGs; however, Vsps are not essential for this binding [61].

After the feeding of *O. hermsi* with *B. hermsii*-infected blood, the bacteria switched from expression of many bloodstream outer surface variable major proteins (Vmps) to a unique protein, variable tick protein (Vtp, Vsp33) [62].

BBK32, RevA and C1-inhibitor

Borrelia burgdorferi expresses at least two fibrinogen-binding proteins, BBK32 [63] and RevA [64]. BBK32 is a protein, whose attenuation does not block the spirochete transmission from the tick to the host [65], but lowers the bacterial loads in different tissues at different time points of infection [66]. *Borrelia burgdorferi* also attaches to endothelium in the vascular system through fibrinogen (Fn) and this interaction becomes stronger with increasing blood flow, allowing the spirochete to overcome fluid shear stress [67]. These stabilizing interactions are sustained by catch bond properties of BBK32 [68]. Following the binding to Fn, BBK32 binds to various kinds of GAGs, including heparin sulphates and dermatan sulphates of the host ECM [69–71]. It also seems to be involved in the modulation of the innate immunity. In particular, BBK32 binds the C1 complex of the classical innate immunity pathway, preventing its activation and thus obstructing classical pathway-mediated *Borrelia* lysis [72].

As *B. burgdorferi* BBK32 mutants are still able to bind fibronectin, an additional Fn-binding protein, RevA, was identified [58]. RevA expression on the *Borrelia* cell surface was upregulated in mammalian host compared to the tick vector. Furthermore, *Borrelia*-infected patients produced anti-RevA antibodies throughout various stages

of Lyme disease suggesting its involvement in Lyme disease establishment and persistence in the host. RevA appears to have multiple binding sites which *Borrelia* uses to bind host cells via Fn [46, 73].

DbpA/DbpB

Decorin-binding proteins A and B (DbpA and DbpB) are adhesins found on the surface of *B. burgdorferi* [20, 45]. These proteins are critical for the virulence of *B. burgdorferi* [74, 75]. New data suggests that the decorin-binding proteins actually do not bind directly to the decorin protein core but interact with decorin via GAGs that are attached to the protein [76–78]. The binding studies of DbpA and DbpB from different *Borrelia* genospecies showed that there are clear differences in the decorin binding activity and that these differences may ultimately lead to the differences in tissue tropism and clinical manifestations associated with particular *Borrelia* genospecies [76, 79]. *In vivo* functional studies demonstrated the importance of DbpA/B adhesins for *Borrelia* invasion of the mammalian host especially in the early stages of infection [80].

Bgp

Borrelia burgdorferi glycosaminoglycan binding protein (Bgp) is a surface-exposed protein on intact spirochetes [70]. Recombinant Bgp bound the same GAG as the whole spirochete, agglutinated erythrocytes and inhibited binding of *B. burgdorferi* to the mammalian cells. A transposon mutant of the *Bgp* gene had less ability to adhere to host endothelial and epithelial cells *in vitro* and to colonize host target tissues leading to the reduced inflammatory manifestation of Lyme disease in the mouse model. The adherence was not fully disrupted due to the existence of other GAG-binding adhesins which facilitate host colonization and also highlights the importance of *Borrelia* GAG-binding ability for the completion of the infection cycle [81]. Although the Bgp attachment to GAG is not essential for disease establishment, the protein appears to be involved in the formation of an initial infectious niche in the host. Different spirochetes strains possess different GAG-binding preferences and their binding ability to multiple cells depends on the GAGs that they express [76].

BmpA

BmpA (*Borrelia* membrane protein A) and its three paralogues B, C, and D are all laminin-binding borrelial outer surface proteins [82]. Like other *Borrelia* surface proteins, BmpA is also antigenic. All bmp genes are located on the *Borrelia* chromosome, arranged in clusters that are differentially regulated [83]. The involvement in the development of arthritis in the mouse model was described for two Bmp proteins, BmpA and BmpB [84].

***Borrelia* adhesins and integrin-mediated interactions**

Borrelia binds to host endothelial cells *via* the interaction of integrins $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, and $\alpha_V\beta_1$ with *Borrelia* surface adhesins [59, 85]. It was also described that the causative agent of relapsing fever, *B. hermsii*, binds to human platelets promoted by the platelet glycoprotein integrin $\alpha_{IIb}\beta_3$ and is diminished by $\alpha_{IIb}\beta_3$ antagonists or by a genetic defect in this integrin [86].

P66

P66 is one of the candidate ligands for β_3 -chain integrins (e.g. $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$) [87]. P66 also functions as a porin [88, 89], and structural predictions, as well as some experimental data, present the molecule as porin assuming the structure of β -barrel [90].

P66 mutants showed a dramatically reduced ability to attach to integrin $\alpha_V\beta_3$ [91]. Endothelial cells responded to wild-type *Borrelia* infection by upregulation of endothelial growth factor compared to a control infection with a P66 deletion mutant. The ability of P66 mutants to transigrate through the cell monolayer was impaired, which suggests the role of P66 in *Borrelia* transendothelial migration, although its porin function does not play a role in the migration process [92].

Mammalian integrins typically contain an RGD (Arg--Gly-Asp tripeptide) consensus sequence in their binding domain, where aspartic acid is a key binding amino acid. P66 lacks this sequence; however, residues 205 and 207 of its 203–209 binding region are both aspartic acid [93]. P66 deletion mutants applied subcutaneously are readily cleared out of the site of infection, which refers to the possible involvement of the innate immune system and confirms the importance of this protein for host colonization together with other studies [94]. However, tick colonization is shown to be P66-independent [94].

BB0172

BB0172 is an outer membrane protein containing von Willebrand factor A domain which mediates intercellular and protein-protein interactions in ECM. It is, for example, involved in the attachment of platelets to the ECM in the site of damaged endothelial epithelium *via* platelet surface glycoprotein [95]. BB0172 showed a weak interaction with ECM-associated fibronectin. Importantly, a strong affinity was observed in the attachment of BB0172 to $\alpha_{IIb}\beta_1$ integrin. Moreover, the affinity was much stronger than the one observed in the interaction of borrelial P66 adhesins with β_3 chain integrins [95].

***Borrelia* adhesins interacting with mammalian complement**

Mammalian innate immunity is alerted by a variety of surface-exposed molecules of invading pathogens. The

first encounter of host antibodies with potentially harmful intruder activates the complement system which assists in tagging of the pathogen for destruction and also acts on pathogen clearance itself by the formation of membrane attack complex. Different pathways of the complement system progress in a cascade-like manner and its brisk response to pathogen invasion must be under the control of regulating mechanisms preventing complement from attacking host cells.

Invading a host organism, the pathogens have evolved different strategies to circumvent the immune response. Many of these strategies are in fact directed against components of the complement system. The most widespread strategy employs molecules recruiting or mimicking the complement regulators, including the direct interaction of pathogens with complement proteins leading to the modulation or inhibition of their function or indirectly to the activation of complement proteins enzymatic degradation [96].

The complement regulators are represented by several serum proteins that are able to dampen the activity of complement and prevent host self-destruction. Two of them, complement factor H (FH) and its splice homologue Factor H-like (FHL) inhibit the alternative complement pathway response using host-specific surface patterns like sialic acid or GAGs and thus promoting self-recognition processes [97, 98].

FH is a plasma glycoprotein containing 9 glycosylation sites [99] bearing complex, predominantly diantennary disialylated, fucosylated, and nonfucosylated glycans at eight of the nine glycosylation sites [100]. Similarly, FHL is also a plasma glycoprotein [101]. Both proteins possess a RGD motif which is assigned cell adhesive properties and thus can modulate cell adhesion. Additionally, FHL promotes anchorage-dependent cell attachment and spreading [102].

CRASPs and ERPs

The two complement regulators, FH and FHL are bound by *Borrelia* surface proteins hence preventing the activation at the central step of the complement cascade. Serum resistant *Borrelia* express adhesins on their surface, which are capable of interfering with different components of the host complement system leading to the modulation of host immune response and hampering the complement-mediated spirochete lysis [103, 104].

The two well-characterized types of complement interfering adhesins, complement regulator-acquiring surface proteins (CRASP) 1 and 2 [105], control the complement activity by binding complement regulating molecules such as FH and FHL-1 [104, 106]. Up to now, five different CRASPs (CRASP-1 to CRASP-5) have been described and each of them presents a different binding ability to FH, FHL-1, or plasminogen [98, 104, 106].

CRASP-1 (CspA, BBA68) has been studied the most extensively. It shows a strong affinity to the complement regulators which inactivate the complement response very efficiently [106, 107].

The expression of CRASP-1 is repressed in the tick vector and increases in the mammalian host, which suggests its role in spirochete transmission and evasion of the host immune response [108, 109]. CRASP-1 also confers serum resistance to *B. burgdorferi*. The role of CRASP-1 in complement inactivation is evident in the CRASP-1 knockout-mutants which inefficiently bound human FHL and attracted complement constituents more readily [110, 111].

Apart from *B. bavariensis*, all studied *Borrelia* species possess CRASP-1 orthologues conferring complement inactivation [112, 113]. The orthologues belong to the same protein family although the encoding genes do not share the same locus with the *B. burgdorferi* CspA [98].

CRASP-2 (CspZ) is another *Borrelia* adhesin binding both FH and FHL-1 independently of CRASP-1 and reinforcing *Borrelia* complement resistance [114, 115]. The CRASP-2 expression fluctuates in a somewhat similar manner to CRASP-1 during the *Borrelia* infectious cycle. Like CRASP-1, CRASP-2 is also upregulated during an established mammalian infection and is able to activate antibody-mediated immune response [116], which makes this adhesin important for the diagnosis of Lyme disease infection. The triggered immune response does not, however, provide the host with protective immunity and has no effect on spirochete dissemination [117].

Three members of the polymorphic Erp (OspE/F-related protein) protein family, ErpA (BBP38, CRASP-5), ErpC (CRASP-4) and ErpP (BBN38, CRASP-3), are plasminogen binding proteins that can simultaneously bind to FH and FH-related proteins [103, 107, 118–122].

The Erp proteins are most probably involved in different reservoir hosts infection due to differential binding abilities of particular Erp paralogues [123, 124]. Despite their complement regulator binding properties, none of the Erp proteins are necessary for the protection of *Borrelia* spirochetes against complement-mediated killing; CRASP-1/CRASP-2 deletion mutants expressing all Erp proteins were susceptible to serum mediated lysis [119, 120, 125].

Erps are not upregulated during *Borrelia* transmission but their expression gradually increases during Lyme disease progression, suggesting their role during mammalian infection [125]. Interestingly, *Borrelia* can regulate the expression of both Erps and CRASPs very dynamically as different isolates of *B. burgdorferi* (*s.l.*) reacted differently to complement-mediated killing [56, 126]. Moreover, some of the Erp members present multiple functions during *Borrelia* infection. For example, ErpX ability to bind complement regulators is complemented by its laminin binding properties [56]. The overlapping activities

of *Borrelia* surface molecules enhance the overall infectious potential of the spirochete.

***Borrelia*-specific host pattern-recognition receptors and lectins**

Toll-like receptors

Recognition of pathogens is mediated by a set of pattern-recognition receptors (PRRs). The group of glycosylated proteins that comprise the Toll or Toll-like receptors family (TLRs) are transmembrane receptors that function as PRRs in mammals [127]. So far, eleven members that potentially participate in the recognition of invading pathogens have been identified in mammalian genomes [128] and glycosylation was shown to have a critical role in TLR presentation on the cell surface [129, 130].

There are several TLR members, whose role in spirochete recognition has been identified. The well-characterized TLR2 is presented on antigen-presenting cells, epithelial and endothelial cells [131]. It was able to recognize a variety of ligands and was important for macrophage activation and further triggering of the immune response in *Borrelia*-infected mammalian hosts when stimulated by OspA [132]. The signal transduction through TLR1/2 in response to *B. burgdorferi* invasion can elicit opposite immunoregulatory effects in the blood and CNS immune cells, affecting the different susceptibility of these compartments to infection [127].

TLR4 is expressed in macrophages and dendritic cells [130] and is upregulated upon *Borrelia* infection or stimulation by OspC [133, 134] and its main ligands are lipopolysaccharides (LPS) from gram-negative bacteria [135]. The role of TLR4 in *Borrelia* recognition remains unclear as *B. burgdorferi* does not express LPS on its surface.

TLR9 is responsible for recognition and further endosomal/lysosomal internalization of CpG motifs in bacterial DNA [136]. This process has been observed in sonicated *Borrelia*, which promoted the activation of murine cells via TLR9 [137].

Nucleotide-oligomerization domain-like receptors

Nucleotide-oligomerization domain-like receptors (NOD-like receptors or NLR) are a group of intracellular PRRs, capable of binding bacterial mucopeptides, the molecules derived from bacterial peptidoglycans [138]. Together with TLRs, NOD-like receptors are crucial for recognition of *Borrelia* species. Contrary to other PRR families, NLRs bind bacterial ligands intracellularly, i.e. they are able to recognize the pathogen-associated molecular patterns (PAMPs) that enter the cell via phagocytosis or through the membrane pores induced during cellular stress [139].

There are several NLR protein members that can bind carbohydrate-associated PAMPs, although only a few of them were directly observed to be involved in Lyme

disease. NOD1 and NOD2 receptors are the most extensively investigated major PRRs [138, 140].

Borrelia-infected primary murine astrocytes upregulated NOD-proteins upon exposure to some TLR-ligands [138], while murine primary microglia infected by *Borrelia* only upregulated NOD2 and not the NOD1 [141]. NOD2 activation by *Borrelia* stimulated inflammatory cytokines release. Their activities are assigned to a host proinflammatory response, although their particular role in Lyme disease establishment remains unknown [142]. NOD2 stimulation by *Borrelia* induces inflammation during the early stages of Lyme disease but induces tolerance and suppresses *B. burgdorferi*-mediated Lyme arthritis and carditis in mice during later phases of infection [143]. *Borrelia* recognition in the host is conferred by the combined action of TLR and NOD2. The activation of both receptors at a time by *Borrelia* species is essential for an effective cytokine release. It has been concluded that TLR2 and NOD2 co-recognition of *Borrelia* surface receptors leads to both induction of a proper immune response and to inflammatory-induced pathology [144].

C-type lectin receptors

A family of calcium-dependent receptors that bind carbohydrate ligands include both soluble and cell-associated (transmembrane) lectins in vertebrates. C-type lectin receptors (CLRs) expressed by dendritic cells are crucial for tailoring immune response to pathogens. The transmembrane type is predominantly expressed by antigen-presenting cells functioning as PRRs recognizing PAMPs in bacteria [128]. Currently, 17 CLR subfamilies are described in vertebrates.

Mannose receptor represents a subgroup of CLRs binding mannose-containing bacterial transmembrane PAMPs. CLRs are involved in the recognition and phagocytosis of several microorganisms including *B. burgdorferi*. In particular, CLRs were upregulated in dendritic cells after *B. burgdorferi* activation and facilitated phagocytosis of *B. burgdorferi* by monocytes and macrophages [128]. However, the recognized borrelial protein is yet to be identified.

Surface glycolipids of *Borrelia burgdorferi*

Borrelia have an unusual composition of glycolipids in their outer membrane; they synthesize mono- α -galactosyl-diacylglycerol (MGalD) and cholesterol derived glycolipids cholesteryl- β -D-galacto-pyranoside, cholesteryl 6-O-acyl- β -D-galactopyranoside (ACG), or cholesteryl 6-O-palmitoyl- β -D-galactopyranoside (ACGal/BbGL-1) [145–147].

The *Borrelia* glycolipids induce inflammatory reactions; in particular, two glycolipids ACGal/BbGL-I and MGalD/BbGL-II, are probably immunogenic [145, 148]. The immunogenic epitope is recognized in the lipid part of the glycolipids [149]. An important constituent of the immunogenic epitope is the α -linked terminally bound

galactose which is recognized by the T-cell receptor of invariant natural killer T cells (NKT) [150]. This then promotes their activation as well as the proliferation of Lyme disease-directed antibodies [151–153] which recognize glycolipids in the cell membrane of *Borrelia* but also *Ehrlichia* [154]. Importantly, the induced antibodies against the glycolipid fraction cross-react with gangliosides, which explains the phenomenon of neuroborreliosis [155].

The glycolipid recognition by invariant NKT cells seems to be an alternative system for innate immune system activation by bacteria lacking LPS, an otherwise typical antigenic determinant of most gram-negative bacteria [156].

Borrelia bind to GalCer (galactosylceramide) on Schwann cells [157], LacCer (lactosylceramide), ceramide trihexoside and gangliosides GD1a and GT1b. Moreover, *Borrelia* displays a specific affinity to disialoganglioside GD1a and trisialoganglioside GT1b carrying sialic acid. The ability to bind such a wide range of glycosphingolipids might provide an explanation for its ability to adhere to a wide spectrum of different cell types [158]. *Borrelia* did not bind gangliosides GM1, GD1b, GM2, GM3 and asialo-GM1 implying the requirement for terminally bound sialic acid in ganglioside recognized epitope and demonstrates the specific character of *Borrelia* and acidic gangliosides interaction. Interestingly, adhesion to GD1a and GT1b, as well as GalCer or LacCer was not compromised by free sialic acid, galactose or lactose, respectively [158, 159]. Conversely, GalCer-binding sites were saturable using free GalCer in CHO-K1 cells preventing spirochetes from attachment [148].

Vector-host glycosylated interactions

Similarly to *Borrelia*, the tick's successful evasion of the host response depends on its ability to conceal its activities from the host immune system. The pursuit of successful feeding drove ticks to equip their saliva with multiple pharmacologically active molecules which feature immunomodulatory activities. The myriad of diverse functions include cytotoxicity, vasodilatation, anticoagulation, anti-inflammation and immunosuppression. The comprehensive list of tick pharmacologically active salivary gland molecules is presented in a recent review [28].

P672 and CCL8

P672 is a chemokine binding protein (evasin). Evasins bind to multiple chemokines of different origin and their effects are thus pleiotropic. To date, several evasins originating in tick saliva have been identified [160, 161] and they inhibit responses of many chemokine sensitive molecules including neutrophils or macrophages, which have been demonstrated in several tick species [28]. P672 was originally identified in *Rhipicephalus pulchellus* and its promiscuous binding abilities assign it 13 different chemokine partners showing different dissociation constants.

Mass spectrometric characterisation revealed the presence of several N-linked glycans and their deprivation negatively influences the affinity of P672 to CCL8, although the underlying mechanism of this observation is yet to be uncovered [162].

Protease inhibitors

Many of the tick salivary proteins are glycosylated [163]. While the exact structure of the glycans attached to these proteins has not been studied, research has concentrated on the role of glycosylation with regard to the recognition of glycans by host immune systems. The importance of the glycan part for antibody recognition was shown for several proteins, such as AamS6 serpin [164], *R. microplus* serpins [165] or evasins 1 and 3 [166] confirming the need to use of glycosylated recombinant proteins in anti-tick vaccine preparations.

For proteins, where the role of glycosylation for the protein function was not confirmed, masking of the tick proteins antigenic epitopes and thus minimization of the immune response was speculated as the role of glycosylation [166].

Serpin 19

Serpin 19 is a serine protease inhibitor identified in the saliva of *Amblyomma americanum*. Serpin 19 displays a broad range of inhibitory activities: it interferes with the host homeostasis, coagulation and the development of inflammatory response. Importantly, the activity of many serine proteases is both positively and negatively regulated when bound to GAGs [167–169] and serpin 19 also contains several predicted GAG binding motives [170]. The functional validation further confirmed its GAG-binding properties and also extended the list of binding partners with heparin sulphate and heparin [170].

Variegins

The inhibition of blood coagulation cascade represents an important property of tick saliva that facilitates successful engorgement on the host. Variegins are small thrombin-binding oligopeptides isolated from *A. variegatum* salivary glands. During tick feeding, variegins bind thrombin and disable its fibrinolytic activity and thus block the blood coagulation cascade. Despite its small size, variegins possess a single O-linked glycan [171]. The synthetic O-glycosylated variegins analogues show significantly higher affinity to thrombin and consequently lower reaction kinetics of thrombin-mediated fibrinogenolysis compared to the non-glycosylated form, confirming the importance of its glycosylation. The functional analysis of the inhibition mechanism using macromolecular docking revealed the formation of some favourable hydrogen bonds between hydroxyl groups of the glycan and the allosterically important sites of thrombin [172].

Glycosylation in the *Anaplasma* infection cycle

Anaplasma is a genus of gram-negative rickettsial bacteria. They are obligate intracellular parasites infecting mammals including many domestic animals. The infection causes a reduction of the animal's body weight, abortions, reduces milk production and frequently leads to death [173–175]. In humans, *A. phagocytophilum* is the only confirmed pathogenic species causing human granulocytic anaplasmosis. Patients suffer from fever, headache, myalgias, chills, leukopenia, thrombocytopenia and liver damage manifested by elevated liver enzymes in serum [176]. The symptoms are usually mild but for some individuals, e.g. patients with a weakened immune system, it can be fatal. The infected vertebrate host serves as a reservoir where the bacterium can proliferate for many years and infect naïve ticks [177].

The main vectors of the genus *Anaplasma* are ticks, especially species of the genera *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma* [178, 179]. The initial phase of the infection during colonization of the host is the recognition of a suitable cell, attachment onto this cell, and entry into it. This process is facilitated by several specialized bacterial proteins (adhesins/invasins) that can recognize host surface molecules including glycans and glycoproteins and initiate signalling cascades to promote pathogen internalization. *Anaplasma* spp. express several surface proteins which are involved in binding to glycosylated host cells receptors and thus in the infection of the host and tick cells. These differ in glycan specificity and importance for the infection of various hosts and host cell types.

Anaplasma glycoprotein-binding surface proteins

As an intracellular pathogen, *Anaplasma* depends on a host cell to survive. *Anaplasma* infects two different types (groups) of organisms: the tick vector and the mammalian hosts, with various cell types being infected by the pathogen. Recognition of the cell type and of the infected organism is provided through binding of surface glycan epitopes or even several epitopes on a glycoprotein molecules.

Two groups of *Anaplasma* surface proteins were shown to recognize tick or host glycoproteins: outer membrane proteins (Omps) and major surface proteins (MSPs).

OmpA belongs to highly conserved genes among *A. phagocytophilum* isolates and is transcriptionally induced during feeding of *A. phagocytophilum*-infected ticks on mice and also during the invasion of mammalian but not tick cells [180, 181]. Pre-treatment of *A. phagocytophilum* or *A. marginale* bacteria with the respective OmpA antiserum reduces their ability to infect mammalian cells [181, 182]. Also, preincubation of mammalian cells with a recombinant ApOmpA effectively inhibits *A. phagocytophilum* infection of host cells.

Glycoproteins containing α 1,3-fucose and either sLex or 6-sulfo sLex on host cells are recognized by the outer

membrane protein A (ApOmpA) of *A. phagocytophilum* [180, 181]. On the other hand, OmpA of *A. marginale* (AmOmpA), a species non-pathogenic for humans, binds only α 1,3-fucose and sLex but not 6-sulfo-sLex glycans. *Anaplasma marginale* also produces AmOmpA in both the infected mammalian and tick cells. Pre-treatment of host cells with sialidase or trypsin reduces or nearly eliminates OmpA adhesion. Therefore, AmOmpA interacts with sialylated glycoproteins via an adhesin-receptor pair. Thus, both AmOmpA and ApOmpA recognize different receptor molecules even though these receptors share some structural similarity and thus provide a similar function to these two bacterial species [182].

Structures of *A. marginale* and *A. phagocytophilum* OmpA proteins are very similar and their binding domains are structurally conserved. The OmpA binding domain was identified within amino acids 59 to 74 and it is responsible for the recognition of α 2,3-bound sialic acid and α 1,3-fucose [180]. A recent study by Hebert et al. [182] describes the OmpA receptor-binding domain between the amino acids 19 to 74.

Another group of surface proteins interacting with host (glycosylated) molecules are the major surface proteins (MSPs) that are involved in the adhesion of host cells and the immunological reaction of the host [183–187]. MSP1 protein with its variants α , β 1, and β 2 and the MSP3 protein are present in *A. marginale*, while MSP2 and MSP4 in both *A. marginale* and *A. phagocytophilum* [188]. The MSP1 complex consists of two polypeptides MSP1a and MSP1b and both polypeptides participate in adhesion processes to both tick cells and bovine erythrocytes [183–186].

Similarly to OmpA, these proteins show glycan-binding activity. *Anaplasma marginale* MSP1 and MSP2 can hem-agglutinate bovine erythrocytes [184] suggesting recognition of some erythrocyte surface saccharide molecules. Recombinant forms of the MSP1 isoforms are glycosylated; MSP1a recombinant glycoprotein contains glucose, galactose, mannose and xylose, while MSP1b contains glucose, galactose and mannose. The functional domain of MSP1a contains tandemly repeated peptides that are important for adhesion to tick cells and bovine erythrocytes. The MSP1a polypeptide backbone alone shows binding to tick cell extract proteins and the glycan in its N-terminus enhances this binding [189, 190]. The MSP2 protein binds to the mammalian PSGL-1 [191] and thus can be responsible for the above mentioned *Anaplasma* recognition of sialic acid on this protein. A hypervariable region is present in the middle of the MSP2 gene which allows the bacterium to express various paralogs of the protein on its surface, possibly enhancing immune system evasion [192, 193]. However, the glycan binding abilities of the various MSP2 paralogs were not studied.

In addition to the above-described receptor molecules, two other proteins, Asp14 and AipA, were found to be acting together with OmpA during the infection of host cells. However, neither of these two proteins were shown to bind glycans nor to be glycosylated [180]. Finally, during the past ten years, other novel *A. phagocytophilum* surface proteins Asp55, Asp62 and APH_1235, with possible function as adhesins and invasins have been identified [194–196]; however, their receptor molecules remain unknown.

Anaplasma-host interactions

A confirmation of *Anaplasma* recognition of host-surface glycans came by Goodman et al. [197] showing binding of *A. phagocytophilum* to the cell surface of the promyelocytic leukaemia cell line HL-60. Bacterial binding to the cell surface correlates with the expression of the sialyl Lewis x (sLex) or a closely related 6-sulpho sLex glycan-containing molecules glycan and α 1,3-fucosylated molecules. On the other hand, α 1,3-fucosylated glycans but not sialylated glycans, are essential for the infection of murine and human mast cells by *A. phagocytophilum* [180, 198]. These glycan epitopes are important for *Anaplasma* *in vivo* as has been shown by Carlyon et al. [199].

The protein part bearing the recognized glycans can be also important; thus, not any glycan molecule is recognized, only the one found on a specific protein. In humans and animal hosts, *A. phagocytophilum* exhibits, amongst others, a tropism for myeloid cells. As an adhesion molecule involved in the binding to the surface of human neutrophils, the P-selectin glycoprotein ligand-1, PSGL-1, has been identified [197, 199–202]. In the case of human PSGL-1, *A. phagocytophilum* cooperatively binds to a short amino acid sequence in its N-terminal region and an O-glycan containing a sialyl Lewis x (sLex) on PSGL-1 (NeuAc α 2,3Gal β 1,4[Fuc α 1,3]GlcNAc) [202] or on another molecule. On the other hand, PSGL-1 is not the major ligand in mice [199, 200]. Thus, the terminal or core α 1,3-fucosylated glycans seem to be a generally recognized receptor, while sialylated glycans and PSGL-1 enhance the infection of diverse types of mammalian host cells.

Anaplasma-vector interactions

In the pathogen-tick relationship, several tick glycosylated molecules can be induced in the presence of a pathogen in the tick tissues and help the pathogen to colonize the tick or enhance its infection. For example, α 1,3-core-fucosylated glycans are required for tick colonization by *A. phagocytophilum* and silencing of the responsible fucosyltransferases results in the absence of *Anaplasma* in the infected ticks. To increase the number of its receptors in the tick, *A. phagocytophilum* induces the expression of α 1,3-fucosyltransferases to enhance the colonization of *I. scapularis* ticks. Therefore, α 1,3-fucose is a unifying

determinant that *A. phagocytophilum* targets to infect its natural murine and arthropod reservoirs and accidental human hosts as well. In addition, the presence or absence of these glycans does not affect the transmission of the pathogen from the tick vector to the vertebrate host. While the infection of the tick by *Anaplasma* depends on the presence of α 1,3-core-fucosylated glycans, these epitopes do not seem to be important for the infection by another tick-borne pathogen, *B. burgdorferi* [203].

Furthermore, tandem repeat peptides of the MSP1a functional domain are important for the adhesion of bacteria to tick cells and the glycosylation of MSP1a probably plays a role during the adhesion of *A. marginale* to tick cells [189, 190].

Colonization of the tick by pathogens depends on the tick life-cycle; one of the crucial steps is the colonization of the midgut or survival in the midgut in the process of the blood meal digestion. For successful colonization, the tick midgut peritrophic matrix (PM) and bacterial biofilms formed in the midgut are critical. The PM forms a barrier between the midgut lumen and the epithelial cells lining the luminal side of the midgut and is formed by a thick matrix of mostly chitin with various proteins, such as chitin deacetylase, and glycoproteins [204]. One of the bacteria depending on the biofilm formation in the *I. scapularis* tick midgut is *A. phagocytophilum*. The presence of this bacterium affects the midgut microbial community and biofilm composition and it also decreases the expression of several genes for the glycoprotein peritrophin, one of the major PM components. This results also in decreased PM thickness. Furthermore, RNAi silencing of these genes significantly enhanced *Anaplasma* colonization of the tick [205]. *Anaplasma* further enhances its chances for a successful colonization of *I. scapularis* ticks by induction of an antifreeze protein (IAFGP) during the infection of ticks [205]. This secreted antifreeze glycoprotein inhibits bacterial biofilm formation through binding to the D-alanine residue of some bacteria peptidoglycan and was induced in response to *Anaplasma* infection [206, 207]. IAFGP expression resulted in thinning of the tick midgut PM and RNAi silencing of *iafgp* gene resulted in the absence of *Anaplasma* in the tick midgut [205].

Tick lectins

Ticks, like other arthropods, lack specific adaptive immunity. To defend themselves against invading microorganisms, ticks use the evolutionarily older nonspecific innate immune system, including both cellular and humoral immune responses. Cellular immune reactions involve haemocytes capable of phagocytosis, encapsulation or nodulation of foreign microorganisms and particles. The humoral immune response involves a range of non-specific pathogen-recognizing defence systems: PRRs,

lectins, complement-like molecules, pro-phenoloxidase activation, haemolymph coagulation factors, antimicrobial peptides, reactive oxygen species, etc. Some of these molecules which function as mediators in the innate immune response are glycosylated and/or may recognize glycan-containing epitopes, e.g. recognition receptors for pathogens, complement-related molecules, or lectins (Table 3). In mammals, lectins play an important role in the recognition of specific glycosylated surface molecules of a variety of pathogens (PAMPs) and subsequent activation of the lectin pathway [208, 209]. MBL or ficolins known to recognize *N*-acetyl groups [210] serve as the recognition molecules, which are further integrated with the MBL-associated serine proteases to trigger the complement activation.

Fibrinogen-related proteins

Invertebrates contain a variety of fibrinogen-related proteins (FRePs), all of them sharing structural similarity with fibrinogen. A common feature of FRePs is their glycan-binding activity as they recognize the invading pathogen through its specific glycan epitopes. Their expression increases upon infection of the invertebrate by parasites or by pathogens [211, 212] with possibly a specific role in complement activation [213]. However, some of the tick FRePs family proteins (such as ixoderins described below) may have various other functions (Table 3).

Dorin M from the soft tick *Ornithodoros moubata*, the first lectin purified and characterized from any tick species, shows a strong similarity to ficolins but lacks the N-terminal collagen domain [214, 215]. Dorin M and its closest homologue OMFREP, also from *O. moubata*, share sequence similarity with the innate immune FRePs Tachylectin 5A and B from the horseshoe crab, *Tachyplesus tridentatus* [215, 216]. It has a binding activity for sialic acid [214], its conjugates and *N*-acetyl-hexosamines. The protein has three *N*-glycosylation sites modified by high-mannose type glycans and core-fucosylated paucimannose glycans [217]. Other FRePs were later identified in the haemolymph of *D. marginatus*, *R. appendiculatus*, *R. pulchellus* and *R. sanguineus* based on the cross-reactivity with sera directed against Dorin M [218].

The hard tick *I. ricinus* contains several FReP encoding sequences in its genome (Ixoderins A, B and C) and their analogues are present in *I. scapularis* as well. While proteins similar to ixoderins A and C are present also in other tick species, Ixoderin B-like proteins are found only in the genus *Ixodes*. All these proteins contain predicted glycosylation sites and they contain the fibrinogen-like domain with carbohydrate-binding properties [213, 215]. In *I. ricinus*, the expression of Ixoderin A is restricted to haemocytes, salivary glands, and midgut while Ixoderin B is only expressed in salivary glands [215]. As expected based on published information on other invertebrate

Table 3 Overview of identified tick lectins. Lectins identified in different tick species are listed including the tissue where the lectin was identified. Lectin binding specificity, its function and molecular weight are also listed if known

Lectin	Species	Tick tissue	Specificity	MW (kDa)	Function	Reference
Galectins (OmGalec)	<i>O. moubata</i>	Haemocytes, midgut, SG, ovaries	Lactosamine-like disaccharides	37.4	Putative functions in tick development, immunity, and vector pathogen interaction	[221]
Dorin M	<i>O. moubata</i>	Haemocytes	<i>N</i> -acetyl-D-hexosamines and Sialic acid specific	na	Pattern recognition molecules	[214]
OMFREP	<i>O. moubata</i>	Hemolymph, salivary glands	Probably similar to Dorin M	na	Probably similar to Dorin M	[215]
Ixoderin A	<i>I. ricinus</i>	Hemolymph, salivary glands, midgut	Peptidoglycan recognition protein?	na	Putative defence protein, identification of self-/non-self tissues	[215, 219]
Ixoderin B	<i>I. ricinus</i>	Salivary glands	Unknown	na	Unknown putative immunomodulatory function	[215, 219]
Hemelipoglycoprotein	<i>D. marginatus</i>	Haemocytes, salivary glands, gut	Galactose- and mannose-binding specificity	290, 2 subunits	Putative innate immunity	[220]
Unknown lectin	<i>I. ricinus</i>	Gut, hemolymph	Sialic acid, <i>N</i> -acetyl-glucosamine	85	Putative recognition molecule	[233]
Unknown lectin	<i>I. ricinus</i>	SGs	Sialic acid	70	Unknown	[233]
TSLP1	<i>I. scapularis</i>	Unknown	Mannan	na	Unknown	[42]
HICLec	<i>H. longicornis</i>	Midgut, ovary	Unknown	60.2	Unknown	[223]
Serpin 19	<i>A. americanum</i>	Saliva	GAGs	43.0	Serine protease inhibitor	[170]

Abbreviations: MW molecular weight, na not available

FRePs, Ixoderins are also involved in defence against pathogens. Namely, Ixoderins A and B are involved in phagocytosis of some pathogens as shown for *Candida albicans* [219]. On the other hand, knockdown of these two Ixoderins did not affect the phagocytosis of the tick-transmitted *B. afzelii* and knockdown of all three Ixoderins does not affect its transmission [219]. The reason can be the missing protein glycosylation and thus the binding site for these lectins on the *Borrelia* surface [37]. Ixoderins and FRePs can be involved in other processes as well; Ixoderin B may be involved in the matrix attachment processes and angiogenesis inhibition. Alternatively, it may antagonize the effect of host ficolin [215].

Finally, one of the tick storage proteins, hemelipoglycoprotein, from several hard tick species seems to share a structural similarity to FRePs with its primary sequence showing a high similarity to the fibrinogen domain [218, 220].

Other tick lectins

OmGalec from the soft tick *O. moubata* is the first member of galectin family identified in ticks with the specificity towards β 1-3 and β 1-4 bound galactose to GlcNAc, and Glc and α 1-3 bound galactose to GalNAc [221]. Similar proteins are also present in *R. appendiculatus* and *I. scapularis* [188]. OmGalec contains two carbohydrate-binding domains which share low sequence similarity and thus possibly possesses a different

saccharide-specificity. The protein is expressed in various life-stages and tissues, with the highest expression in haemocytes, midguts and ovaries [221]. It has been shown that galectins play a vital role in immune homeostasis by being pathogen recognition receptors [222].

C-type lectins are also present in the available tick genomes and transcriptomes [223, 224]. The only characterized C-type lectin from *Haemaphysalis longicornis* (HICLec) contains three various carbohydrate-binding domains. Each of them has been shown to recognize the bacteria *E. coli* and *S. aureus* and participate in the tick defence against gram-negative bacteria, but they do not have a direct effect on bacterial growth. HICLec also affects the blood-feeding process and affects larvae hatching and mortality. Expression of this lectin is increased during blood-feeding and is the highest in the midgut and ovary [223]. In mosquitoes, C-type lectins influence the midgut colonization by bacteria midgut microbiome [225] and facilitate infection with West Nile and dengue viruses [226, 227].

Calreticulin (CRT), a lectin chaperone responsible mainly for the control and proper folding of glycoproteins, is conserved in all tick species and is even used as the biomarker for human tick bites in *I. scapularis* [228]. In blood-feeding parasites, CRTs participate in evasion of the host defence mechanisms, namely the complement by binding the initiator of this pathway, the C1q protein, or factor Xa participating in the blood coagulation [229]. In

mammals, CRT on the surface of neutrophils also binds C1q as well as other immune-related lectins [230]. Similarly, the salivary secreted CRT from *A. americanum* binds host C1q. On the other hand, it does not bind the factor Xa and does not inhibit the activation of the classical complement cascade and host haemostasis. The *A. americanum* CRT shares a very high sequence similarity with other tick CRTs and thus similar functions of tick CRT can be expected [231].

Several other lectins are characterized in *I. ricinus*, but have not been identified to date: the 37, 60, 65, and 73 kDa lectins from midgut showing haemagglutination activity [163, 232]. The 37 kDa lectin has a binding specificity towards β 1-3 glucan, while the 65 kDa protein binds bovine submaxillary mucin, containing a complicated mixture of various glycan structures and more specifically binds free sialic acid. Another lectin is present in haemolymph/haemocytes with a molecular weight of 85 kDa. It is a C-type lectin with specificity towards sialic acid and GlcNAc [233]. Several other lectins with haemagglutination activity have also been described in other ticks including *R. appendiculatus* [234, 235], *O. tartakovskyi*, *O. tholozani* and *A. polonicus* [233].

Tick glycans

Regarding the glycans and glycoproteins of blood-feeding arthropods, several studies describe these molecules using lectin staining and other indirect methods. Lectin studies show the presence of both *N*- and *O*-glycosylated proteins in tick tissues and some glycoproteins have been shown to be antigenic determinants for the immune response of the host [236–239]. In recent years, the direct determination of glycan structures and composition, mostly using mass spectrometry, has also been published, either from tick tissues and cells [203, 240] or purified proteins [220]. The three most interesting glycan structures related to host-parasite interaction and host immune system reaction are described below; representation of these structures in a glycan molecule is shown in Fig. 2. An overview of tick glycans with known structures is listed in Table 4.

Alpha-galactose epitope

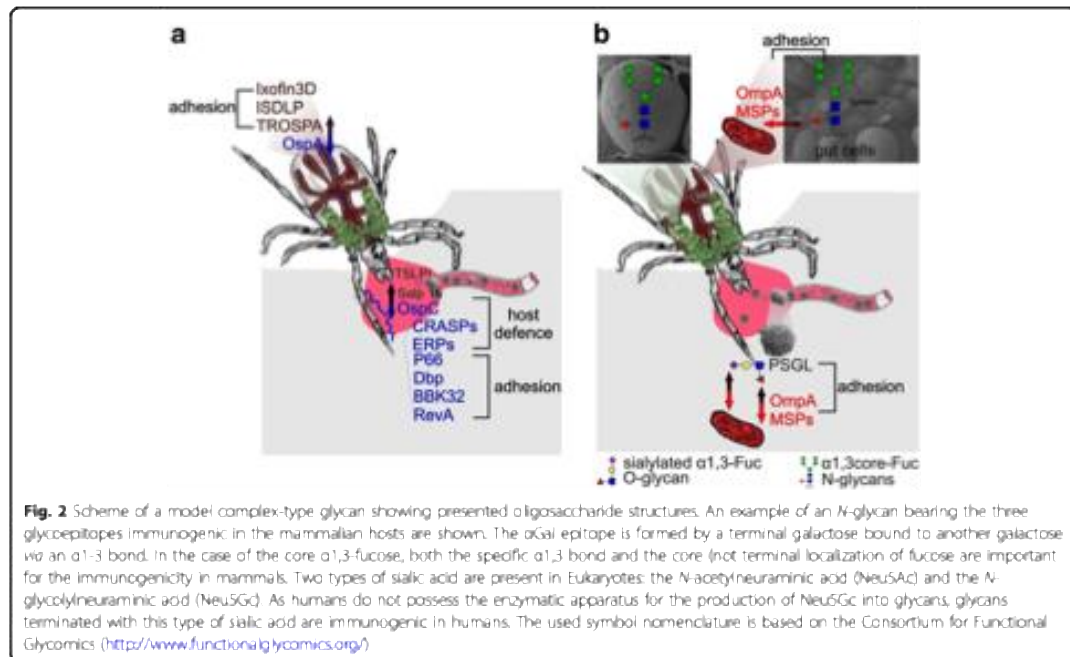
Alpha-galactose epitopes (Gal α 1-3Gal; α Gal) are abundant on glycolipids and glycoproteins of plants, arthropods and non-primate mammals [241]. α Gal is a novel allergen identified first during clinical trials in 2004 in patients treated with cetuximab, a medical preparation for metastatic colorectal cancer treatment. Several cases of hypersensitivity reaction were registered soon after cetuximab administration into the blood due to the presence of α Gal in its structure. The majority of sensitive individuals come from a population in south-eastern USA [242]. Furthermore, the geographical distribution of cases with cetuximab hypersensitivity corresponded

to the distribution of red meat allergy cases and tick prevalence. Additionally, patients with red meat allergy experienced a tick bite in the months preceding the allergy symptoms. The causative agent of the α Gal sensitization in the south-eastern region of the USA is the lone star tick *A. americanum* [243]. Red meat allergy is also linked with *I. holocyclus* tick bite in the Australian population [244]. Conversely, a bite by the *I. scapularis* tick from the same genus in the USA does not seem to result in red meat allergy [243]. Lastly, Chinuki et al. [245] described the allergy development upon *H. longicornis* bite in Japan. Direct evidence on α Gal epitopes presence in *I. ricinus* is provided by Hamsten et al. [246], specifically in the tick midgut. However, the presence of α Gal just in the tick saliva is what is important for patient sensitization. In this regard, the presence of undigested complete host proteins and glycoproteins was described in the tick body and, importantly, in the tick saliva [240, 246, 247] and thus the presence of α Gal originating in the blood of non-mammalian hosts from the previous blood-feeding can be expected in the saliva. The α Gal epitope is only known to be present in the saliva of *A. sculptum*, a tick that until now has not been connected with red meat allergy cases [248].

Core α 1,3-fucosylation

The allergenic core α 1,3-fucose (α 1,3-Fuc) attached on the proximal GlcNAc residue is widely present in plants and arthropods and is one of the well-known possible human allergens as it is usually absent in mammals. It can induce production of specific IgE antibodies associated with IgE-mediated allergic immune responses, which is mostly described for schistosomes or venoms of some species of the order Hymenoptera. However, such a response is not described after a tick bite [249–251]. It is rather surprising, as α 1,3-fucosylated structures are present in the tick salivary glands as well as in saliva of both *I. ricinus* and *I. scapularis* [203, 252]. This can be explained by the structural features of the allergenic epitopes; for example, in the case of core α 1,3-Fuc, terminal GlcNAc weakens the immune response [253]. Additionally, more than one epitope has to be present to trigger the allergic reaction and the presence of blocking IgG4 antibodies against this epitope can lower the immune reaction [249].

The α 1,3-Fuc modification of the *N*-linked glycan core mediates an entrance of one of the tick-transmitted pathogens, *A. phagocytophilum*, into *I. scapularis* midgut cells, but it is not required for the transmission of the pathogen to a vertebrate host. Furthermore, *Anaplasma* increases the expression of α 1,3-fucosyltransferases in the tick, further increasing its ability to infect the tick. On the other hand, the infection of the tick by *B. burgdorferi* was not affected by the presence or absence of core α 1,3-Fuc [203].



Sialic acids

Sialic acids (Sia) are found typically in the terminal position of vertebrate complex *N*- or *O*-linked glycans. In insects, some studies have shown the ability of sialylation [254, 255] and the importance of sialylation for insect-development, even though the abundance of sialylated glycans is very low [256].

N-glycans terminated with Sia are present also in the organs of the tick *I. ricinus*, namely in the gut, salivary glands, ovary and Malpighian tubules [240, 257]. However, the sialylated proteins in the adult ticks originate most probably from the host blood [258]. Hypothetically, sialoglycans present in the tick organs and in the secreted tick saliva can be engaged in molecular mimicry. We suppose that sialic acid is produced also by the tick itself in the ovary and eggs and later in larvae; the exact role of the tick sialylated proteins for the physiology and development of ticks is not yet known (unpublished results). Both eukaryotic types of sialic acids, *N*-acetyl-neuraminic acid and *N*-glycolyl-neuraminic acid (Fig. 2), were detected in the ticks [240].

N-linked glycans of flaviviruses

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus*, can cause serious infections in humans, which may result in encephalitis/meningoencephalitis. The viral single-stranded genomic RNA of positive polarity contains one open reading frame, which encodes

a single polyprotein that is co-translationally and post-translationally cleaved by viral and cellular proteases into three structural and seven non-structural proteins (Table 4) [259].

Flaviviral non-structural proteins

Non-structural proteins of the flavivirus family (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) do not have their precise role elucidated, but they are generally considered as the effectors of viral replication, which occurs in close association with cellular membranes. Dramatic changes in the intracellular membrane structures including convoluted membranes, vesicle packets or paracrystalline arrays were observed as a result of dengue virus (DENV), TBEV or West Nile virus (WNV) replication [260–262]. Recently, NS1, NS4A and possibly also NS2A, were described to be involved in the formation of vesicle packets [263, 264]. Moreover, novel functions in terms of virus-host interactions were recently described for particular NS proteins; for example, TBEV NS5 protein acts as an inhibitor of interferon-activated Jak-STAT signalling [265].

Flaviviral structural proteins

Apart from seven non-structural proteins, the flaviviral genome encodes three structural proteins (C, M, E). The flaviviral nucleocapsid is composed of (+) ssRNA genome and the capsid protein (C), whereas the host-derived

Table 4 Overview of identified tick glycan structures composition. Monosaccharide compositions of the identified N-glycans are shown. Note that in some cases, the same composition can define various structures. For each glycan, the protein or the tick samples is listed, in which it was identified by mass spectrometry

Glycan composition				Protein/sample	Reference
Paucimannose glycans					
HexNAc	Hex	dHex	Sia		
2	3	0	0	Dorin IM (position ⁴¹ NH ₂ , ¹⁷¹ NGS, ¹²⁹ NH ₂) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
2	4	0	0	Dorin IM (position ⁴¹ NH ₂ , ¹⁷¹ NGS, ¹²⁹ NH ₂) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
High-mannose glycans					
HexNAc	Hex	dHex	Sia		
2	5	0	0	Dorin IM (position ⁴¹ NH ₂ , ¹²⁹ NH ₂) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
2	6	0	0	Dorin IM (position ⁴¹ NH ₂ , ¹²⁹ NH ₂) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
2	7	0	0	Dorin IM (position ⁴¹ NH ₂ , ¹²⁹ NH ₂) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
2	8	0	0	Dorin IM (position ⁴¹ NH ₂ , ¹²⁹ NH ₂) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
2	9	0	0	Dorin IM (position ⁴¹ NH ₂ , ¹²⁹ NH ₂) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>D. marginatus</i> Hemelipoglycoprotein	[220]
				<i>I. scapularis</i> salivary gland	[203]
2	10	0	0	<i>D. marginatus</i> Hemelipoglycoprotein	[220]
				<i>I. scapularis</i> salivary gland	[203]
Core-fucosylated glycans					
HexNAc	Hex	dHex	Sia		
2	3	1	0	Dorin IM (position ¹⁷¹ NGS) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
2	4	1	0	Dorin IM (position ¹⁷¹ NGS) from <i>C. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]

Table 4 Overview of identified tick glycan structures composition. Monosaccharide compositions of the identified N-glycans are shown. Note that in some cases, the same composition can define various structures. For each glycan, the protein or the tick samples is listed, in which it was identified by mass spectrometry (Continued)

Glycan composition				Protein/sample	Reference
2	5	1	0	<i>I. scapularis</i> salivary gland	[203]
				Dorin M (position 171 NGS) from <i>O. moubata</i>	[217]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
3	2	1	0	<i>I. ricinus</i> fed female salivary glands	[240]
3	3	1	0	<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
4	3	1	0	<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
4	4	1	0	<i>I. scapularis</i> salivary gland	[203]
4	5	1	0	<i>I. ricinus</i> fed female midgut	[240]
				<i>I. scapularis</i> salivary gland	[203]
4	6	1	0	<i>I. ricinus</i> fed female midgut	[240]
5	3	1	0	<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. scapularis</i> salivary gland	[203]
5	5	1	0	<i>I. ricinus</i> fed female midgut	[240]
6	6	1	0	<i>I. ricinus</i> fed female midgut	[240]
Complex glycans					
HexNAc	Hex	d-Hex	Sia		
3	4	0	0	<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
4	6	0	0	<i>I. ricinus</i> fed female midgut	[240]
4	7	0	0	<i>D. marginatus</i> Hemifipoglycoprotein	[220]
4	8	0	0	<i>D. marginatus</i> Hemifipoglycoprotein	[220]
5	6	0	0	<i>I. ricinus</i> fed female midgut	[240]
6	2	0	0	<i>I. ricinus</i> fed female midgut	[240]
Sialylated glycans (containing either Neu5Ac or Neu5Gc)					
HexNAc	Hex	d-Hex	Sia		
4	5	0	1	<i>I. ricinus</i> fed female salivary glands	[240]
				<i>I. ricinus</i> fed female midgut	[240]
4	5	0	2	<i>I. ricinus</i> fed female midgut	[240]
5	6	0	1	<i>I. ricinus</i> fed female midgut	[240]
5	6	0	2	<i>I. ricinus</i> fed female midgut	[240]

Abbreviations: HexNAc N-acetyl-hexosamine (N-acetyl-glucosamine or N-acetyl-galactosamine), Hex hexose (mannose, glucose, galactose), d-Hex deoxyhexose (fucose), Sia sialic acid (N-acetyl-neuraminic acid, N-glycolyl-neuraminic acid)

envelope contains two glycoproteins, the membrane (prM/M) protein and the envelope (E) protein [266].

The E glycoprotein is localized in the viral envelope and is the main antigenic determinant of TBEV inducing a humoral immune response. It mediates fusion of

TBEV with host cell membrane and thus facilitates the virus entry to the host cell. It forms heterodimers with the prM protein; the prM-E protein interaction is essential for proper folding of E protein [267]. The heterodimers then migrate to the ER membrane and eventually

bud off as nucleocapsid-containing immature virions [260, 268].

The M glycoprotein is an integral part of the viral envelope together with E protein. It forms heterodimers with the E protein and functions as a chaperon ensuring proper folding of E protein [267]. Non-infectious immature virions containing prM/E homodimers undergo maturation process in late trans-Golgi network by cleavage of pr part from the prM protein by host protease furin. The cleavage produces protein M and triggers re-organization of protein E to form homodimers [268, 269].

TBEV proteins glycosylation

The glycosylation of viral proteins increases their folding efficiency and promotes their intracellular transport by the interaction with host lectins [270]. In several viruses that cross the endoplasmic reticulum during their life-cycle, the protein glycosylation was proven important for virus growth, budding, secretion, and pathogenicity (reviewed in [271–273]). So far, only membrane glycoproteins prM/M and E are known to be *N*-glycosylated in TBEV (Table 5) [274–276]. α 1,3-core fucosylated, high-mannose and hybrid *N*-glycans were shown to be present in the E protein of TBEV produced in chicken embryos by affino-blot [277]. E protein glycosylation was also confirmed recently also using cryo-electron microscopy, even though the exact glycan structure was not defined [278]. Another two sites (N130 and N207) in the NS1 protein are *N*-glycosylated in the case of dengue virus [274, 279]. One of these two *N*-glycosylation sites (N207) is present also in the TBEV NS1 protein; however, its glycosylation has not yet been shown (Table 5).

The E glycoprotein is a viral surface protein and thus contains major antigenic epitopes responsible for triggering the host immune system [280]. The *N*-glycosylation site at position N154 is present in the majority of TBEV strains and other flaviviruses. Moreover, a potential N361 glycosylation site is present in TBEV E protein as well.

Depending on the strain, zero to two glycans are attached [280, 281]. The investigation of the presence and position of E protein glycans showed that an increased number of E protein glycans elevate its expression. Conversely, the E protein glycosylation deletion mutants showed reduced E protein production, suggesting the importance of glycosylation for the viral life-cycle [276].

Importantly, the absence of E-protein glycosylation affects the E protein conformation and further the TBEV infectivity only in the mammalian host, but not in the tick vector. Different temperatures in the host (37 °C) and the vector (23 °C) do not affect the stability of the deglycosylated E protein [282]. In light of this evidence, the E protein glycosylation seems to represent one of the factors conferring different vector and host competence. Interestingly, the investigation of mosquito-borne flaviviruses, DENV and WNV, shows that glycans modifying E protein are important for virus propagation in both vector and host cells. However, the role of the particular *N*-linked glycosylation site varies depending on the invertebrate/vertebrate host [283–285]. For example, the importance of *N*-linked glycan at 154 aa of WNV E protein was proved in case of the vector (*Culex pipiens* and *Cx. tarsalis* mosquitoes) as well as the bird host (*Gallus gallus*) [286, 287].

The prM protein encodes for the precursor of membrane protein M and also contains one *N*-linked glycosylation consensus sequence in N32 position (Table 5) [267, 276]. During TBEV maturation, the structural proteins prM and E form heterodimer, where prM has a chaperone-like role in the folding and maturation of E protein [267], although the biological role of TBEV prM glycosylation, has not yet been elucidated. However, Goto et al. [279] suggest the participation of carbohydrate-mediated interaction for prM-E heterodimer formation; glycosylation-deficient mutant of prM reduces the secretion of E protein to 60% in comparison to the wild-type prM. Further evidence for the crucial role of prM glycan was described in the case of WNV.

Table 5 TBEV protein glycosylation overview. List of TBEV proteins and their functions. Identified or predicted *N*-linked glycosylation sites are listed as well. NetNGlyc 1.0 Server was used for *N*-linked glycosylation site prediction

	Protein	Function	<i>N</i> -linked glycosylation	Reference
Structural	C	Capsid protein; forming of nucleocapsid	None	
	prM/M	Envelope protein; E protein chaperone	N32	[265, 302]
	E	Envelope protein; binding and fusion	N154, N361	[263–265]
Non-structural	NS1	Replication	Predicted: N185, N207	
	NS2A	Assembly, replication	None	
	NS2B	NS3 serine-protease cofactor	None	
	NS3	Serine-protease, helicase, replication RNA triphosphatase	Predicted: N160, N499, N555	
	NS4A	Assembly, replication	None	
	NS4B	Assembly, induction of membrane rearrangements	Predicted: N188	
	NS5	Methyltransferase, RNA-dependent RNA polymerase	Predicted: N118, N175, N215	

The prM glycosylation-deficient mutants decreased the formation and release of virus-like particles as well as genome copies. However, the infectivity of prM glycosylation-deficient mutants was not affected in mosquito, avian or mammalian cell lines [284].

In summary, *N*-linked glycosylation of TBEV prM and E proteins represents a multifaceted factor which is involved in many steps of the viral life-cycle, especially in virion assembly/secretion, and host/vector competence. Despite various studies, there are many aspects which need to be elucidated, especially the role of viral protein *N*-linked glycans within tick vectors. Moreover, the presence of other *N*-linked glycans in predicted sites of NS1, NS3, NS4B, and NS5 remains to be determined as well as their potential function.

Conclusions

The recent decades have provided an outstanding amount of new data about glycoconjugates and a growing line of evidence highlights the importance of carbohydrate-based interactions in the complex pathogen-host environment [288]. Glycoconjugates have an enormous structural diversity in the glycan moieties and therefore fulfil a variety of biological roles [289]. Given the fact that glycoconjugates are the major components of the outer surface of animal cells [290], it is likely that all interactions of microbial pathogens with their hosts/vectors are affected to a certain degree by the pattern of glycans and glycan-binding molecules that each produces. Despite the fact that protein glycosylation in the field of tick-borne pathogens has become a subject of increased attention in the last decade [37, 218, 291], there is still a deep knowledge gap regarding the nature and the specific roles of the glycoconjugates in the infectious cycle of these pathogens. All the findings mentioned in this review have tackled the important, yet still inadequately explored, the field of the carbohydrate-based interactions at the pathogen-tick-host interface. The basis of these interactions needs to be further addressed to gain clearer insights into the intricate strategies that the parasites employ to successfully finish their life-cycles. Ultimately, the common goal of scientists working in any field dealing with infectious diseases is to find an effective countermeasure against the particular threat. Ticks transmit a great variety of bacterial, viral and protozoan pathogens and therefore the search for a potent vaccine against each of these parasites costs an enormous amount of effort and money. One of the most promising strategies to cope with all pathogens transmitted by ticks is the development of a general anti-tick vaccine [292]. The potentially important role of sugar moieties in such a tick vaccine has already been suggested, showing, for instance, the tick midgut protein Bm86 to be more immunogenic in glycosylated form than non-glycosylated [291]. However, the progress in this matter is still

insufficient and intense analysis of glycosylation needs to be addressed in future studies in order to be applied to the development of new therapeutics. Modern glycan sequencing technologies and strategies that allow site-specific mass-spectrometric identification of proteins with glycan modifications in a complex biological sample have shown that glycosylation could be much more extensive than previously thought [293].

Abbreviations

ACG: Cholesteryl 6-O-acyl-β-D-galactopyranoside; ACGal/Bb-Gal-1: Cholesteryl 6-O-palmitoyl-β-D-galactopyranoside; AmOmp: *Anaplasma marginale* outer membrane protein; AP: Alternative complement pathway; ApOmp: *Anaplasma phagocytophilum* outer membrane protein; Bgpa: Borrelia GAG-binding protein; CLR: C-type lectin receptor; CP: Classical complement pathway; CRASP: Complement regulator-acquiring surface protein; CRT: Calreticulin; DAF: Decay accelerating factor; DbpA: Decorin-binding protein; DENV: Dengue virus; ECM: Extracellular matrix; Epi: OspE/F-related proteins; Fht: Complement factor H; FHL: Factor H-like; Fln: Fibrinogen; FREP: Fibrinogen related protein; Fuc: Fucose; GAG: Glycosaminoglycan; GalCer: Galactosylceramide; WFGP: *Ixodes scapularis* antifreeze glycoprotein; ISDLP: *Ixodes scapularis* dystroglycan-like protein; LacCer: Lactosylceramide; LP: Lectin complement pathway; LPS: Lipopolysaccharides; MAC: Membrane attack complex; MBL: Mannose-binding lectin; MGalD: Mono-α-galactosyl-diacylglycerol; MSP: Major surface proteins; MW: Molecular weight; na: Not available; NK1: Natural killer T cells; NLN/NOO-like receptors: Nucleotide-digomerization domain-like receptors; Omp: Outer membrane protein; Osp: Outer surface protein; PAMP: Pathogen-associated molecular pattern; PM: Peritrophic matrix; PRR: Pattern recognition receptor; PSGL-1: P-selectin glycoprotein ligand-1; RGD: Arg-Gly-Asp tripeptide, a binding motif of fibronectin; Salp: Salivary protein; SG: Salivary glands; Sia: Sialic acid; TBEV: Tick-borne encephalitis virus; TLR: Toll-like receptors; TROSPA: Tick receptor for OspA; TSLP: Tick salivary lectin pathway inhibitors; Vmcp: Variable major protein; Vsp: Variable small protein; Vtp: Variable tick protein; WNV: West Nile virus

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Authors' contributions

All authors contributed to the manuscript preparation. PV and JS contributed to this work equally. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Consent for publication

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Paper IV

Click-on-Membrane for Detection of Metabolically Labelled Proteins and RNA

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DETECTION OF METABOLICALLY LABELLED PROTEINS AND RNA BY CLICK REACTION ON MEMBRANE

The article is dedicated to the memory of prof. Ing. Rudolf Zahradník, DrSc.

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Introduction

The term Click chemistry was first introduced in the work of Kolb et al.¹. It refers to a type of reactions that are modular, stereospecific, producing high yields and only harmless (intermediate) products. The reactions take place under simple reaction conditions (ideally, they should not be sensitive to the presence of water and oxygen), the product must be easily isolated and stable under physiological conditions¹.

One type of Click reactions is the 1,3-dipolar cycloaddition between the terminal alkyne and azide groups, which results in the formation of the stable triazole². However, this reaction proceeds slowly at physiological temperatures, so the so-called copper-catalyzed azido-alkyne Huisgen cycloaddition (CuAAC, copper-catalysed azide-alkyne cycloaddition) was developed. The use of copper as a catalyst allowed for an acceleration of the reaction and an increase in regioselectivity, since only 1,4-disubstituted products were formed^{3,4}. Click chemistry is now widely used in various research and industrial areas such as pharmaceutical research, biochemistry, polymer chemistry or nanoscience^{5–7}. Click chemistry is one of the methods that can be used to detect, visualize, and identify newly synthesized or differentially synthesized proteins, glycans, lipids and/or nucleic acids⁸.

Bioorthogonal chemistry is defined as a set of chemical reactions that can take place in living organisms and that do not interfere with natural biochemical processes in the cell⁹. It allows visualization of processes in living systems and is available for labelling of almost any type of

biomolecules – proteins, glycans, lipids or nucleic acids *in vivo* and *in vitro*^{9–13}. A widely used combination of bioorthogonal and Click chemistry for protein labelling is called BONCAT (bioorthogonal non-canonical amino acid tagging)⁹.

The BONCAT method is based on the incorporation of modified amino acids carrying a small non-reactive group into newly synthesized proteins. The amino acids used for BONCAT, L-azidohomoalanine (AHA) and L-homopropargylglycine (HPG), serve as a substitute for methionine and are taken up by the cell's translational machinery¹⁴. The requirements for bioorthogonality are also met; the azide and alkyne groups are small, inert, do not occur in living systems and are stable under physiological conditions^{10,15}. Each protein theoretically contains at least one methionine; however, previous studies show that approximately 5 % of human protein sequences contain only the initiating methionine, which can be removed during post-translational modifications⁹.

Different approaches have been used to detect individual bioorthogonally tagged proteins. One possibility is electrophoretic separation of proteins, their electroblotting and detection on the membrane using small protein-binding molecules. These molecules, e.g., enzyme inhibitors, are conjugated to an alkyne/azide and bind covalently to the active site of the enzyme blotted onto the membrane surface. For the detection itself, the CuAAC reaction is used, resulting in a fluorescent label bound to the protein followed by fluorescence detection; or biotin labelling followed by detection with streptavidin conjugated to horseradish peroxidase^{16,17}. A similar approach was used by Ohata et al.¹⁸ for the detection of single proteins; in this case, proteins were directly labelled with an azide or alkyne group followed by binding of a fluorescent dye by the Click reaction. In all these cases, electrophoresis was used primarily to separate the labelled proteins from other small molecules.

The combination of BONCAT with CuAAC is a very powerful and simple tool for visualizing changes in protein synthesis and identifying proteins with altered expression, which represents a major advance in biochemical and molecular biology studies^{9,13,19,20}. For example, Hatzepichler et al.¹³ labelled newly synthesized proteins with AHA and allowed them to react with DBCO-PEG₃-Carboxy-rhodamine 110 using a voltage-assisted Click reaction. The labelled proteins were electrophoretically separated and detected using a fluorescence signal.

The most commonly used method for separation of AHA- or HPG-containing proteins from the reaction mixture (containing unreacted labelling molecules) is precipitation, most commonly using methanol and chloroform²¹. However, this method can lead to the loss of some proteins^{22–24} and can affect the migration of proteins in the gel and cause signal blurring, which is likely to occur due to protein aggregation²⁵.

For the above reasons, we optimized BONCAT using a Click reaction approach on the membrane to detect metabolically labelled AHA or HPG proteins without prior precipitation. We applied the same method to labelled

proteins separated by 2D electrophoresis and, to confirm the feasibility of the method for other biomacromolecules, also for the detection of 5-ethynyl uridine (5-EU)-labelled RNA. We believe that the Click-on-membrane approach is more suitable for the detection of bioorthogonally labelled biomolecules compared to the Click reaction in solution followed by standard methanol/chloroform precipitation.

Experimental part

Detection of metabolically labelled proteins using Click on membrane method

Cell culture and metabolic labelling of proteins

Human glioblastoma cells were cultured in RPMI 1640 medium (Biowest) containing 10 % fetal bovine serum (FBS, Sigma-Aldrich), 1 % L-alanyl-L-glutamine (Biobrom) and 1 % antibiotics (final concentration of amphotericin B 0.25 $\mu\text{g mL}^{-1}$, penicillin G 100 U mL^{-1} , streptomycin 100 $\mu\text{g mL}^{-1}$, Biowest) at 37 °C. Cells were washed twice in sterile PBS (Phosphate Buffer Saline) and incubated for 1 h in modified RPMI 1640 medium without sulphur-containing amino acids (Sigma-Aldrich) containing 0.21 mM cystine (SERVA) and 2 mM L-alanyl-L-glutamine (Biobrom). After methionine depletion, AHA or HPG (both Click Chemistry Tools) was added to the medium at a final concentration of 50 μM and the newly formed proteins were metabolically labelled for 2 h. Cells were washed twice in PBS, released into PBS using a scraper and centrifuged for 10 min at 4 °C and 400 $\times g$. The cell pellet was lysed in lysis buffer (1 % SDS, 0.1 M sodium phosphate buffer, pH 8.0) supplemented with protease inhibitors (HALT Protease Inhibitor Cocktail, Thermo Fisher Scientific). Cell lysates were incubated for 30 min on ice, sonicated and frozen at –80 °C. Protein concentration was determined by the BCA method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific).

Click reaction in solution

Click reaction in solution was carried out according to ref.²⁶. For every 50 mg of proteins, 0.5 mL of Click reaction mixture containing 100 μM CuSO_4 , 0.5 mM THPTA (Click Chemistry Tools), 250 μM sodium ascorbate (SERVA), 100 mM potassium-phosphate buffer, pH 7.0, and 100 μM biotin-PEG₄-alkyne or biotin-picolyl azide (both Click Chemistry Tools) was used in AHA- or HPG-labelled samples, respectively. The reaction was carried out at room temperature for 1 h.

2D electrophoresis

Two approaches were used to remove unreacted reaction components and prepare the protein samples for electrophoresis – precipitation with methanol and chloroform²⁷ and ultrafiltration. For ultrafiltration, Vivaspin 2 Centrifugal Concentrator ultrafiltration columns (10K MWCO, PES, Sartorius) were used according to the manufacturer's instructions. Samples were reduced to 10 % of their original volume and diluted with rehydration buffer.

Precipitated proteins were dissolved in rehydration buffer (7 M urea deionized with Amberlite MB-150 (ref.²⁸), 2 M thiourea, 2 % CHAPS, 40 mM dithiothreitol (DTT), 0.2 % 3–10 Pharmalytes, 0.02 % bromophenol blue (all Sigma-Aldrich)) to a final concentration of 0.4 $\mu\text{g }\mu\text{L}^{-1}$. Samples were centrifuged for 10 min at 13 400 $\times g$ to remove any aggregates formed during precipitation. 125 μL of supernatant was applied to an IPG strip with a linear pH gradient of 3–10 (Bio-Rad) and rehydrated overnight under a layer of mineral oil (Sigma-Aldrich). Isoelectric focusing (IEF) was performed on an IEF100 instrument (Hoefer) at 20 °C (total 13 750 kVh).

After IEF, strips were equilibrated for 10 min in equilibration buffer (8 M urea, 0.375 M Tris, pH 8.8; 2 % SDS, 20 % glycerol) with 2 % (w/v) DTT followed by 10 min in equilibration buffer with 2.5 % (w/v) iodoacetamide (all Sigma-Aldrich). Strips were transferred onto a 10 % acrylamide gel (Mini-PROTEAN TGX Precast Gels, Bio-Rad) and overlaid with 0.5 % low melting point agarose (SERVA) in electrode buffer. A coloured protein standard (10–245 kDa, Applichem) was applied next to the positive end of the strip. Electrophoresis was performed according to ref.²⁹ in a Mini-PROTEAN Tetra Cell apparatus (Bio-Rad).

Electroblot and Click reaction on the membrane

Proteins separated by electrophoresis were transferred to a PVDF membrane³⁰. For samples where the Click reaction was performed in solution prior to electrophoresis and blotting, the membranes were stained and developed. In the case of samples that were not processed by the Click reaction in solution, protein separation by electrophoresis and transfer to the PVDF membrane was followed by the Click reaction on the membrane (see below). All gels after electroblotting were fixed and stained using the Blue Silver method according to³¹. The modified Click reaction was performed on the membrane surface according to ref.²⁹. For one 7.5 cm $\times$ 8.5 cm membrane, 5 mL of Click reaction mixture was used and the reaction was run for 1 h at room temperature. During the optimization of the method, biotin-PEG₄-alkyne was used at a concentration range of 5–100 μM , after optimization a concentration of 10 μM was used. After labelling, membranes were washed twice with PBS-T (PBS with 0.05 % Tween-20), blocked in Roti-Block blocking reagent (Carl Roth) in PBS-T and incubated in streptavidin conjugated with alkaline phosphatase (AP). This was followed by incubation with biotinylated anti-streptavidin antibodies and a second time with streptavidin-AP (all Vector Laboratories, 1:1000 dilution in Roti-Block). This three-step detection provides a highly sensitive detection with low background (unpublished results). The resulting signal was induced in developing buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl_2 , pH 9.0) with 330 $\mu\text{g mL}^{-1}$ BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 165 $\mu\text{g mL}^{-1}$ NBT (*p*-nitroblue tetrazolium chloride) (both Applichem). The reaction was stopped after inducing a sufficiently strong signal by washing the membrane in distilled water.

Detection of metabolically labelled RNA by the Click reaction on the membrane

Cell culture and metabolic labelling of *de novo* synthesized RNA

A human medulloblastoma cell line (DAOY ATCC HTB-186, ref.³²) was cultured in RPMI 1640 medium containing 10 % FBS, 1 % antibiotic-antimycotic, 1 % L-alanyl-L-glutamine and 50 nM β -mercaptoethanol. Cells were cultured at 37 °C in an atmosphere with 5 % CO₂. Cells were seeded in six-well plates at a density of $5 \cdot 10^5$ cells per well. 5-EU was added to the cells at a final concentration of 1 mM and metabolic labelling was performed at intervals of 30 min, 2 h, 4 h and 6 h. The experiment was performed in biological triplicates.

RNA isolation

Cells were washed in PBS and lysed by adding 1 mL of RNA Blue reagent (Top-Bio) per well. Total RNA was then isolated according to the manufacturer's manual. RNA pellets were dissolved in diethyl pyrocarbonate-treated water. RNA concentration and purity were checked with a Nanophotometer Pearl spectrometer (Implen).

Electrophoresis and blotting

RNA was separated on a 1.2 % agarose denaturing gel buffered with MOPS (3-(*N*-morpholino) propanesulfonic acid) containing 6–7 % formaldehyde and visualized using the GelRed dye (Biotium). Capillary blotting of RNA was then performed on PVDF or nylon membrane (Cytiva) using 20× SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0). The membrane was fixed with UV light (120 mJ cm⁻²) and washed with 0.1 M potassium phosphate buffer, pH 7.0.

Click-on-membrane

Membranes were incubated in Click reaction mixture containing 10 μ M picolyl-biotin-azide for 1 h in the dark at room temperature. The membranes were then blocked (Roti-Block) and labelled with a triple antibody system (goat anti-biotin antibody, AP-conjugated horse anti-goat antibody, AP-conjugated rabbit anti-goat antibody). Chemiluminescence was detected using the CDP-Star kit (Novex); a development buffer containing 330 μ g mL⁻¹ BCIP and 165 μ g mL⁻¹ NBT (both AppliChem) was used for colorimetric signal induction.

Results and discussion

The standard BONCAT procedure is based on the analysis of proteins that undergo the Click reaction in solution. This approach is widely used^{9,10,13,33}, however, the Click reaction performed in this way may not always lead to clear results. One of the disadvantages of the in-solution reaction is the need to add an excess of the low molecular weight reagent, which should be removed from the sample before further analysis, as its presence may lead to false positive or biased results. This is an additional step that

may affect the result. One possibility is precipitation²⁵ but this can lead in some cases to the formation of insoluble protein aggregates causing insufficient protein focusing during IEF³⁴. Therefore, we investigated the possibility of performing the Click reaction on the membrane after electrophoresis.

The first step was to optimize the amount of biotin-alkyne used for the Click reaction performed on the membrane. The click reaction in AHA-containing samples was performed for 1 or 3 h with different concentrations of biotin-alkyne (100 μ M, 50 μ M, 20 μ M, 10 μ M, 5 μ M). A decrease in biotin-alkyne concentration did not significantly affect the signal intensity, nor was it necessary to proceed to an extension of the incubation time (fig. 1). All subsequent experiments using the Click on the membrane method were carried out in a reaction mixture containing 10 μ M biotin-alkyne for 1 h.

AHA-labelled proteins after Click reaction in solution were analysed by 2D electrophoresis and western blot. The gel and membrane showed insufficient separation of proteins throughout the gel (fig. 2A, B). The use of HPG instead of AHA had no beneficial effect (fig. 2C, D). Biotin-labelled proteins were visualized using a three-step detection (fig. 2B, D). 2D electrophoresis is a method highly sensitive to incomplete protein focusing, which manifests as protein streaks across the entire range of separation in one direction or the other and incorrect protein profiles instead of focused protein spots (ideally in the form of dots). Attempts to improve the results and remove protein aggregates by ultrafiltration, repeated centrifugation, precipitation, or the use of deionized urea²⁸ have also failed to

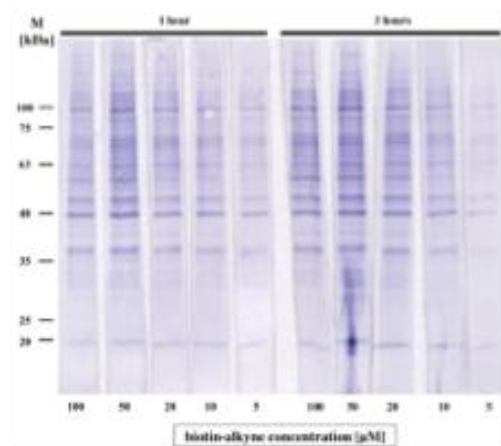


Fig. 1. Optimization of the detection of AHA-labelled proteins by the Click-on-membrane method; 1 μ g of protein was separated on a 10 % acrylamide gel and blotted PVDF membrane. Click reactions were performed with the indicated concentrations of biotin-alkyne for 1 or 3 h. The newly synthesized proteins were detected by a three-step detection using AP-conjugated streptavidin and biotinylated anti-streptavidin antibodies followed by chromogenic detection.

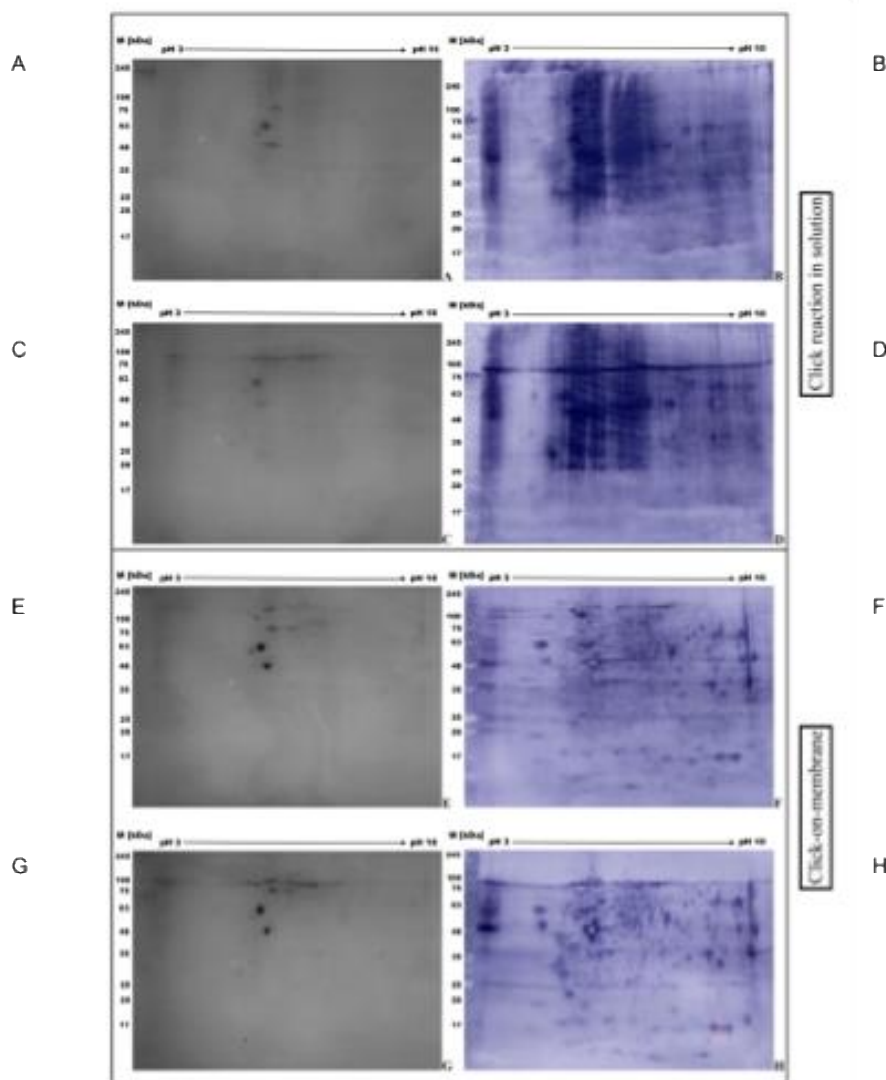


Fig. 2. Comparison of 2D electrophoretic profiles and detection of AHA- or HPG-containing proteins labelled by Click reaction in solution or Click-on-membrane approach; 50 μ g of proteins after the Click reaction in solution (A-D) or before the Click reaction (E-H) were separated by IEF followed by SDS-PAGE. The newly formed proteins in A, B, E, and F were labelled with AHA, and the proteins in C, D, G, and H were labelled with HPG. The separated proteins were transferred to PVDF membrane and the gels were stained by the Blue Silver method (A, C, E and G). Click reactions on the membrane were performed on the blotted proteins in F, H. Membranes (B, D, F and H) were blocked and newly synthesized proteins were detected by a three-step detection with streptavidin-AP and biotinylated anti-streptavidin antibodies followed by chromogenic detection

produce any satisfactory improvement (data not shown). Difficulties in the separation of bioorthogonally labelled proteins (especially small proteins and peptides) in the gel have been documented in other publications¹⁸.

Therefore, we proceeded to perform the Click reaction on the membrane instead of the Click reaction in solution prior to electrophoretic separation and electroblotting. After cell lysis, the AHA-containing protein samples were

processed in the same manner as standard samples for analysis by 2D electrophoresis, and the Click reaction was performed on blotted proteins on the membrane surface (fig. 2F). Compared with the protein profile after the Click reaction in solution (fig. 2B), the protein spots were sharper and more focused, and the presence of poorly separated proteins in the form of streaks was significantly reduced. Similar results were observed using HPG (fig. 2D, H). One reason for these observed differences may be the change in pI of the modified proteins after biotin-alkyne binding³⁵ or the formation of protein aggregates³⁴. An analogous effect was observed for both 1D SDS-PAGE and 2D electrophoresis (unpublished data).

Detection of newly synthesized RNA by Click-on-membrane

After the successful use of the Click reaction on the membrane for the analysis of AHA or HPG labelled proteins, we verified the suitability of this method for other bioorthogonally labelled biomolecules. As an example, we tested the detection of 5-EU labelled RNA. DAOY cells were incubated with 1 mM 5-EU for 30 min, 2, 4, and 6 h. The purity and integrity of the isolated RNA were verified by spectrophotometric and electrophoretic methods. Staining of RNA with GelRed reagent in denatured agarose gel demonstrates its high integrity (fig. 3A). Incorporated 5-EU was detected by the Click-on-membrane reaction with 10 nM picolyl-biotin-azide followed by a three-step detection of biotin using antibodies. 5-EU incorporated in nascent RNA was visualized using chemiluminescence (fig. 3B) and chromogenic substrate (fig. 3C). Figure 3B shows that the intervals (except the 30 min interval) are sufficient to incorporate detectable amounts of 5-EU in the case of 45S pre-rRNA. A nonspecific signal originating from the GelRed fluorescent dye in the 18S and 28S rRNA region is observable on the membranes for all samples (fig. 3B, C).

Metabolic labelling of *de novo* synthesized RNA is widely used, often using radioactive nucleosides with incorporated radioisotopes of tritium (³H) or phosphorus (³²P) followed by electrophoretic separation and transfer of the separated nucleic acids to a nylon or nitrocellulose membrane and detection of the newly formed RNA by autoradiography^{36,37}. Click chemistry serves as a non-radioactive alternative. Fluorescence *in situ* detection of newly formed RNA with incorporated 5-EU has been described³⁸ and widely used^{39,40}, whereas detection of *de novo* synthesized RNA on membrane by Click reaction has not been described so far.

In this work, we have shown that the Click-on-membrane method can be successfully applied to detection of 5-EU-containing RNA, and is similarly applicable to the detection of other bioorthogonally labelled biomacromolecules. However, the procedure is only suitable when using a more durable PVDF membrane, as a weak signal and high background were observed when using a nylon membrane (data not shown).

Conclusion

Bioorthogonal chemistry is one way how to modify various biomolecules in their native environment. The combination of bioorthogonal and Click chemistry is widely used to label and analyse newly synthesized proteins,

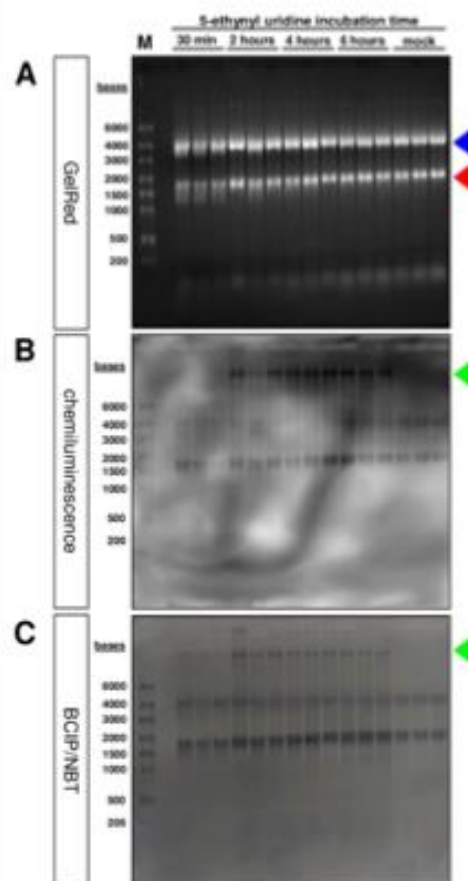


Fig. 3. Detection of newly synthesized RNA by Click-on-membrane method; Total RNA containing 5-EU was separated in a 1.2 % denaturing agarose gel and visualized by the GelRed dye (A). RiboRuler High Range RNA ladder (M) was used as a size standard. Arrows point to 28SrRNA (blue) and 18SrRNA (red). (B) RNA molecules were subsequently transferred from the gel to a PVDF membrane, fixed with UV light and labelled using the Click-on-membrane approach. RNA synthesised *de novo* was detected by triple labelling using streptavidin-AP and biotinylated anti-streptavidin followed by chemiluminescence detection. 45S pre-rRNA is indicated by the green arrow. (C) The membrane was further used for colorimetric signal detection using BCIP/NBT. Non-specific signal caused by the fluorescent dye Gel Red is visible in the 18S and 28SrRNA region in (B) and (C)

but the commonly used sample processing procedure often results in a significant reduction in the quality of the protein profile during 2D electrophoresis.

Our described combination of bioorthogonal chemistry with the Click-on-membrane method is applicable for the detection of newly synthesised proteins and RNA that are bioorthogonally labelled, separated by electrophoresis and transferred to a PVDF membrane.

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Abstract

Bioorthogonal chemistry provides one of the possibilities to modify various biomolecules in their native environment. The combination of Click chemistry with the BONCAT method (bioorthogonal non-canonical amino acid tagging) is widely used for tagging and analysis of newly synthesized proteins, which are clearly distinguishable from the pre-existing protein pool. However, the commonly used procedure results in low quality 2D electrophoretic profiles. We put a lot of effort into obtaining clear

results using a standard Click protocol, with a negligible effect. Here we describe a Click-on-membrane approach which we successfully used not only to monitor *de novo* protein synthesis but also to detect newly synthesized RNA.

Paper V

Immunogenic and cross-reactive carbohydrate determinants in ticks and other arthropods

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Review article

Immunogenic and cross-reactive carbohydrate determinants in ticks and other arthropods

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Abstract

Ticks are parasites that feed on the blood of humans and animals, using it as their sole source of food. Due to their lifestyle, ticks at each life stage transmit various tick-borne pathogens while feeding on their hosts. Unlike mosquitoes – another important group of pathogen-transmitting arthropods – ticks can feed on the same host for prolonged periods, which underscores the importance of tick mechanisms that inhibit or reduce the host's defence mechanisms and, consequently, allow the transmission of pathogens. This unique tick-host-pathogen triangle requires the involvement of various tick molecules to mediate interactions with both host and pathogen molecules at multiple levels. This review highlights the glycan molecules identified in various tick species that can trigger an immune response and thus primarily affect the interaction with the host.

As the enzymes involved in Golgi-mediated glycan maturation differ between ticks, insects, and their vertebrate hosts, these differences contribute to the glycan profiles observed in ticks. These differences in glycan structures influence the interactions ticks have with their hosts and pathogens. On one hand, they may participate in molecular mimicry and mechanisms that lower the host's immune reaction, blood clotting, and other defence mechanisms due to their different structures, but on the other hand they may also trigger the host's defence mechanisms, such as participating in delayed red meat allergy and facilitating the transmission of pathogens.

Tick glycobiology remains largely unexplored and deserves more attention, especially considering the potential of glycans and glycoproteins as targets for anti-tick vaccines.

Keywords: α 1,3-core fucose; α Gal; β 1,2-xylose; N-glycolylneuraminic acid; Tick

Highlights:

- Tick glycans shape key interactions within the tick-host-pathogen triangle.
- Tick-specific glycans can modulate or trigger host immune and defence responses.
- Some tick glycans contribute to red-meat allergy/ α -Gal syndrome.
- Tick glycobiology offers promising targets for next-generation anti-tick vaccines.

Abbreviations:

α -Gal – Galactose- α 1,3-galactose epitope; AGS – α -Gal syndrome; CCD – cross-reactive carbohydrate determinant; CMP – cytidine monophosphate; Fuc – fucose; Gal – galactose; GalNAc – N-acetylgalactosamine; GlcNAc – N-acetylglucosamine; HRP – horseradish peroxidase; MALDI TOF-TOF – Matrix-Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight; Man – mannose; Neu5Ac – N-acetylneuraminic acid; Neu5Gc – N-glycolylneuraminic acid; SG – salivary gland; Xyl – xylose

Introduction

Ticks are blood-sucking ectoparasites that transmit pathogens harmful to humans and livestock. The complex interactions between the transmitted pathogens, ticks, and their hosts require these parasites to produce various immunologically active molecules that influence the dynamics between the tick and its host, as well as between the tick and its tick-borne pathogens. Most interactions are mediated by proteins; how-

ever, protein-bound glycans in ticks may influence allergic responses, pathogen transmission, and modulation of the host immune system. In addition, some glycan moieties can trigger host immune responses due to shared structural motifs with other allergens, referred to as cross-reactive carbohydrate determinants (CCDs) (Aalberse et al., 1981). Moreover, tick salivary glycans can trigger host immune responses (e.g., basophil-dependent pathways) that inhibit subsequent feeding (Allen, 1973). Certain glycans, beyond classical CCDs, contribute to acquired tick resistance in some non-natural

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hosts, resulting in reduced engorgement, impaired feeding, or tick death upon re-exposure (Allen, 1989; summarised in Wikel, 1996). Overall, immune responses against antigenic structures in tick tissues, including glycans, can damage these tissues or interfere with salivary molecules, thereby reducing tick feeding efficiency. The presence of such molecules and the resulting immune response targeted against tick structures during blood feeding could hinder blood feeding.

Despite technological advances, research on tick glycans has been limited. A Web of Science search with 'tick + glycan', 'tick + glycoprotein', and 'tick + saccharide' found 88 publications, excluding studies on tick-borne viruses without glycosylation or pathogen interactions. Even recently, only a few publications have addressed this topic each year. The discovery of α -Gal syndrome (AGS) has underscored the importance of glycans as cross-reactive carbohydrate determinants (CCDs) and their immunological importance for both ticks and hu-

mans, bringing tick glycan research to the forefront. This review summarises information on known CCDs present in ticks and their effects on human health.

Cross-reactive carbohydrate determinants and their immunological relevance

During feeding, ticks introduce a complex mixture of salivary molecules into their host, including glycoproteins rich in carbohydrate motifs. Several of these motifs can act as cross-reactive carbohydrate determinants (CCDs). CCDs are structures which are not produced by humans; therefore, their structural foreignness can elicit immune responses mediated by various antibody classes and subclasses. The known CCD epitopes include N-glycan α 1-3 linked core fucose (α 1-3 core Fuc), galactose α 1-3 linked to galactose (α -Gal epitope), sialic acid variant N-glycolyneuraminic acid (NeuGc), and core N-glycan β 1-2 linked xylose (Xyl) (Fig. 1) – Platts-Mills et al. (2021).

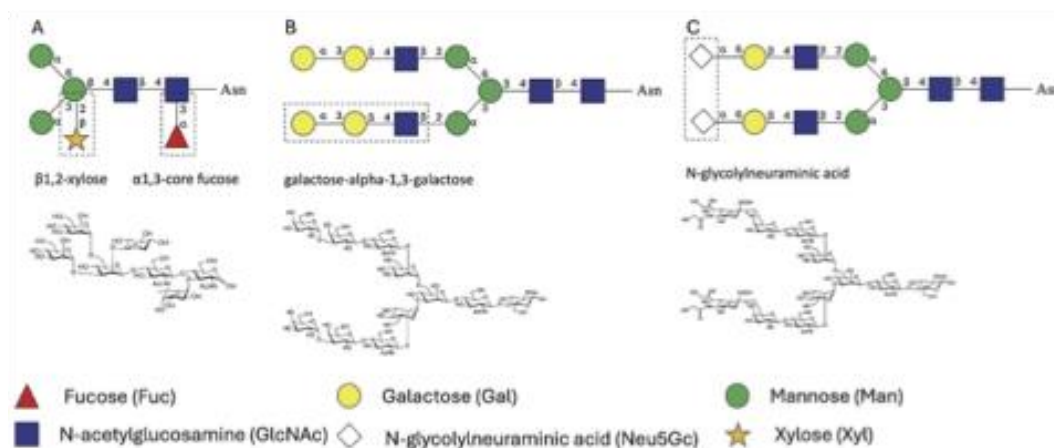


Fig. 1. Visualisation of the cross-reactive carbohydrate determinants structures; the specific epitopes are highlighted by frames in the structure of an N-glycan. Structures are shown both according to the Symbol Nomenclature for Glycans (Neelamegham et al., 2019) and as Reeves projections. **A** – paucimannosidic N-glycan with linked immunogenic α 1-3 linked core fucose (α 1-3 core Fuc) and β 1-2 linked xylose (Xyl). **B** – N-glycan containing α -Gal epitopes, and **C** – complex N-glycan with N-glycolyneuraminic acids (NeuGc). Symbol description of individual monosaccharides is shown.

Despite the rise of mass spectrometry analyses of various invertebrate glycomes, the knowledge of tick glycomes is, to date, limited to particular tick species and organs of tick nymphs and adults. Salivary glands and the gut have been heavily studied in *A. americanum*, *I. ricinus*, and *I. scapularis* ticks. High-mannose, paucimannose, complex glycans, and a small amount of hybrid N-glycans, all with two or three antennae, were detected in ticks (Park et al., 2020; Pedra et al., 2010; Vancova et al., 2012). In *I. scapularis* and *A. americanum* individuals, N-glycans containing α 1,3-core Fuc were shown (Park et al., 2020; Pedra et al., 2010). In *I. ricinus* and *A. americanum*, N-glycans with terminal Neu5Gc and Neu5Ac were present (Park et al., 2020; Vancova et al., 2012). A unique combination of CCDs in one glycan was observed in *A. americanum* salivary glands: N-glycans containing, for example, both α -Gal and β -Xyl, α -Gal and α 1,3-core Fuc, or two α -Gal epitopes (Park et al., 2020).

Although CCDs are commonly discussed in the context of IgE, humans also produce glycan-specific IgG (notably IgG1 and IgG3) and IgM against a broad range of carbohydrate

epitopes (Schneider et al., 2015), with antibody isotypes and clinical relevance varying by structure.

For α 1,3 core fucose, IgE levels are very high, but clinical relevance is generally low, despite frequent IgM and IgG1 production (Bardor et al., 2003) and elevated IgG1 in CCD positive individuals (Román-Carrasco et al., 2020); nonetheless, the biological activity of anti- α 1,3 core Fuc IgE has been demonstrated (Plum et al., 2023). Polyclonal IgE that recognise α 1,3 core fucose usually also recognise core N-glycan bound xylose due their presence in the same glycan molecules (Aalberse et al., 1981).

In contrast, α -Gal-specific IgE is directly responsible for allergic and anaphylactic reactions, as was shown for cetuximab (reviewed in Platts-Mills et al., 2021). While anti- α -Gal IgG and IgM are naturally present in humans due to gut microbiota, IgE triggered by α -Gal sensitisation is responsible for the allergic/anaphylactic reaction (Ailsworth et al., 2025; de la Fuente et al., 2019). Patients with red meat allergy have higher IgG1 and IgG3 anti- α -Gal antibody titres, while non-allergic patients have elevated IgG4 (Kollmann et al., 2017).

In patients with xenotransplants, α -Gal triggers IgG and IgM responses, but not IgA (Galili et al., 1993). Recently, tick feeding was shown to induce a strong IgG response to the α -Gal epitope in both mice and humans (Ailsworth et al., 2025; Hils et al., 2024).

NeuGc is another antigenic epitope, for which IgE antibodies are not detected, and differing titres of IgA, IgG, and IgM antibodies are naturally present in humans. The combination of these antibodies – together with the incorporation of NeuGc into human tissues – is responsible for (chronic) inflammation and resulting symptoms (Hutton et al., 2025; Padler-Karavani et al., 2008).

From a clinical perspective, IgE antibodies against CCDs may yield false-positive allergy test results because they bind structurally similar glycans attached to various molecules, particularly in the case of anti- α 1,3 core Fuc found in plant allergens. Failure to recognise this phenomenon can result in an incorrect diagnosis and inadequate treatment (Ebo et al., 2004; Mari, 2002).

For a long time, anti-glycan antibodies were considered clinically insignificant, as proteins are considered the main allergenic epitopes. However, recent findings of type I allergies, which are caused by glycan structures, changed the view of this problem (Commings et al., 2009). Detailed insights into red meat allergy driven by IgE antibodies targeting the α -Gal have revealed that carbohydrate-mediated reactions may cause life-threatening systemic anaphylaxis.

Sensitisation to α -Gal after a repetitive tick bite can trigger anaphylaxis or other allergic symptoms upon the administration of α -Gal-containing drugs (as described for the chimeric human-mouse monoclonal antibody cetuximab) (Chung et al., 2008). These new understandings challenge previous beliefs and confirm the significant clinical importance of specific carbohydrate allergens.

α 1,3-core fucose (α 1,3-core Fuc)

Of the many possible types of glycan fucosylation, only one belongs to CCDs. Besides O-glycans, N-glycans can be fucosylated either at the core as α 1,6-core Fuc, a characteristic feature of mammals, and α 1,3-core Fuc found mainly in plants and insects – and additionally as a terminal modification of N-acetylgalactosamine (GalNAc) in the N-acetyllactosamine (LacdiNAc, GalNAc β 1-4GlcNAc) structure, incorporating an α 1,3 fucose or the Lewis X epitope found also in mammals. α 1,3-core Fuc in N-glycans is the sole fucose-containing CCD and is well-documented in invertebrates. It has been identified, in combination with the α 1,6-core Fuc attached to N-glycans, for example, in mosquitoes and honeybees (Hykollari et al., 2019; Kubelka et al., 1993, 1995; Kurz et al., 2016). Based on previous research, the main honeybee venom allergens with α 1,3-core Fuc were identified as phospholipase A2 (Api m 1), hyaluronidase (Api m 2), and icarapin (Api m 10) (summarised in Burzyńska and Piasecka-Kwiatkowska, 2021). Other identified molecules, for example, basic peptide melittin (Api m 4), dipeptidylpeptidase IV (Api m 5) (Blank et al., 2010), Api m 6 (Michel et al., 2012), and vitellogenin (Api m 12) (Blank et al., 2013), seem to be protein-based allergens (Köhler et al., 2014). Phospholipase A2 from *Apis mellifera* displays core-difucosylated N-glycans (thus containing the immunogenic α 1,3-core Fuc) along with terminal fucosylation (Kubelka et al., 1993; Staudacher et al., 1992). Glycans with multiple antennae, which have unusually attached fucose, were also described (Hykollari et al., 2019). The study of Seismann et al. (2010) explored allergenic cross-reactivity of hyaluronidases (Api m 2 and Ves v 2a/b). Screening venom-sensitive sera revealed that Api m 2

has allergenic significance beyond carbohydrate epitopes. In contrast, Ves v 2b primarily exhibits carbohydrate-based reactivity, raising questions about its role in allergic reactions.

Tick glycans containing α 1,3-core Fuc were shown to be involved in the tick-pathogen relationships. The significance of α 1,3-core Fuc was highlighted by Pedra et al. (2010), who demonstrated that α 1,3-core fucosylated glycans are crucial for the survival of *Anaplasma marginale*, the bacteria responsible for anaplasmosis, in ticks. They also found that this bacterium modifies the expression of three α 1,3-fucosyltransferases. Furthermore, silencing fucosyltransferases significantly decreases the colonisation of tick cells by *Anaplasma* (Pedra et al., 2010). MALDI TOF-TOF analysis of SG (salivary gland) from fed nymphs revealed N-glycans with m/z values corresponding to α 1,3-core Fuc. Protein extracts from SGs and midguts also showed a strong signal after western blot with anti-HRP (horseradish peroxidase) antibodies that recognise the α 1,3-core Fuc (Pedra et al., 2010). Hebert et al. (2017) assume that AmOmpA, *A. marginale* outer membrane protein A, targets fucosylated residues on tick ISE6 cells. The hypothesis was supported by Vimoniš et al. (2022) in the *D. andersoni* primary midgut cell culture system. The authors showed that expression of tick α 1,3-fucosyltransferases A1 and A2 is regulated by *A. marginale* infection of midgut cells. Moreover, the amount of α 1,3 core Fuc, along with the sensitivity of uninfected *D. andersoni* gut cells, was lowered after the silencing of α 1,3 fucosyltransferase A2.

Detailed analysis of α 1,3-fucose and α 1,6-fucose attached to the core of N-glycans was done in partially fed *Ixodes ricinus* females (Vancova and Nebesarova, 2015). A unique combination of fluorescence and scanning electron microscopy localised α 1,3- and α 1,6-core Fuc on the basement membranes of acini types II and III, as well as in the small secretory granules within the cytoplasm of "F" cells. Nevertheless, only α 1,6-core Fuc was detected in secretory granules of acini type II, the place from which the active molecules are released to the host during the feeding.

The N-glycome of *Amblyomma americanum* salivary glands and carcasses was shown to display a high proportion of fucosylated glycans, with both core α 1,3- and core α 1,6-linked Fuc observed, occasionally appearing in difucosylated forms (Park et al., 2020).

Proteins bearing α 1,3-core fucosylated N-glycans thus seem to be evolutionarily conserved. They present similarly to other arthropods and may potentially be excreted to the host. At the same time, it was shown that tick-borne pathogens co-evolved utilising this epitope present in the vector but not in the hosts.

Galactose- α 1,3-galactose (α -Gal)

Generally, the Galactose- α 1,3-galactose is a saccharide that is attached terminally to N-glycans by the α 1-3 galactosyltransferase that attaches Gal to the N-acetyllactosaminyl group (Gal β 1-4GlcNAc-R) (Galili et al., 1988). α -Gal is an oligosaccharide widely present in glycoproteins/glycolipids of non-primate mammals, certain bacteria, and parasites, including ticks (summarised in Wilson et al., 2024), but it is absent in humans, apes, and Old-World monkeys because of the inactivated α 1-3 galactosyltransferase. For that reason, α -Gal appears foreign to the immune systems of humans, apes, and Old-World monkeys.

In ticks, the α -Gal epitope was described in species all over the world from the genera *Amblyomma* (Araujo et al., 2016; Crispell et al., 2019; Murangi et al., 2022; Park et al., 2020), *Haemaphysalis* (Chinuki et al., 2016), *Hyalomma* (Mateos-Her-

nandez et al., 2017; Valcárcel et al., 2024), *Ixodes* (Hamsten et al., 2013), and *Rhipicephalus* (Mateos-Hernandez et al., 2017; Murangi et al., 2022). Its presence in ticks could cause α -Gal syndrome in humans upon tick feeding. AGS is triggered by tick bites, which introduce α -Gal into the body and sensitise the immune system. After this, eating red meat or exposure to products containing α -Gal can cause IgE-mediated allergic reactions (Van Nunen et al., 2009). A key study that fills a major gap in understanding how tick bites lead to delayed allergic reactions to mammalian meat demonstrates that *Ixodes scapularis* ticks endogenously synthesise α -Gal. The heterologous expression of identified galactosyltransferase genes *b4galt7*, *a4galt-1*, and *a4galt-2* induced α -Gal synthesis, and gene knockdown experiments in ticks demonstrated that these genes are essential for endogenous α -Gal production (Cabezas-Cruz et al., 2018).

Given the typical course of anaphylactic reactions, AGS is delayed by 1 to 6 months after tick exposure; moreover, research indicates that IgE levels specific to α -Gal rise with increasing numbers of tick bites (Hashizume et al., 2018). An allergic reaction to α -Gal can also be caused by ticks that do not specialise on humans as hosts, such as pigeon ticks (*Argas* spp.) (Rolla et al., 2018).

The range of clinical symptoms of AGS in humans is wide, spanning from the asymptomatic course, through mild symptoms, to severe anaphylactic shock. Many sensitised individuals remain asymptomatic or exhibit isolated gastrointestinal problems; others report urticaria, skin pruritus, and/or shortness of breath/coughing/wheezing (Binder et al., 2023; summarised in Commins, 2020). In certain individuals, the allergic reaction can lead to life-threatening anaphylaxis (Commins and Platts-Mills, 2009; Commins et al., 2009), and a fatal case only four hours after eating beef has already been described (Platts-Mills et al., 2025). This case also shows the importance of severe abdominal pain as a sole recognisable symptom of the severe anaphylactic reaction. AGS is also connected with the non-allergic symptoms. AGS, characterised by increased IgE levels against α -Gal, appears to be associated with cardiac diseases. Notably, it is associated with a higher risk of developing atherosclerotic plaques (Vernon et al., 2022; Wilson et al., 2018, 2019). Additionally, patients with AGS who receive bovine or porcine valves face a greater mortality risk compared to those who receive mechanical valves (Hawkins et al., 2016). AGS is regarded as the first allergic disease caused solely by a carbohydrate, triggering IgE-mediated allergic reactions.

α -Gal epitope was identified in vitellogenins and α -2-macroglobulin protein groups of *I. ricinus* larvae and adults, including in saliva (Apostolovic et al., 2020). Antibodies against α -Gal recognised this epitope in SG of *A. americanum* and

I. scapularis. Moreover, α -Gal-containing protein extracts activated basophils from patients allergic to red meat (Crispell et al., 2019). An unusual combination of α -Gal and core-Fuc, α -Gal and Xyl, and di α -Gal structures were identified in SG of *A. americanum* (Park et al., 2020). Because SGs contain high levels of α -Gal, the authors also suggest that α -Gal may mimic proteins secreted into non-primate hosts, with α -Gal potentially acting as an allergy sensitizer in the atypical human host (Contreras et al., 2023; Park et al., 2020). Female and male *A. americanum* ticks fed on human blood showed significantly higher α -Gal levels in their salivary glands than those fed on bovine, rabbit, or dog blood (Maldonado-Ruiz et al., 2023). Moreover, α -Gal levels increase over the course of feeding, reaching a peak within 3 days. Large tick-to-tick variation and differences between various field-collected ticks of α -Gal levels were observed. It was reported that host-derived factors may modulate glycosylation patterns in ticks, which could help explain the differences in sensitisation observed in α -Gal syndrome (Maldonado-Ruiz et al., 2023). Cabezas-Cruz et al. (2018) also demonstrated that silencing of galactosyltransferases responsible for α -Gal attachment (*b4galt7*, *a4galt-1*, and *a4galt-2*) impaired tick feeding. Moreover, α -Gal levels increased during *Anaplasma phagocytophilum* infection. The authors conclude that ticks can produce endogenous α -Gal, and its expression is needed for tick feeding and controlling bacterial infection.

The presence of host protein (immunoglobulins), adsorbed in the tick midgut and subsequently secreted into the tick saliva, supports the idea that some of the α -Gal in tick saliva comes from a mammalian blood meal (Jasinskas and Barbour, 2005). Endogenous production of α -Gal and incorporation of α -Gal-containing constituents from a mammalian blood meal were also concluded in *I. ricinus* (Fischer et al., 2020). α -Gal production together with the responsible galactosyltransferases was shown in *I. scapularis*, together with the confirmation of their importance for tick physiology (Cabezas-Cruz et al., 2018).

N-glycolylneuraminic acid (Neu5Gc)

Sialic acids are an abundant terminal modification of glycans in mammals and higher Eukaryotes; among them, N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid are the major forms (Lewis et al., 2022). The hydroxyl group that distinguishes Neu5Gc from Neu5Ac is located on the glycolyl part of the substituent attached to the nitrogen at position C5. The conversion of Neu5Ac to Neu5Gc is catalysed by CMP-N-acetylneuraminic acid hydroxylase (Fig. 2), which is mutated in humans; therefore, they are unable to synthesise it (summarised in Angata and Varki, 2002). For that reason, Neu5Gc is highly immunogenic in several ways.

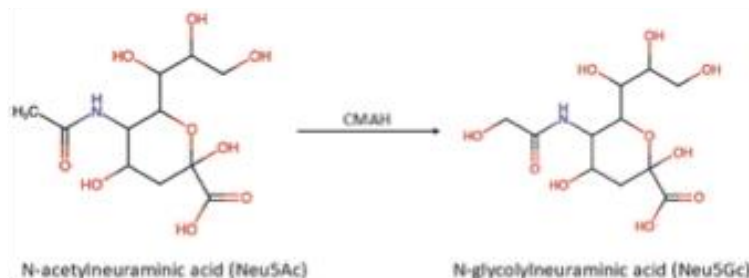


Fig. 2. The chemical structures of Neu5Ac and Neu5Gc. Schema for the enzymatic conversion of Neu5Gc from Neu5Ac via the action of CMP-N-acetylneuraminic acid hydroxylase, an enzyme which is inactive in humans.

Firstly, due to the dietary uptake, Neu5Gc is displayed on human cells. Neu5Gc is present in human tissues in minimal amounts due to the consumption of red meat, milk products, etc. Mammalian-derived glycoproteins are absorbed into the human body via micropinosis (Banda et al., 2012; Bardor et al., 2005; Malykh et al., 2001), digested, and amino acids, as well as saccharides, are subsequently metabolically incorporated into newly synthesised glycans (Banda et al., 2012; Bardor et al., 2005; Bergfeld et al., 2012; Samraj et al., 2015; Tangvoranuntakul et al., 2003). Therefore, human tissues present a "foreign" but cell-bound antigen. Secondly, humans naturally develop circulating anti-Neu5Gc antibodies that arise due to persistent exposure to dietary Neu5Gc and possibly through microbial presentation of Neu5Gc-containing glycans (summarised in Altman and Gagneux, 2019). Thirdly, when anti-Neu5Gc antibodies bind Neu5Gc, as a foreign antigen, this coexistence seems to worsen diseases mediated by chronic inflammation, like cancer, cardiovascular diseases, and autoimmunity (summarised in Alisson-Silva et al., 2016; Dhar et al., 2019; Okerblom and Varki, 2017; Samraj et al., 2014, 2015; Varki, 2017).

In cancer tissues, Neu5Gc was found at high levels (Malykh et al., 2001; Samraj et al., 2014, 2015) and seems to be a potential biomarker in oncology (Cao et al., 2022; Teng et al., 2022). The dietary source, antigen properties of the epitope itself, and ubiquity/abundance on the cell surface render Neu5Gc a distinctive antigen, deserving further attention and investigation.

In ticks, researchers identified traces of N-glycans containing Neu5Gc and Neu5Ac in the gut of partially fed *I. ricinus* females using MALDI TOF/TOF (Vancova et al., 2012). Further MS/MS analysis showed the presence of the complex glycans containing two antennae with one or two Sialic acids (Sias) and three antennae with one Sia. Localisation of NeuGc using immunolabelling with gold particles showed its presence in the basement membranes and cytosolic structures of SG acini, as well as in digestive cells and the gut lumen of partially fed ticks. Considering the structure of glycans, their placement within tick tissues, and the type of tick samples, the authors hypothesise about the host origin of the identified glycans. The host-origin theory of most of the sialic acid in ticks was supported by Sterba et al. (2014), who quantified Sia levels in tick tissues. Using a commercial Sia quantification kit, the total Sia concentration was determined in the gut, SG, and OV, expressed as mmol Sia per gram of protein. Conversely, the enzymatic assay indicated that the amount of tick-origin Sia was approximately 1,000 times lower. By comparing the total Sia amount with the metabolically produced one, the authors conclude that the majority of the Sia in tick tissues is of host origin (Sterba et al., 2014). Trace amounts of terminal NeuGc attached to the terminal galactose in male SGs were also shown in *A. americanum* (Park et al., 2020). The enzymes necessary for NeuAc production were identified in *I. ricinus* and *I. scapularis*, and the expression and activity of sialyltransferase needed for Neu5Ac attachment to glycans was confirmed. However, the presence of CMP-N-acetylneuraminic acid hydroxylase necessary for Neu5Gc production was not confirmed in ticks (Sterbova et al., 2026). Thus, the exact role of sialylated glycoproteins in ticks remains to be elucidated.

β 1,2-xylose

Generally, xylose is a typical CCD found in plants that does not occur in human and mammalian glycans (van Ree et al., 2000). Therefore, it is immunologically foreign to those organisms and, in sensitised patients, can cause an increased IgE anti-

body titre. Structurally, xylose is attached in N-glycans in the position β 1,2- to the core Man, and it could accompany core α 1,3-fucose or α -Gal epitope.

In invertebrates, Xyl, together with core α 1,3- and α 1,6-fucose, has been reported in *Schistosoma* (Khoo et al., 2001; Smit et al., 2015) and in snails (Lommerse et al., 1997). Xyl-syltransferases responsible for the attachment of Xyl residue were identified in *C. elegans* (Hwang et al., 2003) and in *D. melanogaster* (Lee et al., 2013; Wilson, 2002a, b).

The presence of β 1,2-xylose in ticks was reported by Crispell et al. (2019), who did MS profiling of glycans in saliva and salivary glands of various North American tick species. The structures containing core-mannose pentasaccharide modified by Xyl were found in *I. scapularis*, *A. americanum* and *A. maculatum* in unfed and partially fed females and also in the saliva of these species. A deep MS analysis of *A. americanum* N-glycome revealed the presence of complex glycans, with Xyl attached to the core-mannose in 8% of the total N-glycan profile in unfed females, 5% in females fed for five days, and 16% in males. A unique combination of Xyl and α -Gal was also detected (Park et al., 2020).

Conclusion

Recent research has highlighted the significant immunological functions of glycan epitopes in ticks. These unique carbohydrates, absent in humans, can trigger immune responses, occasionally leading to allergic reactions or false-positive allergy-test results. Moreover, they play critical roles in the complex triad of tick-host-pathogen interactions, potentially affecting pathogen transmission, tick survival, and host sensitisation to allergens. Evidence of endogenous synthesis and host-derived glycan incorporation demonstrates the dynamic nature of tick glycomes. Although previously considered clinically insignificant, these glycan structures are now recognised as crucial elements in allergy and immunology, with promising potential as future vaccine or diagnostic targets.

An important aspect of CCD epitopes' presence in tick tissues and saliva is their immunogenicity, which is in contradiction to the need of the tick to lower the defence responses of the host during blood feeding. We hypothesise that the presence of various inhibitory signals, like the presence of sialylated glycoproteins of tick or host origin (summarised by Vechtova et al., 2018; Wickramasekara et al., 2008), may be one of the reasons why ticks transport undigested host proteins during blood feeding from the gut to the salivary glands and saliva as a form of molecular mimicry.

To better understand glycan-based relationships within the tick-host-pathogen triangle, further studies spanning multiple levels are needed. The molecular biology-based level could focus on the identification and characterisation of glycan-modifying enzymes, as well as the description of corresponding glycosylation pathways, as in the work of Cabezas-Cruz et al. (2018). Mass spectrometry analyses characterising glycomes from various tick species, their developmental stages, and organs, could extend knowledge at the glycomics-based level – as in the work of Park et al. (2020). Finally, exploring the ecological and physiological factors underlying host- and tick-dependent expression of CCDs could shed light on differences in CCD expression, as illustrated by the case of α -Gal reported by Maldonado-Ruiz et al. (2023). Furthermore, detailed characterisation of tick cell lines, including their glycomic profiles, would enhance their use as models for tick research (Bell-Sakyi et al., 2018).

Ongoing research is crucial for deepening our understanding of tick-derived glycan biology, developing effective strategies to prevent tick-borne diseases and manage allergies, and advancing novel, animal-free medications for patients with AGS (Altomari et al., 2025). The importance of such research is evidenced by the alarming increase in the number of cases of anaphylactic reactions. In the US, hundreds of thousands of cases are reported (Thompson et al., 2023), while substantial case numbers are also documented in Asia, Australia, or Europe, including the Czech Republic (Müller et al., 2023), Austria (Pisazka et al., 2019), Germany (Fischer et al., 2017), Poland (Brzozowska et al., 2021), or Italy (Villalta et al., 2017). The reason for this rapid increase is not yet known (van Nunen, 2025). Anti- α -Gal vaccines were proposed to lower anti- α -Gal IgE production and basophil activation (summarised in Propst and Thompson, 2025; Saunders et al., 2024), as well as to serve as a pan-vector and pan-pathogen vaccine (summarised in Hodžić et al., 2020). Until such a vaccine is developed, avoidance of tick bites, together with the elimination of red meat and beef-originating foods, is the only means to prevent AGS (Commins et al., 2011; summarised in Propst and Thompson, 2025; Young et al., 2021).

In recent years, awareness and clinical relevance of cross-reactive carbohydrate determinants (CCDs) have also increased within the Czech Republic, and anaphylactic reactions have been described in Czech patients (Müller et al., 2023). Several clinical laboratories now offer testing for a range of CCD epitopes, including facilities such as Thomayerova Hospital, St. Anne's University Hospital, the General University Hospital in Prague, and selected EUC Clinics.

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Ethical aspects and conflict of interest

The authors have no conflict of interest to declare.

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Paper VI

Click chemistry reveals sialylated glycoprotein biosynthesis in *Ixodes ricinus* ticks and tick-derived cells

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Click chemistry reveals sialylated glycoprotein biosynthesis in *Ixodes ricinus* ticks and tick-derived cells

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ABSTRACT

Sialic acid commonly decorates the surface of cells and secreted proteins of Eukaryotes. It plays roles in cell signalling and adhesion, affects immune reactions and alternative complement pathways, and neurogenesis. While sialic acid is a saccharide present in high abundance in vertebrates, its minor representation was reported in arthropods and among medically necessary parasites.

Ixodes ricinus tick is a significant vector of pathogens causing severe infections in humans and livestock. It has been known mainly through indirect evidence that sialylated glycoproteins are present in tick tissues. As a blood-feeding parasite, the host's blood, full of sialylated molecules, could be the source of such glycoproteins within the tick's body. To detect solely the tick sialylated glycoproteins, we used an azide-modified precursor of sialic acid to be metabolically incorporated by the tick cell line IRE/CTVM20 originating in this tick or by ticks if the corresponding biosynthetic pathway is present and active.

Our *I. ricinus* genome screen revealed two sialyltransferase genes, which were expressed in all ticks' life stages, and we confirmed sialyltransferase activity in tick cells. We combined *in vitro* feeding along with Click chemistry to track the presence of sialylated glycoproteins from fed female ticks through their eggs to the larvae. Similarly, sialylated glycoproteins were produced by the IRE/CTVM20 cell line. Thus, we confirmed the ability of ticks to produce their own sialylated glycoproteins in addition to those originating from the host blood.

1. Introduction

Protein glycosylation is a biochemical process in which a mono- or oligosaccharide is covalently attached to a target molecule. This process is crucial for various biological functions, such as protein folding and stability, cell-to-cell communication, immune response, and host-pathogen recognition (summarised in [1]).

Glycans consist of various monosaccharide subunits and can often contain complex branched chains. They are found throughout the evolutionary chain, from simple bacteria to complex organisms, including green plants, arthropods, and humans. Sialic acid (Sia) is the unified name for a group of negatively charged hexose monosaccharides characterised by a nine-carbon skeleton, in Eukaryotes mostly N-acetylneuraminic acid (Neu5Ac) or N-glycolylneuraminic acid. Sia biosynthesis involves several intermediate steps. First, the precursor uridine diphosphate-N-acetylglucosamine is processed through N-

acetylmannosamine, N-acetylmannosamine-6-phosphate, Neu5Ac-9-phosphate, and Neu5Ac into the donor monosaccharide cytidine monophosphate-N-acetylneuraminic acid (CMP-Neu5Ac) [2], which is linked to the target glycan by sialyltransferase.

On the one hand, it has long been known that sialylated glycoproteins occur frequently and in high concentrations in vertebrate tissues, where they are present on the surface of many cell types or are secreted (summarised in [3]). However, during the past years, it has been shown that they also exist in specific cases in arthropods and bacteria [4,5]. Sia plays numerous essential roles in vertebrates: cell signalling, cell adhesion, influencing the alternative complement pathway [6,7], and other parts of the immune system [8], neurogenesis [9] or protection of the organism from invading pathogens [10,11].

On the other hand, only a limited representation of sialylated glycans was reported in invertebrates. Among them, insects remain the best-studied group. Cicada *Philaenus spumarius* was one of the first insects

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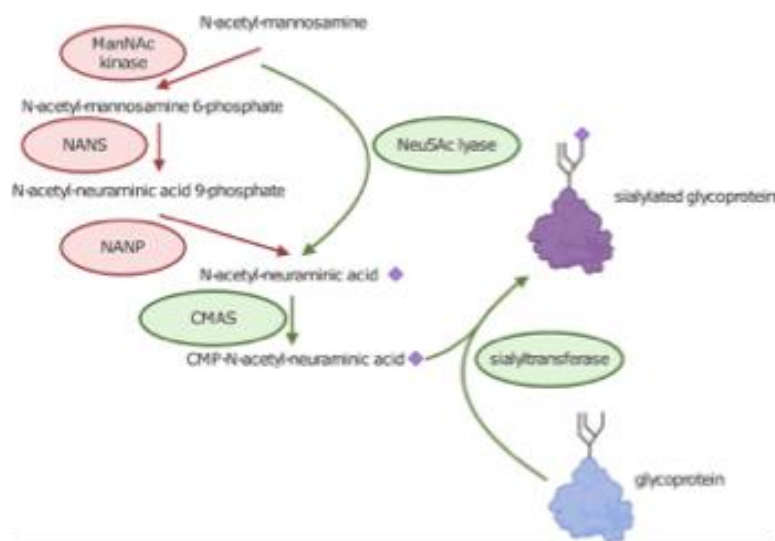


Fig. 1. Sia metabolic pathways. Sia can be produced from ManNAc either by ManNAc kinase, *N*-acetylneuraminate synthase (NANS), and sialic acid phosphatase (NANP) or directly by Neu5Ac lyase. While ManNAc kinase, NANS, and NANP were not found in the *I. ricinus* published genomes, a Neu5Ac lyase-like protein was identified. Furthermore, CMP-Neu5Ac synthetase and two putative sialyltransferases were identified as well, thus completing the metabolic pathway from ManAc/ManAz to Neu5Ac/Neu5Az and to sialylated glycoproteins.

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in which sialylated structures were identified [12]. Glycans with α 2,6-linked Neu5Ac and α 2,8-linked polySia were shown to be present in *P. spumarius* concrement vacuoles of the Malpighian tubules, and Sia was also confirmed in larvae [12]. In the honeycomb moth *Galleria mellonella*, Sia was detected in larvae in its lactonized form of Neu₅Gc₇Ac₂ [13]. In *Bombyx mori*, α 2,6-sialyltransferase activity was detected in various organs in different life stages [14]. Sia was also detected in the insect model organism *Drosophila melanogaster* [15], and glycoprotein sialylation was later confirmed using metabolic labelling [16]. During its embryonic development, an α 2,6-sialyltransferase is expressed [17] and plays a dominant role in the development and function of the *Drosophila* neural system [18]. In mosquito *Aedes aegypti* tissues, Sia and sialylated structures were detected, and the responsible α 2,6-sialyltransferase was identified. Furthermore, Sia was important for recognising mosquito salivary gland tissues by the dengue virus [19]. In contrast, sialyltransferase activity was not detected in various insect cell lines [20,21].

Ticks are, after mosquitoes, the second most important vector of pathogens, in terms of the number of species they transmit [22]. They live a parasitic lifestyle on hosts, whose blood is their only food. Blood feeding on the host is required for all life stages transformation, i.e. from larva to nymph, from nymph to adult, and for egg laying [23].

Protein glycosylation is vital in the relationship between the tick, its host, and tick-borne microorganisms. For example, α 1,3-core fucosylation is necessary for the infection of *Ixodes scapularis* by the pathogenic bacterium *Anaplasma phagocytophilum* and its transmission to the host [24]; further information was reviewed in [25]. Ticks, similarly to other invertebrates, usually produce high-mannose or hybrid-type N-glycans [25]. The ability of tick haemolymph proteins to specifically bind Sia and the presence of Sia-binding proteins in ticks and tick cell lines were reported decades ago (summarised in [26]). Dorin M, a Sia-specific lectin, was characterised in the soft tick *Ornithodoros moubata* [27,28]. It is mainly expressed in salivary glands, and due to its ability to bind sialic acid, its potential role in tick innate immunity and pathogen transmission is discussed [29].

Sia in ticks was detected in salivary glands using Sia-specific lectins

MAA II and SNA [30]. Later, mass spectrometry analysis confirmed the presence of sialylated glycans in tick gut and salivary glands, and their specific localisation was observed using electron microscopy in the basement membrane of salivary glands [31]. However, the structure of identified glycans did not correspond to the tick origin due to their complex nature.

The possibility of host origin of sialylated molecules was proved via quantification of total Sia compared to bioorthogonally labelled sialylated molecules [32]. Experiments showed that most of the Sia in adult *I. ricinus* females and tick cell cultures originates in the host. Small amounts of tick-origin sialylated glycans were found in salivary glands and ovaries and were localised in oocytes [32]. Bioorthogonal chemistry in combination with Click chemistry has become a powerful tool in recent years for studying post-translational protein modifications, including glycosylation [33]; it has also been used to study tick-borne pathogens and tick glycosylation [34].

Based on our previous results, we aimed to confirm the ability of *I. ricinus* ticks and tick cell lines to produce sialylated glycoproteins and verify their presence in tick samples.

Firstly, we examined the *I. ricinus* genome for the presence of enzymes involved in CMP-Neu5Ac biosynthesis (KEGG pathway M00922, [35]), which is a substrate for sialyltransferase (SiaT). Further, the expression of the identified enzymes in transcriptomes was verified. Then, we assessed the sialyltransferase activity in the tick cell line model. Finally, we used bioorthogonal chemistry to track the sialylated molecules of tick origin in the tick cell line and adult tick females, eggs, and larvae. To verify the production of sialylated proteins, we used metabolic labelling with a precursor of sialic acid, *N*-azido-mannosamine (ManNAz, used as a tetraacetylated precursor), which is metabolised to *N*-azido-neuraminic acid (NeuNAz, [36,37]), followed by the Click reaction [38], to distinguish the tick sialylated glycoproteins from sialylated proteins originating in the host blood. ManNAz was added to blood for *in vitro* artificial tick feeding or to tick cell line growth media. Tick females fed on ManNAz-spiked blood were left to lay eggs, which allowed us to trace NeuNAz from fed females through eggs to larvae. We confirmed the presence of sialylated molecules of tick origin in the IRE/

CTVM20 tick cell line and tissues using fluorescence microscopy and western blotting.

2. Materials and methods

All procedures were performed at ambient temperature if not otherwise stated.

2.1. Identification of sialic acid biosynthesis pathway members and sialyltransferase genes in the *Ixodes ricinus* genome

The presence of enzymes participating in Sia metabolism and attachment to proteins was searched within the published *I. ricinus* genomes and transcriptomes. First, the Sialic acid biosynthetic pathway members, including ManNAc 6-kinase (MNK EC:2.7.1.60), *N*-acetylneuraminase (*N*-acetylneuraminic acid-9-phosphate) synthase (NANS EC:2.5.1.56/57), and sialic acid (*N*-acetylneuraminic acid-9-phosphate) phosphatase (NANP EC:3.1.3.29) were searched for, see Fig. 1. As no genes bearing similarity to the known enzymes of this pathway were found, we searched for an *N*-acetylneuraminase lyase-like enzyme (EC:4.1.3.3) potentially performing an alternative reaction of Sia synthesis from ManNAc. Additionally, two more enzymes, completing the pathway, were searched for: CMP-sialic acid synthetase (CMAS EC:2.7.7.43) and sialyltransferases (SiaT, EC:2.4.3.1 and EC: 2.4.3.6). For more information on the pathways, see the Kyoto Encyclopedia of Genes and Genomes (KEGG), which includes three other tick species (namely *Dermacentor silvarum*, *Rhipicephalus sanguineus*, and *R. microplus*, [35]). The searched data included 1) our Oxford Nanopore Technology polished long read assembly (BioProject ID PRJNA1028502); 2) previously published assemblies of two *I. ricinus* isolates (BioProject ID PRJNA816462); 3) and our comprehensive transcriptomic data from multiple life stages of *I. ricinus* (GenBank ID GIDG00000000, [39]).

For the *N*-acetylneuraminase lyase-like enzyme, sequences were identified in EukProt v3 [40] using hmmssearch of the HMMER suite v3.3.2 [41] and the PTHR12128 Panther database Hidden Markov Model (HMM) profile. These sequences were complemented with reference *N*-acetylneuraminase lyase sequences from KEGG (K01639) and reannotated with kofamscan v1.3.0 with KEGG profiles v2022-04-03 [42]. Following clustering with MMseqs2 (easy-linclust-min-seq-id 0.5-c 0.5-cov-mode 1-cluster-mode 2; [43]), the sequences were aligned with Muscle (v5.1; REF) and trimmed with trimAl (v1.2rev59, parameter-gt 0.3; [44,45]). The Maximum Likelihood tree was calculated using FastTree (v2.1.11, substitution model WAG; [46]) or IQ-TREE (v2.3.4, substitution model WAG + I + R10, 1000 ultra-fast bootstrap replicates and 1000 SH-like aLRT replicates; [REF PMID: 32011700]). Finally, the dataset was manually pruned to retain curated homologs of *N*-acetylneuraminase lyase and 4-hydroxy-tetrahydrodipicolinate synthase, two of which showed a phylogenetic relationship in our analyses, and reanalysed as above.

Annotated ST3Gal from *Clona savignyi* (GenBank ID CAF25172.1) and ST6Gal from *Varroa destructor* (GenBank ID ADR32072.1) were used to search the mites and ticks nr protein database. The best hits found in the *I. scapularis* genome were used to find their orthologs in the *I. ricinus* transcriptome. Both transcripts were used to search for their homologs in the genomic context in the *I. ricinus* genome assembly. The best hits were extracted, and their exon-intron structure was identified by mapping the transcripts to the extracted genomic contigs using Bowtie2 in Geneious Prime v2025.0.2.

Protein alignments were prepared from translated TSA sequences and the identified closely related proteins in Geneious Prime 2024.0.7 using the BLOSUM62 substitution matrix and affine gap costs with a gap open penalty of 12 and a gap extension penalty of 3 (<https://www.genieious.com>).

2.2. Expression of sialyltransferase gene in *Ixodes ricinus* genome

ST3Gal and ST6Gal transcripts presence and expression were verified in transcriptomic data from multiple life stages of *I. ricinus* (GenBank ID GIDG00000000, [39]), which includes eggs, larvae, fed nymphs, unfed females, and fed females; thus, this dataset also comprises the early life-stages of *I. ricinus*. The read counts were normalised across libraries to produce a catalogue of stage-specific transcripts and are expressed as normalised read counts.

Next, RNA-seq data retrieved from the Sequence Read Archive published in [47] (BioProject ID PRJNA395009) were used to search for ST3Gal and ST6Gal expression profiles across different *I. ricinus* life stages (unfed and partially fed nymphs, males, and unfed and partially fed females). Raw reads were aligned, and counts were extracted using Salmon v1.10.1 [48]. Cross-sample normalisation by library depth and composition was done using DeSeq2 v 1.46.0 [49].

2.3. Tick cell lines

The *I. ricinus* embryo-derived tick cell line IRE/CTVM20 provided by the Tick Cell Biobank [50,51] was cultured in Leibovitz' L-15 medium supplemented with 20% foetal bovine serum (Cytiva), 1% antibiotics/antimycotic mix (all Biosera), 2 mM glutamine, and 10% tryptose phosphate broth (Merck) in 2 ml volumes in sealed, flat-sided cell culture tubes (Nunc, Rochester, USA) at 28 °C as described before [52].

2.4. Ticks

Ixodes ricinus ticks were collected during the summer by flagging in České Budějovice. The ticks were kept in the tick-rearing facility at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences. Here, the ticks were stored in glass containers with vents at high relative humidity and a temperature of 26 °C.

2.5. Blood

For *in vitro* feeding, fresh bull blood collected at a slaughterhouse in České Budějovice was used. The blood was treated with an anti-clotting agent, heparin (lithium salt, Merck) at a concentration of 15 U/ml and was stored at 4 °C until use.

2.6. Sialyltransferase activity

IRE/CTVM20 cells from one cultivation tube (approximately 10⁶) were centrifuged at 600 RCF and washed with 1 × PBS (137 mM NaCl, 1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 2.7 mM KCl, pH 7.4) three times. The cells were then resuspended with RIPA buffer (0.1% SDS, 1% NP40, 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% sodium deoxycholate), mixed with HALT protease inhibitors (Thermo Fisher Scientific) and sonicated for 15 min on ice. Sonicated samples were then vortexed for 10 min and centrifuged for 10 min at 13000 RCF. The concentration of the proteins was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Sialyltransferase activity was measured with a Sialyltransferase activity kit (R&D Systems) in a 96-well plate. Briefly, the optical density of phosphate standards was measured at 620 nm, and the phosphate standard curve was prepared. A sialyltransferase assay was prepared by mixing 1.25 mM CMP-sialic acid (Merck) as a donor substrate, 2.5 mM Asialofetuin (Merck) as an acceptor substrate, and 10 ng/μl of coupling phosphatase 2. The reaction was initiated by adding IRE/CTVM20 cell lysate in a protein concentration of 1500 ng/μl as a working sialyltransferase solution. α-2,3-Sialyltransferase from *Pasteurella multocida* (Merck) in a 0.1 ng/μl concentration and human medulloblastoma DAOY cell lysate (DAOY HTB-186; American Type Culture Collection; in a protein concentration of 1500 ng/μl in RIPA buffer) were used as positive controls; mammalian cell lysate was used instead of an insect cell line as sialyltransferase activity is naturally

present in mammalian cells contrary to insect cell lines. Negative control and Phosphatase 2 controls were prepared according to the manual instructions. The microplate was incubated at 37 °C for 4 h. Then, Malachite Green Reagent A, deionised water, and Malachite Green Reagent B were added to each well. After 20 min incubation at room temperature, the optical density at 620 nm was measured, and the product formation was calculated according to the manufacturer's instructions. A positive reading (after the subtraction of the blank) served as a confirmation of a detectable sialyltransferase activity, rather than directly comparing the activity between the samples, thus, a statistical analysis was not included.

2.7. Metabolic labelling of tick cell line proteins

Tetraacetylated *N*-azidoacetyl- α -mannosamine (ManNAz, Thermo Fisher Scientific or Click Chemistry Tools) or *N*-(4-pentynoyl) mannosamine (ManNAI, Click Chemistry Tools) dissolved in DMSO (Merck) was added to a tick cell culture to a final concentration of 100 μ g/ml; DMSO in the same volume was added to the control samples as a negative control. Experiments were performed at least in biological triplicates.

Cells were seeded at a density of 10^6 cells per tube in a 4.5 ml medium and cultivated for five days. Next, cells were harvested separately from each cultivation tube and seeded for a fluorescent detection of NeuNAz and a cell viability assay as described below. The remaining cells were pelleted at 600 RCF for 5 min, the supernatant was removed, and the cells were washed three times by resuspending the cell pellet in phosphate-buffered saline, pH 7.4 (PBS) and spinning at 600 RCF for 5 min. The cell pellet was stored at -20 °C until further use. Next, the cell pellet was lysed in 50 mM Tris-HCl with 1% SDS, pH 8.0 and HALT protease inhibitors (Thermo Fisher Scientific).

2.8. Cell viability assay

Harvested tick cells were seeded in a 24-well plate (TPP) at a density of 1.5×10^5 cells/well in 1 ml of cultivation media, sealed with parafilm to prevent excessive evaporation of media and cultivated for one day in an air-tight humid chamber at 28 °C. The following day, alamarBlue Cell Viability Reagent (Thermo Fisher Scientific) was added to a 1 $\times$ working concentration. The blank sample (1 ml of 1 $\times$ alamarBlue in cultivation media) was added to a separate well. The plate was sealed with parafilm, covered with aluminium foil to prevent fluorescence bleaching, and placed in an air-tight humid chamber at 28 °C. The change of the colour indicator was monitored continuously for 13 h after the alamarBlue addition. When a visually detectable colour change from dark blue to purple was observed, the medium was collected, the contained cells were pelleted at 600 $\times$ g for 5 min, and the fluorescence of the supernatant was measured in technical triplicates per sample in a 96-well plate (TPP) using an Infinite M200 Microplate Reader (Tecan). The relative viability of treated cells was related to the viability of DMSO controls and the untreated culture.

2.9. Metabolic labelling of tick proteins — *in vitro* tick feeding

Before use, 5 μ g/ml gentamicin, 1 mM ATP, and 100 U/ml Nystatin (all from Merck) were added to the blood (final blood concentrations). ManNAz was added to a final concentration of 1 μ g/ml or 10 μ g/ml in the blood for metabolic labelling. As a control, blood with DMSO was used.

The preparation of the tick feeding units followed the published work of Kröber and Guerin [53,54] with two differences: the incubation period before *in vitro* feeding was omitted, and the stimulus using animal fur was not used. The glass plate was wrapped with food foil and fixed with adhesive tape. Cellulose papers (Kodak) were placed on the top of this foil and fixed with tape at opposite ends. A silicone mixture containing E4 silicone (Wacker), FL white colour paste (Wacker), DC 200 silicone oil (Fluka/Honeywell), and 150 g/kg hexane (Merck) was

prepared and spread in a 60–80 μ m thin layer over the paper. The mixture was left to dry for 14 h. The feeding units were coated on the bottom side with a thin layer of E4 silicone, pressed onto the membrane, and left to adhere for 4 h. The feeding units were then released with a scalpel, and the film on the bottom side of the membrane was removed. These feeding units were then used with six-well plates (TPP).

To each well of the six-well plate, 3.1 ml of blood was added. The blood was enriched with ManNAz in DMSO to a concentration of 10 μ g/ml, or with a corresponding volume of DMSO in control wells. Ten tick females and five males were placed in each feeding unit, plugged with a breathable bandage (Supplementary File S1). The panel was then placed in a water bath heated to 37 °C and covered to maintain high humidity. The blood was changed every 12 h, and during each change, the panel was cleaned with distilled water and PBS containing Nystatin at a concentration of 100 U/ml. Ticks were fed for 7–9 days in this manner until the spontaneous release of the tick occurred.

After feeding, ticks were surface-disinfected with 70% ethanol for 10 s, washed three times with sterile water, and left at high humidity until oviposition or dissected. Salivary glands, gut, Malpighian tubes, and ovaries were removed from three females partially fed on blood complemented with ManNAz or DMSO. 15 eggs were collected after 3, 7, and 14 days. Larvae were collected and processed immediately after hatching. Experiments were performed in biological triplicates.

2.10. Preparation of tick tissues and cell lysates for SDS-PAGE, western blot analysis, and Click reaction

Sterilised and washed individual tick organs and eggs were homogenised in lysis buffer (100 mM sodium phosphate, 1% SDS, protease inhibitors (Thermo Fisher Scientific) using TissueLyser II homogeniser (Qiagen) at 30 beats/s for 15 min. Subsequently, the samples were sonicated in a sonication bath at 4 °C for 15 min, vortexed for 15 min, and centrifuged at 14,000 RCF for 5 min at 4 °C. The supernatant was pipetted into a new microtube, and protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were kept at -20 °C for further use.

Click reaction with proteins from tick tissues was prepared according to [55], i.e., 50 μ l of tissue lysate containing 50 μ g of proteins was mixed with 382.5 μ l of 100 mM potassium phosphate buffer, pH 7.0. Subsequently, 10 μ l 5 mM Click reactant, 25 μ l deionised water, 7.5 μ l of 1:2 premix of 20 mM CuSO₄ and 50 mM Tris(3-hydroxypropyl)triazolylmethylamine, and 5 mM sodium ascorbate were added. For NeuNAz-containing samples, biotin-alkyne or Alexa 488-alkyne was used; for NeuNAI-containing samples, biotin-azide or Alexa 488-azide was used. The mixture was incubated for 1 h at 37 °C. Click reaction with proteins from the IRE/CTVM20 tick cells was performed according to the manual for the Click-IT Protein Reaction Buffer Kit (Thermo Fisher Scientific).

Both types of samples were then precipitated with the methanol-chloroform technique described in the Click-IT Protein Reaction Buffer Kit (Thermo Fisher Scientific). The dried proteins were then dissolved in 1 $\times$ Laemmli loading buffer with DTT (SERVA).

2.11. SDS-PAGE and Western blotting

Protein extracts from ticks, organs, eggs, larvae, and cells were prepared for SDS-PAGE similarly. An appropriate amount of 4 $\times$ reducing Laemmli SDS sample buffer (Thermo Fisher Scientific) was added to the processed samples. The samples were heated to 75 °C for 5 min and centrifuged at 5000 RCF for 5 min. SDS-PAGE was performed according to the protocol published previously [56]. Protein marker (Protein Marker VI (10–245) prestained (AppliChem) and PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific)) was applied together with the samples to the 12% acrylamide gel. The separation was carried out at 120 V for approximately 75 min.

The gel was then rinsed with distilled water, and blotting was carried

out according to protocol [57]. Tick organs and egg samples were blotted for 12 h at 3 V using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). The blotting of samples from tick cells was carried out for 1 h at 20 V using a semi-dry blotter (Cleaver Scientific, United Kingdom).

After electroblotting, the PVDF membranes were washed with distilled water. First, the membranes were blocked with 5% non-fat milk/PBS for 1 h. After blocking, they were incubated with 5% non-fat milk/PBS with Anti-Alexa Fluor 488 made in rabbit (Vector Laboratories) diluted 1:1000 for 1 h. Subsequently, they were washed 3× in PBS-T for 10 min each. After washing, membranes were incubated with Anti-Rabbit IgG conjugated with Alkaline Phosphatase/Horseradish Peroxidase (Vector Laboratories) diluted 1:1000 in 5% non-fat milk/PBS solution for 1 h. This was followed by washing again with PBS-T 3× for 10 min. Finally, the membranes were split over with 10 ml AP developing buffer (100 mM TRIS, 0.1 mM NaCl, 10 mM MgCl₂) with 100 µl of BCIP/NBT (both final concentration 50 mg/ml in 100% dimethylformamide (BCIP) or 70% dimethylformamide (NBT)) or HRP developing buffer (100 mM Tris-HCl pH 7.2, 0.6 mM 3,3'-diaminobenzidine, 0.01% H₂O₂) and left on the workbench until a signal developed. The membranes were then rinsed with distilled water and left on the bench to dry. Separated proteins were stained using the Blue Silver method [58].

2.12. Preparation of tick tissues and IRE/CTVM20 tick cell line with ManNAz for fluorescence microscopy and Click reaction

Harvested tick cells were seeded in 8-well flexible tissue culture inserts (FlexiPERM slide, Sarstedt) attached to a microscopy slide (5 × 10⁴ cells/well) and cultivated for two more days.

Tick cells were fixed with Roti-Histofix (Carl Roth) for 15 min and permeabilised with 0.25% Triton X-100/PBS for 15 min. Samples were then washed with 1% BSA/PBS for 10 min, and the Click reaction was performed with 1 µM Alexa Fluor 594 Alkyne (Thermo Fisher Scientific) according to the manufacturer's protocol using Click-IT Cell Reaction Buffer Kit (Thermo Fisher Scientific). Subsequently, samples were washed 3× with PBS. After this step, autofluorescence was quenched with ammonium chloride (50 mM NH₄Cl/1% BSA/PBS) 2 × 10 min, and the cells were 3× washed 10 min with 1% BSA/PBS and 3× in PBS and left to dry. Vectashield Mounting Medium containing DAPI (Vector Laboratories) was added, and the cell samples were covered with cover glass. The edges of the glass have been coated with nail polish and stored at 4 °C until further analysis.

Unless otherwise stated, eggs and larvae sections were prepared at RT. First, the samples were washed 3× with PBS for 15 min. Eggs were fixed with 4% formaldehyde/0.1% glutaraldehyde/PBS solution for 60 min, while larvae were fixed for 120 min. Samples were washed 3× for 15 min with PBS/20 mM glycine. 10% gelatine preheated to 37 °C was added, mixed thoroughly, and left for 10 min at 4 °C. The larvae and eggs in the gelatine were then placed in a 2 cm long rectangular form containing Tissue Freezing Medium. In this form, it was deep frozen using liquid nitrogen. After complete freezing, the sample was peeled out, and the part containing the eggs/larvae was cut into 5 µm-thin sections for eggs and 4 µm-thin sections for larvae at −24 °C, which were heat-fixed to microscopy slides. This was followed by permeabilisation with 0.25% Triton X-100/PBS for 20 min. Click reaction was performed using the Click-IT Cell Reaction Buffer kit (Thermo Fisher Scientific) per the manufacturer's protocol with 1 µM Alexa Fluor 488 Alkyne (Thermo Fisher Scientific). The sections were blocked with 3% BSA/PBS for 60 min. After this step, autofluorescence was quenched for 20 min with ammonium chloride (50 mM NH₄Cl/1% BSA/PBS). Next, the sections were washed three times with PBS/1% BSA for 10 min. A 1% BSA/PBS solution containing rabbit Anti-Alexa Fluor 488 (Vector Laboratories, 1:1000 dilution) was applied, with incubation for 1 h. This was followed by washing three times with 1% BSA/PBS for 10 min each. A 1% BSA/PBS solution containing Anti-rabbit DyLight 549 (Vector Laboratories,

1:1000 dilution) was applied, and the sections were incubated for 1 h. In case of lectin labelling, samples were incubated with biotinylated GNL lectin (*Galanthus nivalis* lectin, Vector Laboratories) diluted in 1% BSA/PBS 1:2000 for 1 h, washed 3× for 10 min with 1% BSA/PBS and incubated 1 h with Streptavidin DyLight 488 (Vector Laboratories) in 1% BSA/PBS diluted 1:2000. Sections were washed thrice in 1% BSA/PBS for 10 min and three times in PBS for 10 min. After this step, they were left on the bench for 20 min to dry. Samples were coated with Vectashield Mounting Medium containing DAPI and covered with a coverslip.

2.13. Fluorescence and confocal microscopy

Sections of eggs and larvae were observed under an Olympus Fluoview FV-10i confocal laser scanning microscope and viewed using FV-10i-SW software. The acquired images were processed and edited with the FV10-ASW 1.7 Viewer software.

Samples of tick cells were viewed using an Olympus BX53 (Olympus, Tokyo, Japan) fluorescence microscope equipped with a DP73 camera. Using OLYMPUS cellSens Standard software, images of cells with ManNAz staining were taken at the exposition time of 4–8 ms, images with DMSO control staining were taken at the exposition time of 8–12 ms, and DAPI staining was photographed at the exposition time of 15 ms. Images from the fluorescence microscope were processed using ImageJ software [59].

2.14. Mass spectrometry identification of proteins and prediction of N-glycosylation sites

Protein bands selected from SDS-PAGE gels were excised and processed using Pierce Trypsin Protease (Thermo Fisher Scientific). The resulting tryptic peptides were purified using StageTips containing C18 solid-phase extraction discs (CDS Empore). Purified peptides were dissolved in 60 µl of 3% acetonitrile and 0.1% formic acid and subsequently analysed by LC-MS. Chromatographic separation was performed on an UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) coupled online to a timsTOF Pro mass spectrometer (Bruker Daltonics), as described in [60].

Protein identification was carried out using MaxQuant (version 2.6.3.0) with the integrated Andromeda search engine [61]. Searches were performed against the *Drosophila ricinus* protein database downloaded from UniProt (21 August 2023; 61,426 entries), supplemented with the MaxQuant contaminant database. Default parameters for the TIMS-DDA workflow and Bruker TMS instruments were used, as described in [60]. Trypsin/P was specified as the protease, with up to two missed cleavages allowed. Carbamidomethylation of cysteine was set as a fixed modification, while protein N-terminal acetylation and methionine oxidation were defined as variable modifications. Peptide spectrum matches (PSMs) and protein identifications were filtered using a target-decoy strategy at a false discovery rate (FDR) of 1%. N-glycosylation sites were predicted using NetNGlyc — 1.0 [62].

3. Results and discussion

Sialylated glycans are essential in various cellular processes in Eukaryotes, mainly in the animal kingdom. While Sia is abundantly present in vertebrates, it is less prominent in invertebrates and medically important parasites. The presence of Sia in arthropods has been a topic of debate for a long time; however, Aoki and colleagues provided direct evidence of arthropod sialylated glycans using mass spectrometry analysis in the *Drosophila melanogaster* embryo in 2007 [63]. SiaT was reported in the *D. melanogaster* genome [17] with proven expression and activity, and the importance of sialylated glycans for neural tissues during development [18]. Furthermore, sialylation pathway enzymes are necessary for heat and oxidative stress tolerance and for maintaining the normal levels of voltage-gated sodium channels in the neural system.

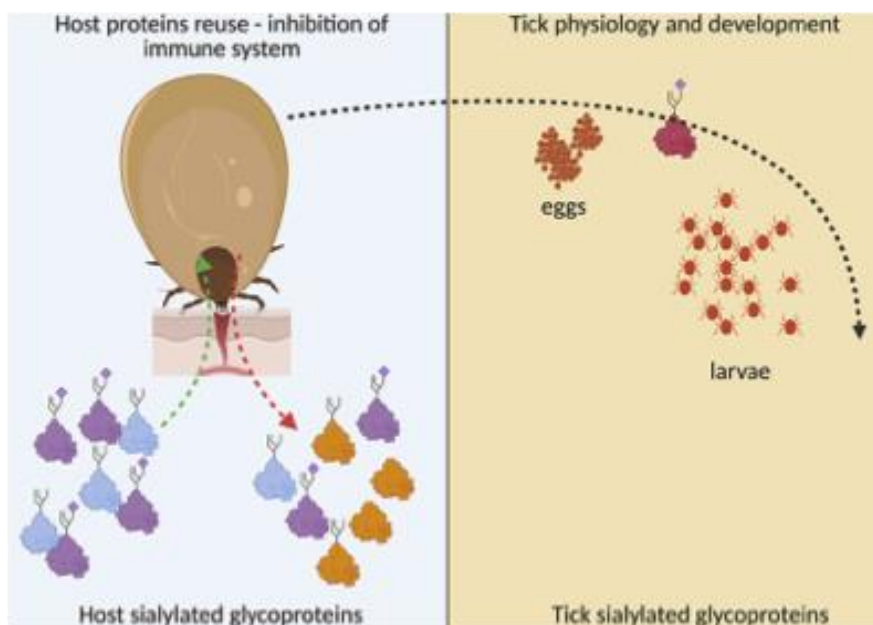


Fig. 2. The two possible roles of sialylated glycoproteins in tick physiology. A) During blood feeding, host non-sialylated (blue) and sialylated (magenta) glycoproteins are uptaken by the tick. To lower the activation of the host immune system, the tick can release the host's sialylated glycoproteins together with its own bioactive molecules (orange) to the host. Possibly, ticks can release their own sialylated glycoproteins into saliva, but this has not been confirmed yet. B) Ticks produce their own sialylated glycoproteins, primarily in the embryonal and larval stages, which are required for embryonal development.

Created in BioRender. Sterba, J. (2025) <https://BioRender.com/xn41j14>.

In addition, the findings of Scott and colleagues [64] unveiled a distinct two-part sialylation pathway that facilitates communication between glial cells and neurons, regulating neural excitability and stress tolerance.

Sialylated glycans/glycoproteins in tick tissues have been continuously investigated in the last few decades. Initially, Sia was identified in *Ixodes ricinus* salivary gland cells using SNA and MAA II lectins, suggesting Sia in $\alpha 2,3$ -(MAA II and SNA) and $\alpha 2,6$ -(SNA) glycosidic bonds [30]. Subsequent studies confirmed the presence of Sia in the gut using Western blot and mass spectrometry techniques [31]. However, these methods cannot definitively determine the origin of the detected sialylated glycoproteins.

As ticks are blood-feeding ectoparasites, several factors suggest a host origin for sialylated molecules. Firstly, ticks feed primarily on mammalian blood rich in sialylated glycoproteins. These compounds can persist in the tick for long periods of time [65]. Moreover, the gradual digestive process in ticks allows prolonged exposure to ingested material, contributing to their remarkable resistance to starvation [66,67]. Secondly, the MS analysis revealed that sialylated glycans in ticks exhibit two or three antennae, with Sia binding to terminal regions on galactose. This characteristic pattern aligns with the hybrid and complex N-glycans typical of vertebrates [23].

Besides the more detailed information on the presence of sialylated glycoproteins and their role in *Drosophila*, there are only a few reports on the presence of sialic acid or sialylation in Arthropods, with some reports on the absence of sialyltransferase activity in insect cell lines and yet more scarce information on sialylation in ticks. Especially in blood-feeding parasites, confirmation of endogenous production of sialic acid and sialylation of proteins is important, taking into account blood rich in sialylated proteins as the sole source of food and survival of intact host proteins of the digestion process; as an example, host proteins or they possible fragments were detected in salivary glands of ticks, which

means they survived the digestion in the midgut, were transported to the haemolymph and next to the salivary glands. Among other proteins, several sialylated serum proteins were identified, such as transferrin, $\alpha 2$ -macroglobulin, or fibrinogen [68–70]. A more or less specific recognition of some host proteins and their transport to tick organs is suggested by the presence of host proteins also in other organs, such as transferrin in the ovaries [71]. Overall, the utilisation of host (glyco)proteins for some physiological functions, such as immune system suppression [68] or participation in the regulation of feeding site defence mechanisms [69], can be suggested.

Thus, the presence of sialylated glycoproteins in tick tissues could serve to host immune system inhibition in the case of reuse of the host blood sialylated glycoproteins or participate in the development of tick (early life stage) tissues in the case of sialylated glycoproteins produced by the tick itself (Fig. 2). Until now, there has been no direct evidence of ticks to sialylate proteins, nor confirmation of the enzymatic pathways required for this process.

3.1. ManNAs processing enzymes and sialyltransferases in the *Ixodes ricinus* genome

There are two known possible Sia synthetic pathways within Eukaryotes (Fig. 1); firstly, a three-step pathway in which enzymes ManNAc 6-kinase (MNK EC:2.7.1.60), *N*-acetylneuraminase (*N*-acetylneuraminase-9-phosphate) synthase (NANS EC:2.5.1.56/57), and sialic acid (*N*-acetylneuraminase-9-phosphate) phosphatase (NANP EC:3.1.3.29) are involved; the other is comprised of *N*-acetylneuraminase lyase-like enzyme (EC:4.1.3.3). Both these pathways result in the production of Neu5Ac from ManNAc. Next, CMP-Neu5Ac is produced by CMP-sialic acid synthetase (CMAS EC:2.7.7.43), and Neu5Ac is transferred to a glycoprotein by sialyltransferases (SiaT, EC:2.4.3.1 and EC: 2.4.3.6). Identification of these enzymes in the tick genome could

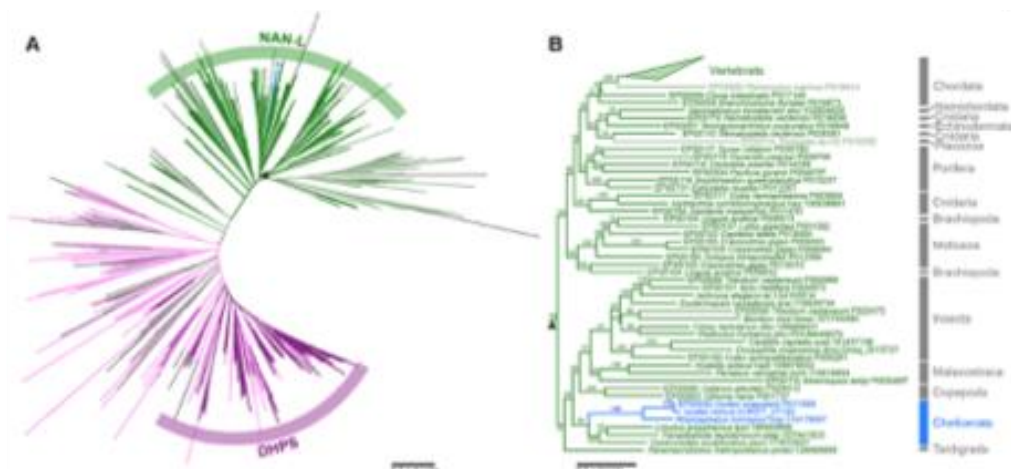


Fig. 3. Phylogenetic position of the tick *N*-acetylneuraminase lyase-like sequences within the dihydrodipicolinate synthase superfamily. Sequences were identified using the PTHR12128 Panther database Hidden Markov Model (HMM) profile and pruned to retain curated homologs of 4-hydroxy-tetrahydrodipicolinate synthase (DHPS) and *N*-acetylneuraminase lyase (NAN-L). A, Maximum Likelihood tree calculated using FastTree (v2.1.11). Dark- and light-coloured branches indicate high- and low-confidence enzyme homologs, respectively, as identified by kofamscan against a library of KEGG HMM profiles (purple — DHPS, green — NAN-L; low-confidence meaning non-significant best hits). Grey marks sequences identified as other enzymes by kofamscan. Blue colour and an asterisk indicate the position of tick NAN-L-like sequences within a high-confidence Chelicerata clade. B, Maximum likelihood tree calculated using IQ-TREE (v2.3.4) with the NAN-L subset of sequences, collapsed to show Animalia sequences. Branch support represented by UltraFast Bootstraps. Root roughly corresponds to the node highlighted with an arrowhead in panel A.

confirm the possibility of ManNAz use for bioorthogonal labelling of intrinsically produced sialylated glycoproteins in ticks.

We could not find evidence for canonical sialic acid synthesis via MNK, NANS, and NNP in either 1) our Oxford Nanopore Technology polished long read assembly (BioProject ID PRJNA1028502); 2) previously published assemblies of two *I. ricinus* isolates (BioProject ID PRJNA816462); and 3) and our comprehensive transcriptomic data from multiple life stages of *I. ricinus* (GenBank ID GIDG000000000, [41]). Neither of the three tick species in the KEGG database [35], namely *Dermacentor silvarum*, *Rhipicephalus sanguineus*, and *R. microplus*, encodes any homologs of this pathway. Instead, all these species encoded an annotated *N*-acetylneuraminase lyase-like enzyme (Fig. 3, Supplementary Files S2, S3, S4), a distant homolog of 4-hydroxy-tetrahydrodipicolinate synthase (EC:4.3.3.7), which is a pyruvate and L-aspartate-4-semialdehyde condensing enzyme. We posit that the tick *N*-acetylneuraminase lyase is capable of a reverse, analogous condensing reaction using pyruvate and *N*-acetyl-D-mannosamine as substrates and, thus, is the enzyme responsible for Sia biosynthesis in ticks. For *I. ricinus*, four sequences encoding parts of the Neu5Ac lyase-like gene can be found in the Transcriptome Shotgun Assembly (TSA) sequence database; furthermore, *N*-acetylneuraminase lyase GenBank ID XM_002410163 can be found in *I. scapularis*, a closely related tick. The alignment of the translated products of these sequences and closely related proteins from other ticks and Arthropods can be downloaded from Supplementary File S5.

Next, CMP-Neu5Ac-synthetase was identified in the *I. ricinus* databases. In the TSA database, CMP-Neu5Ac-synthetase fragments were found in five assemblies (GIXL01003653.1, GFVZ01001309.1, GIDG01027528.1, GANP01000285.1, GCJO01002967.1); additionally, this gene was found in five assemblies in the Whole Genome Shotgun database (JBDHMMW010008074.1, JAZHYW010054697.1, JBDHMMV010017173.1, JXMZ02167595.1, JAZHYW010010247.1). For the closely related *I. scapularis*, a gene encoding this enzyme and two protein entries were found (XP_029849120, EEC15406). Alignment of the translation of the TSA sequences and the closely related proteins from ticks and other Arthropods can be downloaded from the

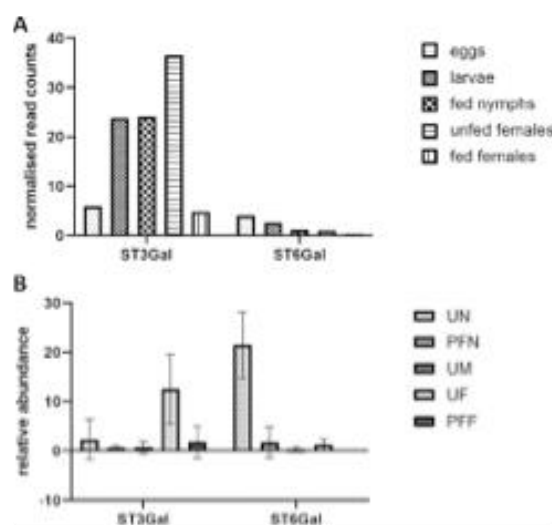


Fig. 4. (A) Sialyltransferases expression in *I. ricinus* life stages, including the eggs and larvae. Normalised read counts of the putative SiaT3 and SiaT6 are shown for the dataset comprising five *I. ricinus* life stages, including eggs and larvae. This dataset was primarily used to confirm the expression of the two genes throughout the life of the tick. (B) Sialyltransferases transcripts relative abundances in the transcriptomes of late *I. ricinus* life stages. Relative abundances of the putative SiaT3 and SiaT6 are shown for the dataset comprising five life stages, including eggs and larvae. The highest expression can be seen in the unfed life stages (nymph and female).

Supplementary File S6.

Table 1

Sialyltransferase activity in the tick cell line lysate, human cell line lysate, and commercially available SiaT. A mammalian cell lysate with a detectable sialyltransferase activity and a pure sialyltransferase were used as controls. Protein concentration is shown in ng/ μ l; 0.1 ng/ μ l of the pure sialyltransferase was used to obtain measurable results. Mean of specific activity with standard deviation is shown.

	IRE/CTVM20 cell lysate	DAOY lysate	α -2,3-Sialyltransferase from <i>Parasaccharibacter multiscidis</i>
Concentration (ng/ μ l)	1500	1500	0.1
Specific activity (pmol/min/ μ g)	1.588 $\pm$ 0.194	3.341 $\pm$ 0.509	197,030 $\pm$ 6703

3.2. Sialyltransferases in the *Ixodes ricinus* genome

Two putative sialyltransferase genes were identified in the *I. ricinus* genome and uploaded to GenBank; a putative beta-galactoside alpha-2,3-sialyltransferase 1 (ST3GAL) GenBank ID PQ468783 encoding the putative enzyme GenBank ID XJQ68691 (partial sequence) and a putative beta-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL) GenBank ID PQ476023 encoding the putative enzyme GenBank ID XKB59570 (partial sequence).

The alignment of the two protein sequences and the closely related proteins from ticks and other Arthropods can be downloaded from the Supplementary Files S7 and S8.

3.3. Sialyltransferase (SiaT) expression

After confirming the presence of the sialyltransferase genes in the *I. ricinus* genome, we verified the production of mRNAs encoding these genes.

First, a dataset comprising all the various life stages, including eggs, larvae, fed nymphs, unfed females, and fed females [39], was used to verify whether the two identified sialyltransferases were expressed. The

expression of both genes was confirmed across all life stages, including eggs and larvae and was depicted as normalised read counts (Fig. 4A).

Next, raw reads of five *I. ricinus* life stages, unfed nymph (UN), partially fed nymph (PFN), unfed female (UF), partially fed female (PFF), and unfed male (UM) [49], were mapped to the two SiaT sequences, cross-sample normalised and mean relative abundances calculated from normalised read counts from 3 biological replicates of the respective transcripts were extracted. Both sialyltransferases were expressed in the various life stages, with the highest expression in the unfed stages (nymph and female) (Fig. 4B, transcripts per million values provided in Supplementary File S9, differential analysis in Supplementary file S10).

3.4. Sialyltransferase activity

To confirm the expression data and demonstrate a detectable sialyltransferase activity, we measured sialyltransferase activity in tick cell lysates using the Sialyltransferase Activity Kit; the removal of phosphate from cytidine 5'-monophosphate (CMP) released upon sialyltransferase transfer of Sia from the CMP-Sia donor to glycans was measured. The optical density was measured after incubation of CMP-sialic acid as a donor, asialofetuin as an acceptor, and IRE20 cell lysate as the working sialyltransferase solution. As positive controls, a human DAOY cell line naturally expressing sialyltransferase and a purified sialyltransferase were used (Table 1).

After 4 h of incubation, sialyltransferase activity was documented in the IRE/CTVM20 cell lysate with a calculated specific activity of 1.588 $\pm$ 0.194 pmol/min/ μ g, which was less than half the calculated activity in the DAOY cell lysate. Commercially available SiaT showed activity several orders higher, as expected, namely 197.03 $\pm$ 6.7 nmol/min/ μ g, after subtraction of the values corresponding to the blank, the positive values confirm the presence of sialyltransferase activity. Compared to the tick cell lysate, a lysate from a naturally sialyltransferase-expressing cell line, DAOY, was used. A reaction containing a pure sialyltransferase was also performed, confirming the test's functionality. From this experiment, we conclude that sialyltransferases expressed in tick cells are active and can sialylate proteins. In addition, the *Ixodes* tick genome encodes a complete pathway from ManNAc to sialylated glycoproteins

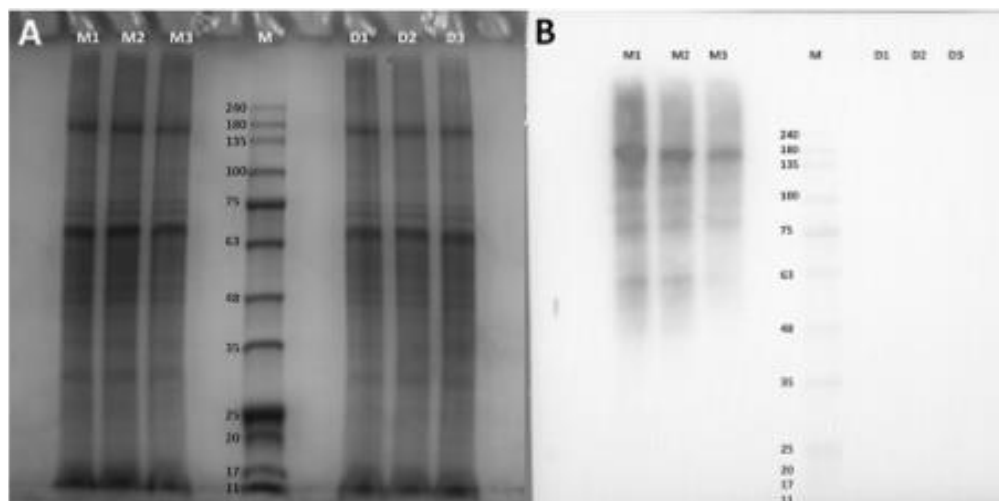


Fig. 5. (A) SDS-PAGE visualisation of IRE/CTVM20 cell protein lysates prepared after the fifth day of incubation of IRE cells with 100 μ g/ml of ManNAc in DMSO and with DMSO as a control. 8 μ g of protein lysate was loaded into each well. (B): Chemiluminescence detection of NeuNAc in IRE 20 tick cells on western blot membrane using anti-Alexa 488 primary antibody and anti-rabbit HRP secondary antibody. IRE/CTVM20 cell protein lysates were prepared after the fifth day of incubation of IRE cells with 100 μ g/ml of ManNAc in DMSO and with DMSO as a control. Experiments were done in biological triplicates.

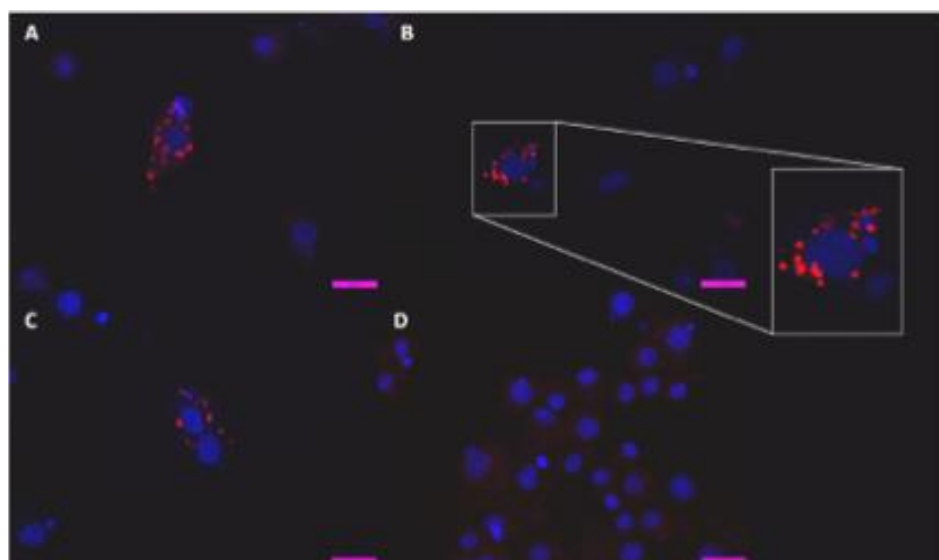


Fig. 6. Detection of NeuNAz in IRE/CTVM20 tick cells. IRE/CTVM20 tick cells incubated for 7 days with ManNAz (10 µg/ml) dissolved in DMSO (A, B, C) and DMSO control (D), both visualised after click reaction with Alexa Fluor 594 Alkyne (red colour). The samples and controls were done in biological triplicates. Nuclei were stained with DAPI (blue colour).

(Fig. 1).

3.5. Detection of NeuNAz-containing glycoproteins in IRE/CTVM20

After confirming the sialyltransferases expression and activity in ticks, we detected sialylated proteins of tick origin using a bio-orthogonally labelled precursor of NeuAc, *N*-azidomannosamine (ManNAz) or *N*-(4-pentynyl)mannosamine (ManNAI). This approach helps

avoid side effects [72,73] that can arise from the removal of Sia caused by the mutation of sialyltransferases or by cleavage by sialidase. These precursors have been previously used for bioorthogonal labelling and subsequent detection of sialylated glycans in various organisms, including ticks [32], and other precursors, such as *N*-levulinoylmannosamine [74], were also used. 100 µg/ml ManAz or ManNAI was added to the cultivation medium to detect sialylated glycoproteins metabolically produced by the tick cells. The presence of glycoproteins

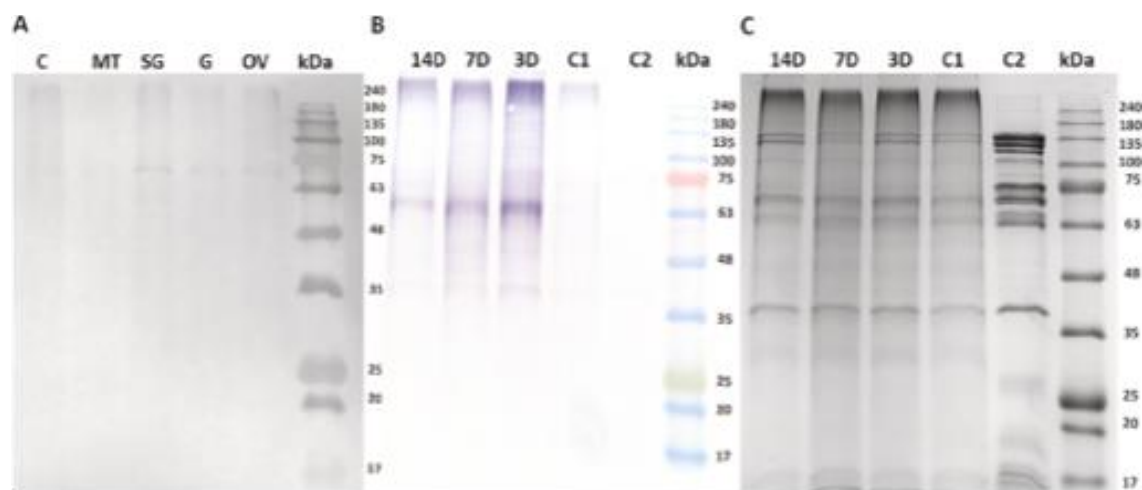


Fig. 7. (A) Detection of NeuNAz in organs and eggs of *Ixodes ricinus* female fed *in-vitro* with blood with ManNAz (10 µg/ml) on western blot membrane using anti-Alexa 488 primary antibody and anti-rabbit secondary antibody. MT — Malpighian tubules, SG — salivary glands, G — gut, OV — ovary, C — the whole lysate of a female tick fed *in vitro* with DMSO in blood. (B), (C): Detection of NeuNAz produced in eggs from a female tick fed *in vitro* with blood with ManNAz (10 µg/ml) on western blot membrane using anti-Alexa 488 primary antibody and anti-rabbit secondary antibody. 14D — eggs collected 14 days after oviposition, 7D — eggs collected 7 days after oviposition, 3D — eggs collected 3 days after oviposition, C1 — protein lysate from eggs from *I. ricinus* female fed *in-vitro* with DMSO with click reaction, C2 — protein lysate from eggs from *I. ricinus* female fed *in-vitro* with DMSO without click reaction.

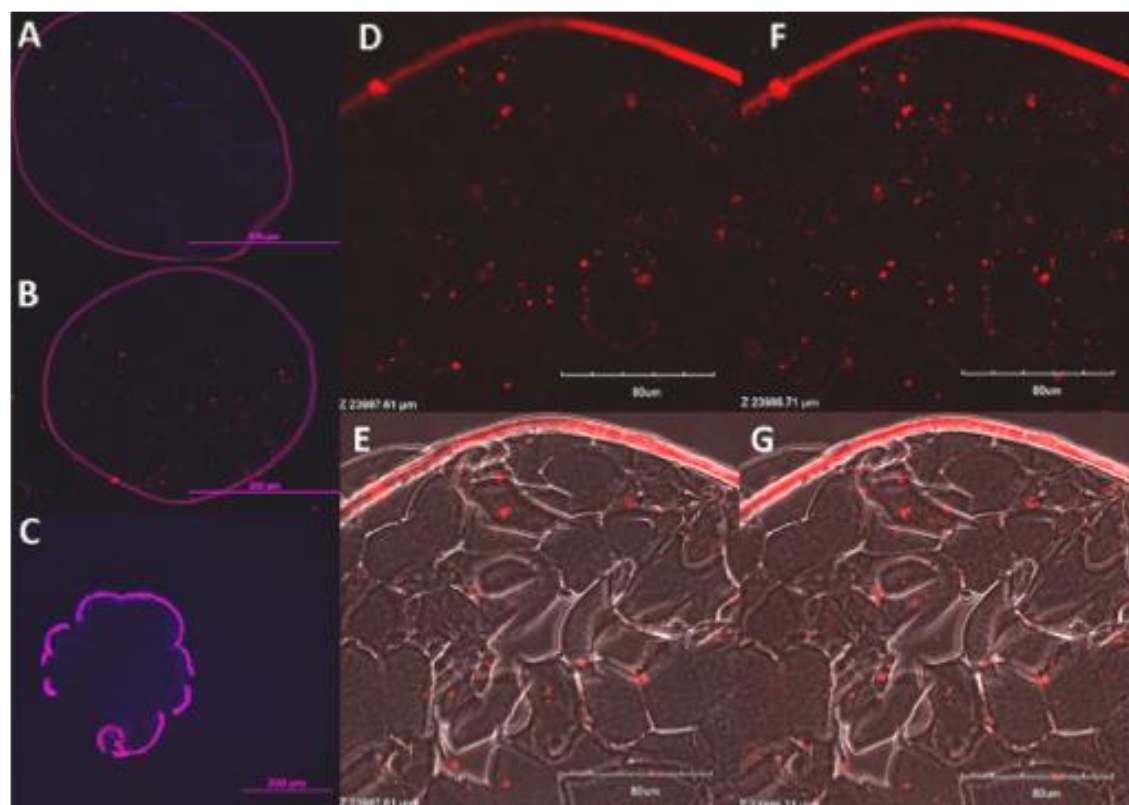


Fig. 8. Detection of NeuNAz produced in eggs from *I. ricinus* female fed *in-vitro* with blood with ManNAz (10 $\mu\text{g}/\text{ml}$) (A, B) using fluorescent microscopy. The eggs were collected 14 days after oviposition. Eggs from a female tick fed with DMSO without ManNAz were used as a control (C). ManNAz was reacted with Alexa Fluor 488 Alkyne. Next, rabbit Anti-Alexa Fluor 488 and Anti-rabbit DyLight 549 (red colour) were used for visualisation. Nuclei were stained with DAPI (blue colour, A, B, C). The inner structure of the egg was interspersed with the Nomarski contrast (E, G).

incorporating the metabolised NeuNAz or NeuNAI was verified using two different Click reactants — biotine-azide or -alkyne, and Alexa 488-azide or -alkyne and subsequent detection with either a Streptavidin-HRP conjugate or a combination of rabbit anti-Alexa 44 antibodies and anti-rabbit-HRP conjugated secondary antibodies (Supplementary File S11). Protein bands corresponding to the detected NeuNAI or NeuNAz-containing proteins were identified using mass spectrometry, and their putative N-glycosylation sites were listed (Supplementary File S12). Because the protein profiles and protein identifications were similar in samples with incorporated NeuNAz or NeuNAI, ManNAz was used in subsequent experiments.

The addition of ManNAz did not significantly affect the tick cell viability and metabolic activity (Supplementary File S13). After the incorporation of ManNAz, metabolised to NeuNAz to tick glycoproteins, newly created sialylated glycoproteins were labelled with Alexa Fluor 488 alkyne using the Click reaction and visualised by western blot analysis (Fig. 5) [75]. DMSO without ManNAz was added to the tick cell culture as a control.

After 5 days of incubation, several sialylated glycoproteins ranging from approximately 48 kDa to approximately 245 kDa were detected in the cell lysate in each triplicate with a very strong signal of a protein with an apparent molecular weight of ca. 150 kDa (Fig. 5B); these proteins are of low abundance as can be seen from the comparison to Coomassie-stained proteins (Fig. 5A).

A detailed visualisation of sialylated NeuNAz-containing

glycoproteins produced by the tick cells revealed their presence in the IRE/CTVM20 cell line (Fig. 6, Supplementary File S14). Tick cells were incubated with ManNAz (10 $\mu\text{g}/\text{ml}$) for 7 days. After the Click reaction with Alexa Fluor 594 Alkyne and DAPI staining, the cells were directly imaged without additional staining. Cells incubated with DMSO without ManNAz were used as a control. Experiments were done in biological triplicates. A positive reaction was observed in the cytoplasm of some cells, confirming a low protein sialylation in ticks. Vacuoles and regions surrounding the nucleus and within the cytoplasm were stained. An overview of positively stained cells is in the Supplementary file S14.

3.6. Detection of NeuNAz-containing glycoproteins in *Ixodes ricinus* eggs and female organs by Western blot and fluorescence microscopy

Ixodes ricinus females were fed *in vitro* on blood containing 1 or 10 $\mu\text{g}/\text{ml}$ ManNAz. Several fed females were dissected to obtain salivary glands, microtubules, gut, and ovaries. Furthermore, eggs were collected 3, 7, or 14 days after oviposition by females fed on blood with both ManNAz concentrations.

Using the Click reaction with Alexa Fluor 488 Alkyne and subsequent antibody staining, NeuNAz-containing proteins were detected by Western blot. A similar glycoprotein profile is detected at both these ManNAz concentrations in the fed blood; however, the signal is considerably stronger at the higher concentration. Thus, the higher concentration used did not affect the profile of detected glycoproteins

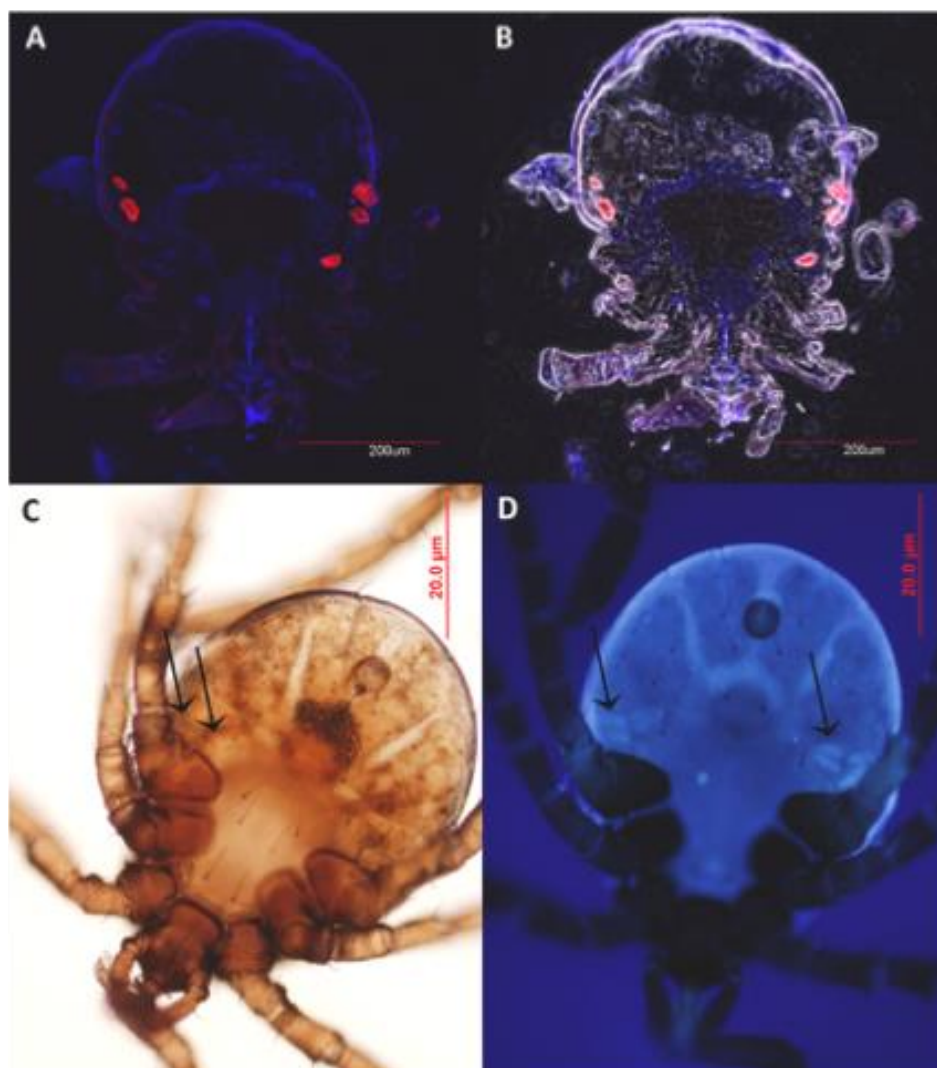


Fig. 9. The dorsal (A, B) and ventral (C, D) view of *I. ricinus* larva originating from an egg from a female fed *in vitro* with blood containing ManNAz (10 $\mu\text{g}/\text{ml}$). ManNAz was reacted with Alexa Fluor 488 Alkyne. Next, rabbit Anti-Alexa Fluor 488 and Anti-rabbit DyLight 549 (red colour) were used for visualisation (A, B). Nuclei were stained with DAPI (blue colour, A, B, D). A — DAPI and DyLight 549 filters, B — DAPI and DyLight 549 filters interspersed with Nomarski contrast, C — light phase, D — DAPI filter.

but ensured a higher proportion of proteins that had incorporated NeuNAz. For this reason, we used the concentration 10 $\mu\text{g}/\text{ml}$ in the further experiments (Supplementary File S15).

In the organs collected from females fed on blood with 10 $\mu\text{g}/\text{ml}$ ManNAz, a sialylated glycoprotein with an approximate molecular weight of 70 kDa was detected in salivary glands, gut, and ovaries by Western blot (Fig. 7). The whole lysate of a female tick fed *in vitro* on blood containing only DMSO without ManNAz was used as a control.

Based on reports from other invertebrates and following the detection of NeuNAz-containing proteins in the ovaries (Fig. 7A), we expected the presence and importance of sialylated glycoproteins in the development of the embryo and/or egg. Therefore, we performed further analyses on the eggs at various time points after oviposition (embryos at various stages of development).

Three fed tick females were left to hatch, and the 15 eggs were collected for each time interval at 3 days, 7 days, and 14 days after oviposition, and the presence of tick-self sialylated proteins was visualised (Fig. 7B). The highest amount of sialylated glycoproteins was present in 3 day old eggs, whereas the intensity of detected proteins decreased from the 3-day-old to the 14 day old day after oviposition, showing the highest production of sialylated glycoproteins early in the development. Besides the protein with an approximate molecular weight of 70 kDa, other proteins with only a light signal also appeared.

As controls, eggs from females fed with blood with DMSO were used. The eggs were collected three days after oviposition, 15 eggs per group. C1 represents protein lysate after the Click reaction and subsequent precipitation, C2 represents protein lysate without the Click reaction; these controls were used to verify an absence of artificial positive

staining resulting from the Click reaction or the precipitation of proteins. The efficiency of NeuNAz incorporation into glycoproteins was assessed by comparing protein profiles of eggs laid by females fed blood supplemented with either 1 µg/ml or 10 µg/ml ManNAz. Following the Click reaction, NeuNAz-labelled proteins were detected by Western blotting (Supplementary file S15). The resulting protein profiles were comparable between the two conditions; however, a stronger signal was observed in eggs from females fed blood containing the higher ManNAz concentration. These results confirm the suitability of using the higher ManNAz concentration, which remains below concentrations previously reported to be toxic.

The presence of NeuNAz in eggs/embryos was further confirmed by confocal microscopy in thin sections of eggs 14 days after oviposition. NeuNAz signal was detected in cell membranes and vacuoles inside the cells (Fig. 8A, B, red colour), nuclei were stained with DAPI (Fig. 8A, B, C, blue colour). The inner structure of the egg was interspersed with the Nomarski contrast (Fig. 8E, G).

The highest content of tick-produced sialylated glycoproteins in the larval stage agrees with findings in *Drosophila*, showing the importance of sialylated glycoproteins and sialyltransferase activity for the development of the nervous system in the embryonic stage and the highest abundance of sialylated glycans in the early embryo stage [17,18]. In this regard, protein sialylation is also not surprising in tick cell lines, as these are derived from tick embryos [51]. 15 eggs per one interval were left to develop until larvae hatched, and 5 µm thin sections of larvae were prepared as for eggs. The slices were then prepared identically to the egg sections. In larvae, sialylated glycoproteins were localised in specific structures on both sides of the larva body (Fig. 9A, B, red colour); a positive signal was not detected throughout the body nor in plasma membranes. These structures do not correspond to a described specific organ within the larval body; their localisation seems to correspond to structures stained by DAPI located in the vicinity of the anterior legs observed using an Olympus BX-60 microscope (Olympus, Tokyo, Japan) (Fig. 9C, D, arrows).

Regarding the potential inhibition of host immune system activation by sialylated glycoproteins (Fig. 2, summarised in [8]), an ability to selectively recognise host sialylated proteins, transport them to the salivary glands, and excrete them into saliva would be beneficial for ticks. Therefore, we plan to decipher the ability of ticks to recognise and selectively transport host sialylated glycoproteins in the future.

4. Conclusion

The metabolic pathway to produce sialic acid and for protein sialylation was identified in the tick *Ixodes ricinus*, and protein sialylation was confirmed in both the ticks (females, nymphs, larvae, eggs) and the tick cell line IRE/CTVM20 derived from *I. ricinus* embryos using bi-orthogonal and Click chemistry. A higher abundance of tick sialylated glycoproteins was detected in the early embryonic stage, suggesting a possible role of sialic acid in tick development. A more detailed description of sialic acid's role and processes related to the host sialylated glycoproteins within the tick and their relation to tick physiology must be elucidated.

CRediT authorship contribution statement

Jarmila Sterbova: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Conceptualization. **Jaroslav Ondrus:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation. **Zoltan Fussy:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Pavlina Vechtova:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Libor Grubhoffer:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Jan Sterba:** Writing – review & editing,

Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.152305>.

Data availability

Data will be made available on request.

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3. Discussion

Over the last few decades, there has been a boom in technologies and methods concerning increasing sensitivity and specificity. As a result, the research on glycosylation and other post-translational modifications is coming to the forefront. Glycosylation is a posttranslational modification in which monosaccharides are covalently attached to the mature glycan. Glycans are not encoded directly in the genome, but they provide significant proteomic diversity and can confer additional functions to a single protein (summarised in Critcher et al., 2022). Continued investigation of glycosylation has underscored its growing importance. As a common protein modification (in Eukaryotes), it plays a role in many cellular processes in both vertebrates and invertebrates, as well as in interactions among hosts, parasites, and pathogens (summarised in He et al., 2024a).

Ticks are blood-sucking parasites, meaning their only source of food is the host's blood. During their life cycle, most of the Ixodid ticks change from larva to nymph to adult after each feeding. Ticks also transmit pathogens of medical and veterinary importance, and, due to their parasitic lifestyle, they produce a variety of active molecules that affect the hosts, the ticks and the pathogens they transmit. Among them, glycosylated proteins and enzymes of the glycosylation pathways deserve greater attention, as they play a key role in cell-cell communication, development, pathogen transmission, and human disorders.

Sialic acid, a negatively charged nine-carbon saccharide, is a specific, multifunctional molecule present mainly in vertebrates, including humans. Research on sialylated glycoproteins and Sia itself has focused mainly on humans and other higher animals, because it has long been known that Sia covers the surfaces of most vertebrate cells and is involved in their cellular processes. In invertebrates, however, their occurrence, diversity, and function are more variable and historically overlooked.

This thesis provides a comprehensive overview of Sia in the *I. ricinus* tick, the most common tick in the Czech Republic and a parasite of humans and addresses the challenge of distinguishing host- and tick-origin sialylated molecules.

The origin of *I. ricinus* Sia

To date, Sia has been described in organs of a few arthropod species - in Malpighian tubules of cicada *P. spumarius* (Malykh et al., 1999), in prothoracic glands of honeycomb moth *Galleria mellonella* (Karaçalı et al., 1997), in *D. melanogaster* embryo (Aoki et al., 2007), in the haemolymph of the silkworm *B. mori* (Soya et al., 2017), in *A. aegypti* tissues (Cime-Castillo et al 2015), and in a mosquito cell line (He et al., 2024b).

As ticks receive mammalian blood as their only source of nutrients, the origin of sialylated glycans is debatable because they could come from both the host and the tick source. The origin of sialylated glycans in ticks is thus a key question in tick glycobiology.

Research over the last few decades has shown that host-derived proteins are present in both tick

tissues and saliva during and after feeding. These are acquired from the blood meal and selectively retained, transported, or even re-secreted. For example, Tirloni and colleagues (2014) identified 68 bovine proteins in *R. microplus* saliva alongside tick proteins, and Liu and colleagues reported 40 host-derived proteins in *H. flava* haemolymph (Liu et al., 2022).

We firstly reported the observation of Sia in SG and in the gut of partially fed *I. ricinus* females using an electron microscope (Vancova et al., 2012). In combination with MS analysis and western blot using a specific antibody/lectin, two Sia types were detected, Neu5Ac and Neu5Gc. However, some evidence suggests that the detected molecules are of host origin. The structure of the identified sialylated glycans, whether bi- or triantennary, resembles that of mammalian glycans more than that of sialylated glycans found in *Drosophila* that feature sialic acid residues attached to only one antenna (Aoki et al., 2007). Park and colleagues (2020) also identified both Neu5Gc and Neu5Ac glycans composed of two or three branches in *A. americanum* that correspond to our findings; nevertheless, the origin of the identified glycans was not elucidated. Moreover, NeuGc attached to the end of four glycan branches was found in *A. americanum* males.

The structure of Sia glycans of *I. ricinus* differs from the sialylated glycans detected in partially fed females of *A. americanum* tick (Park et al., 2020), as the sialylated glycans from *I. ricinus* females lack α 1,3-core fucose. α 1,3-core fucosylation, a characteristic feature of invertebrates, belongs to the group of so-called cross-reactive carbohydrate determinants that are responsible for the IgE production in humans. Humans lack α 1,3-core fucose, so their immune system considers this epitope foreign. α 1,3-core fucosylated glycans have also been observed in *I. scapularis* salivary glands, but without Neu5Gc/Neu5Ac (Pedra et al., 2010) and with Neu5Ac in *A. americanum* partially fed female (Park et al., 2020).

The host origin of the reported NeuGc in *I. ricinus* tissues is supported by its localisation in digestive cells, in the cytoplasm and in small vacuoles. Their path was recorded as it passed through the gut basement membrane (Vancova et al., 2012), which is consistent with the observations that several host glycosylated proteins can survive tick digestion and may be incorporated into tick tissues or transported into saliva (Ackerman et al., 1981; Fujisaki et al., 1984; Valenzuela, 2002; Wang & Nuttall, 1994). Host plasma proteins typically found in ticks include albumin, immunoglobulins (IgG, IgM), complement components, fibrinogen, and other clotting factors; some of them also bear Sia in their glycans. For example, transferrin can carry up to four Sia on its N-glycans (van Dijk et al., 1994), and about 5 % of IgG molecules feature core-fucosylated biantennary complex glycans with two Sia residues (Routier et al., 1998). In human serum, sialylation of plasma protease C1 inhibitor, orosomucoid, haptoglobin, caeruloplasmin, α 1-antitrypsin, and many other glycoproteins has been identified (Alley & Novotny, 2010; Taniuchi et al., 1981). Regarding other potential mammalian hosts for ticks, Neu5Gc occurs in horses, as well as in deer and cattle, being highly variable among species (Contò et al., 2025;

Nemanichvili et al., 2022).

Neu5Gc was also detected in low amounts in the gut of unfed tick females (Vancova et al., 2012). As hard ticks can digest the host blood gradually, which is made possible by the tick's specialised slow intracellular digestion based on lysosomal degradation. This enables intact host proteins to persist in the tick environment, allowing ticks to survive without feeding for extended periods. Additionally, host-derived proteins can stay in the tick body for extended periods, lasting several months after feeding and moulting (Wickramasekara et al., 2008).

Neu5Gc was detected in acini type III (cytoplasm) and in f cells (basal labyrinth and microvilli) in SGs, suggesting the possible excretion from haemolymph to saliva. Moreover, Neu5Gc was stained in granular acini type II (basement membrane), which is responsible for the secretion of many tick salivary components. Finally, the different intensity of anti-Neu5Gc staining was reported between tissue homogenates of fed and unfed *I. ricinus* females in comparison to guinea pig serum (Vancova et al., 2012).

To address the question of the origin of sialylated glycans, we used two different biochemical quantification methods – the thiobarbituric acid assay (TBA; Warren, 1959) and metabolic labelling using NeuNAz to determine the content of Sia in ticks, tick organs, and a tick cell line (Sterba et al., 2014). Thus, we compared the amount of total Sia content to tick-derived metabolically created Sia (NeuNAz). The TBA method, compared with NeuNAz quantitation, showed Sia levels several orders of magnitude higher in all tick tissues and homogenates. The Sia content measured by the TBA assay ranged from 1.3 to 8.5 $\mu\text{mol Sia/g}$ of protein in tick organs and approximately 60 $\mu\text{mol Sia/g}$ of protein in whole tick homogenates; the gut was washed to remove the lumen, which contains the ingested blood. This difference can be explained by the nature of the TBA assay, which measures total Sia released after hydrolysis and therefore includes both host-derived and endogenous pools, and the inclusion of the gut lumen in the whole tick homogenate (Sterba et al., 2014). In contrast, metabolic labelling with NeuNAz specifically reflects newly synthesised sialylated glycoconjugates (Chang et al., 2009). The NeuNAz level was about 3 orders of magnitude lower in OV and SG, and even less in the gut and in whole tick homogenate (Sterba et al., 2014). Taken together, Sia levels were lower in individual organs than in the whole tick homogenate and, overall, were markedly higher than the tick-produced NeuNAz content. This indicates that the whole tick – particularly the gut – contains substantial amounts of sialylated molecules derived from the host, whereas sialylated proteins produced by the tick itself are more abundant in the salivary glands and ovaries. The lower levels of NeuNAz compared to total Sia strongly support the conclusion that the majority of sialylated glycans present in ticks originate from the host blood meal rather than from tick biosynthesis. This interpretation is consistent with our previous study, which demonstrated that ticks internalise sialylated glycans (derived from the host) and transport them from the gut to other organs, including the salivary glands (Vancova et al., 2012).

The amount of Sia and NeuNAz was also examined in the tick cell line IRE/CTVM19. Sia content exceeded that in the full tick homogenate, reaching 86 μmol Sia per gram of protein (Sterba et al., 2014). The presence of Sia in a tick cell line is not surprising and can be linked to the binding of sialylated proteins from bovine serum in the culture medium to the tick cells. However, it was not possible to quantify NeuNAz in the cell lysate (Sterba et al., 2014). Later, we confirmed NeuNAz in the IRE/CTVM20 cell line after optimising conditions for metabolic incorporation and bioorthogonal labelling (Sterbova et al., 2026b).

As mentioned above, to differentiate between host- and tick-derived sialylated molecules, we used a bioorthogonally labelled Sia precursor, N-azido-mannosamine (ManNAz). ManNAz, containing a small inert azido-group, can be metabolised to N-azido-neuraminic acid and incorporated into glycans, provided that the corresponding synthetic pathway is present. Despite the predominance of host-derived glycans, the presence of NeuNAz in tick tissues and IRE cells suggests that ticks possess a functional capacity for de novo sialylation, and corresponding genes can also be found.

In Eukaryotes, there are two possible pathways for the synthesis of Neu5Ac. The classical pathway includes ManNAc-6-kinase (EC:2.7.1.60), N-acetylneuraminate-9-phosphate synthase (EC:2.5.1.56/57), and sialic acid (N-acetylneuraminate-9-phosphate) phosphatase (EC:3.1.3.29). The less common synthetic pathway involves the N-acetylneuraminate lyase-like enzyme (EC:4.1.3.3). ManNAc (N-acetylmannosamine) enters both pathways as a precursor and is metabolised to Neu5Ac. Then, CMP-sialic acid synthetase (CMAS EC:2.7.7.43) creates CMP-Neu5Ac, which is transferred to the mature glycan by SiaT (sialyltransferase) (EC:2.4.3.1 and EC: 2.4.3.6). Searching for the various databases – our Oxford Nanopore Technology polished long read assembly (BioProject ID PRJNA1028502, previously published assemblies of two *I. ricinus* isolates (Bioproject ID PRJNA816462) and our comprehensive transcriptomic data from multiple life stages of *I. ricinus* (GenBank ID GIDG000000000) (Vechtova et al., 2018), we did not find homologues of these enzymes. Additionally, none of the *D. silvarum*, *R. sanguineus*, and *R. microplus* species listed in the KEGG database (Kanehisa et al., 2024) possess homologues of this enzymatic pathway.

On the other hand, an annotated N-acetylneuraminate lyase-like enzyme was found in all of them. This enzyme is a homolog of 4-hydroxy-tetrahydrodipicolinate synthase (EC:4.3.3.7), a pyruvate and L-aspartate-4- semialdehyde condensing enzyme capable of Neu5Ac synthesis from ManNAc. We propose that this enzyme can perform a reverse condensation reaction, similar to its usual activity, by using pyruvate and N-acetyl-D-mannosamine as substrates. Based on this, we suggest that this enzyme can synthesise Sia in ticks. In *I. ricinus*, four sequences corresponding to fragments of a Neu5Ac lyase-like gene are present in the Transcriptome Shotgun Assembly (TSA) database. Additionally, in the closely related species *I. scapularis*, an N-acetylneuraminate lyase sequence is available under GenBank ID XM_002410163.

For glycosylation, CMP-Neu5Ac-synthetase is then required for the attachment/activation of Neu5Ac as a next step. This enzyme or its fragments were identified in five TSA assemblies (GIXL01003653.1, GFVZ01001309.1, GIDG01027528.1, GANP01000285.1, GCJO01002967.1) and in five assemblies in the Whole Genome Shotgun database (JBDHMW010008074.1, JAZHYW010054697.1, JBDHMOV010017173.1, JXMZ02167595.1, JAZHYW010010247.1). Furthermore, two genes and protein entries were found in the closely related *I. scapularis* (XP_029849120, EEC15406).

Attachment of Sia to glycans is the final step in protein sialylation. We confirmed the expression of two SiaT isoforms in various *I. ricinus* developmental stages as well as in IRE/CTVM20 cell lysate and SiaT activity in the tick cell line (Sterbova et al., 2026b). The putative α 2,3-sialyltransferase (GenBank EEC07371) and α 2,6-sialyltransferase (GenBank ADO17789) were also found in the genome of *I. scapularis* and in *A. americanum* (JAT98667.1) (Park et al., 2020). In other Arthropods, SiaT has been identified in the *D. melanogaster* genome (Koles et al., 2004), where its expression and functional activity have been confirmed, along with the crucial role of sialylated glycans present in neural tissues during development (Repnikova et al., 2010).

To test our assumption that ticks can synthesise their own sialylated glycans, we used metabolic labelling in combination with biorthogonal chemistry. Using this approach, we were able to detect tick-derived Sia in every life stage – from fed *I. ricinus* females through eggs to larvae and nymphs. As a precursor, ManNAz in tetraacetylated form (Ac₄ManNAz) was used. It has already been applied as a bioorthogonal probe to label and subsequently detect sialylated glycans in various organisms, including ticks (Sterba et al., 2014) and *Drosophila* (Granell et al., 2011). Other precursors, such as N-levulinoylmannosamine (Charter et al., 2000), have been successfully used previously (Du et al., 2009; Prescher & Bertozzi, 2006).

For our purpose, ManNAz was added to bovine blood during in vitro feeding or to the tick cell growth medium. DMSO instead of ManNAz was used as a control. Generally, this modified sugar precursor competes with the natural substrate ManNAc in the Sia biosynthetic pathway and is converted to NeuNAz. NeuNAz is then incorporated into newly synthesised sialylated glycoproteins, introducing a small, bioorthogonal azido group derived from the administered ManNAz. This azido group can subsequently be covalently coupled via a triazole ring (Tornøe et al., 2002) using a copper-catalysed 1,3-dipolar cycloaddition (Click reaction) with Alexa fluorophores carrying an alkyne group. NeuNAz incorporated into tick glycoproteins was thus tagged with Alexa Fluor 488 and subsequently detected using Western blotting and fluorescence/confocal microscopy. Metabolically derived sialylated glycoproteins were detected in IRE cells and in various stages of *I. ricinus* (Sterbova et al., 2026b).

In the tick cell line, the signal from NeuNAz was localised particularly in vacuoles and areas close to the nucleus and inside the cytoplasm of some cells, which suggests that Sia is produced in small

amounts. Newly synthesised proteins visualised by western blot ranged from approximately 45 kDa to 150 kDa; for several of them, MS identification was performed alongside the prediction of glycosylation sites (Sterbova et al., 2026b).

In eggs collected at various time intervals after oviposition, glycoproteins with NeuNAz were detected by Western blot. Additionally, a change in the amount of these glycoproteins was observed, with the highest levels at egg laying, gradually decreasing until the larvae hatched. It is therefore possible that these glycoproteins play a certain role in embryogenesis. This result corresponds to research on sialylated glycoproteins in the fruit fly. Here, Sia was detected during embryonic development, where it plays a role in nervous system development (Roth et al., 1992). We further examined the presence of NeuNAz in eggs/embryos and larvae using confocal microscopy. A positive signal was detected at the cell membranes and in numerous scattered clusters within vacuoles (Sterbova et al., 2026b).

The larval stage was prepared as 5- μ m-thick sections, in which the signal was displayed in specific structures located on both sides of the larval body. Although larval anatomy is only partially described in the available literature, the structures appear to correspond to structures stained with DAPI located in the vicinity of the anterior legs, as observed using an Olympus BX-60 microscope (Sterbova et al., 2026b). The elevated levels of sialylated glycoproteins produced by ticks during the larval stage are consistent with observations in *Drosophila*. Studies by Koles and colleagues (2004) and Repnikova and colleagues (2010) highlight the crucial role of sialylated glycoproteins and sialyltransferases in nervous system formation during embryogenesis, as well as the highest occurrence of sialylated glycans in early embryos. Accordingly, the occurrence of protein sialylation in tick-derived cell lines is expected, given that these lines originate from tick embryos (Bell-Sakyi et al., 2007).

In the *I. ricinus* tick and the tick cell line, our recent studies have identified sialyltransferase activity and demonstrated the metabolic incorporation of a Sia precursor into tick glycoconjugates, confirming that ticks can synthesise their own sialylated molecules. Notably, the distribution of NeuNAz suggests that endogenous sialylation is more active in early developmental stages, particularly in eggs and larvae. Moreover, we quantified and compared the total amount of Sia and the amount of metabolically created Sia (NeuNAz). The amount of host-derived Sia exceeded that of tick-derived Sia. We showed that tick gut and salivary glands contain NeuGc glycans, pointing to the host origin of the detected molecule.

Immunologically interesting glycans

In parallel to our research on Sia, glycomic studies appeared to be a very relevant tool for revealing and describing the mechanisms of pathogen transmission and host-parasite interactions. Despite growing interest in protein glycosylation and arthropod glycans, major knowledge gaps remain

to be filled regarding the nature and specific functions of glycoconjugates in the tick-pathogen-host triad. Ticks transmit a broad range of bacterial, viral and protozoan pathogens, making pathogen-specific vaccines expensive and difficult to develop. A promising alternative is a general anti-tick vaccine, potentially exploiting tick glycoproteins such as the midgut antigen Bm86, which is more immunogenic in its glycosylated than in its non-glycosylated form (de la Fuente et al., 2006). Vaccines based on this antigen, TickGARD and Gavac, protect cattle against *R. microplus* by reducing tick numbers and decreasing egg production (Willadsen, 2008). Additionally, an anti-tick vaccine based on recombinant Ferritin 2 and native tick protein extract showed promising results (Hajdusek et al., 2010; Knorr et al., 2018).

Modern glycomic approaches have revealed that tick glycosylation is more extensive than previously assumed, and unusual combinations of glycans are present (Park et al., 2020). Some glycan epitopes are increasingly recognised as immunologically relevant, contributing to allergies and offering promising targets for diagnostics, therapies, and broad anti-tick vaccines. Among them, CCDs absent in humans have emerged as clinically relevant epitopes that contribute to allergy, anaphylaxis (e.g., α -Gal syndrome), and diagnostic interference. One prominent example is the α -Gal epitope, which is involved in delayed allergy to red meat associated with tick bites known as α -Gal syndrome. AGS cases have been reported in increasing numbers worldwide, but the reason for this rapid rise remains unclear. From a clinical standpoint, anti- α Gal vaccines have been proposed both to reduce anti- α Gal IgE production and basophil activation and as potential broad-spectrum vaccines targeting multiple vectors and pathogens (summarised in Petry et al., 2026).

The presence and immunogenicity of CCD epitopes in tick tissues and saliva pose an obvious paradox, as ticks need to suppress the host's defensive responses to feed successfully. Our hypothesis is that inhibitory signals, including sialylated glycoproteins derived from the tick or the host, can modulate the immune response and, through molecular mimicry, mask highly immunogenic tick-specific glycans. Ticks may transport undigested host sialylated proteins and glycans from the gut to their salivary glands and saliva. This may allow them to present host-like glycan patterns at the feeding site, helping to suppress host immune activation while still utilising immunogenic carbohydrate complexes for other functions (Sterbova et al., 2026a; Vancova et al., 2012).

4. Conclusion and future prospects

Over recent years, the occurrence of ticks, tick-borne pathogens, and tick-borne diseases has increased worldwide. Due to climate change, various tick species are spreading to higher elevations and into areas where they were previously not found (Daniel, 1993; Daniel et al., 2003), thereby posing a threat to a greater number of people, wild animals, and cattle. Due to the increasing incidence of tick-borne diseases and AGS, the development of new strategies to control their life cycles is a hot topic. To gain a deeper understanding of the complex interactions between parasites, pathogens, and hosts, it is essential to examine the role of individual components at the molecular level. More recent work at the tick-host-pathogen glycobiology interface highlighted the broad importance of glycans and glycan-binding molecules in vector competence and in the development of anti-tick vaccines (summarised in Vechtova et al., 2018).

In this study, focused on sialylated glycoproteins from the *Ixodes ricinus* tick and cell line, NeuAc and NeuGc were shown to be present in tick saliva/salivary glands, and the gut, and their host origin was suggested. Their transport from the blood meal through the gut into the haemolymph and salivary glands was visualised. Subsequent research quantified Sia in different tick organs and tick cell lines, showing that host sialylated molecules dominate in gut and whole-body homogenates, whereas metabolically incorporated Sia is more enriched in salivary glands, ovaries, and embryo-derived tick cell lines. Additionally, genomic and transcriptomic analyses identified tick sialyltransferases, CMP-Neu5Ac synthetase, and an N-acetylneuraminate lyase-like enzyme, supporting a non-canonical endogenous Sia biosynthetic pathway. Using a bioorthogonal precursor (ManNAz) and Click chemistry, the study provided evidence that ticks synthesise their own sialylated glycoproteins, particularly during embryonic and larval stages and in tick-derived cell lines. The tick glycome emerges as a dynamic, composite system shaped by both endogenous biosynthesis and the incorporation of host-derived glycoconjugates. Sialylated glycoproteins of host origin seem to be recycled, potentially even reattached to tick proteins, while endogenously produced sialylated glycoproteins seem especially important during early tick development. This complexity underlies key aspects of tick biology, including long-term survival without feeding, immune evasion at the feeding site, pathogen transmission and the induction of allergic responses in the host.

In parallel, the coexistence of immunogenic CCDs with inhibitory, often sialylated, glycoproteins from both host and tick sources supports a model in which ticks use molecular mimicry and glycan-mediated signalling to suppress host defences while still eliciting carbohydrate-specific immune responses. As tick ranges expand with climate change and tick-associated allergies and infections continue to rise, a deeper understanding of tick glycobiology, including sialic acid metabolism, glycan-mediated interactions and immunogenic carbohydrate epitopes, will be essential for designing new diagnostic tools, vaccines and therapies, and for improving the management and prevention of tick-borne infections and associated allergies such as AGS.

The roles and mechanisms in the glycobiology field in ticks remain incompletely understood; therefore, translating this knowledge to benefit humans remains a challenge for research. To complete the overall picture of Sia in the *I. ricinus* tick, one crucial piece of this puzzle remains to be solved: the question about host-derived Sia in tick tissues and cells. Therefore, our future research will aim to clarify how ticks selectively recognise and transport host sialylated glycoproteins, how endogenous and host-derived glycans are integrated into the tick glycome and how these processes impact immune modulation and pathogen transmission.

5. References

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6. *Curriculum vitae*

Curriculum vitae

CONTACT INFORMATION

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Email: sterbova@prf.jcu.cz
Phone: +420-38-903-6220
Date and place of birth: 17. 9. 1985, Uherské Hradiště

EDUCATION

2005-2008 **BSc. in Biology**, Faculty of Science, USB, České Budějovice
2009-2011 **MSc. in Experimental Biology**, Faculty of Science, USB, České Budějovice
26. 1. 2011 **RNDr. in Experimental Biology**, Faculty of Science, USB, České Budějovice
2011- **Ph.D. in Integrative Biology**, Faculty of Science, USB, České Budějovice
(February 2014 – February 2017; February 2018 – February 2021 Recognized period of parenthood)

Attended courses:

2012 AKK course Professional medical laboratory methods at the Institute for Postgraduate Medical Education, Prague
2011 Basics of Scientific Work, CAS Brno

Students' grants (principal investigator):

2008 Isolation and biochemical characterization of fibrinogen-like proteins (FREPs) from the hemolymph of the tick *Dermacentor marginatus* – Student Grant Agency of the Faculty of Science USB
2009 Localization of FReD (Fibrinogen-related domain) proteins in tissues of the tick *Dermacentor marginatus* by electron and immunofluorescence microscopy methods and their biochemical characterization – Student Grant Agency of the Faculty of Science USB
2010 Characterization of N-glycome of nymphs of the tick *Ixodes ricinus* – Student Grant Agency of the Faculty of Science USB
2012 Characterization of fucosylated glycans of *Ixodes ricinus* ticks using sugar analogues – Grant Agency of the University of South Bohemia
2013 Passage of host-sialylated glycoproteins through tick body – Grant Agency of the University of South Bohemia

International internships:

2014/2015 (3 m): Genomics group, SaBio (Sanidad y Biotecnología) research group, National Wildlife Research Institute IREC (CSIC-Universidad de Castilla – La Mancha), Ciudad Real, Spain
2010 (1 m) National Centre for Glycomics and Glycoproteomics, Department of Chemistry, Indiana University, Bloomington, IN, USA

WORK EXPERIENCE

2011 - 2020 R&D scientist, Institute of Parasitology, Biology Centre, ASCR, České Budějovice (February 2014 – May 2016, maternity/parental leave, February 2018 – August 2020 maternity/parental leave)
2013 – 2014 R&D Specialist, GEN-TREND, s. r. o.,
2022 – 2024 R&D scientist, Institute of Parasitology, Biology Centre, ASCR, České Budějovice
2016 – Research assistant, Department of Chemistry, Faculty of Science, USB, České Budějovice (February 2018 – August 2020 maternity/parental leave)

2021-2023 Member of the covid testing team – PCR and antigen testing, sample collection

Teaching Activities

2011 – 2013 Basic and Advanced biochemistry Laboratory – CZ and EN
2022 – Glycobiochemistry CZ and EN (part of the lectures)
2025 – Basic and Advanced biochemistry Laboratory – CZ and EN

Students (co-supervisor)

2010 – 2013 Rita Urbanová – Purification of hemlipoglycoprotein from *Dermacentor marginatus haemolymph*
2011 – 2014 Hana Zavadilová – Detection and localization of fucosylated glycans in tick cell lines (*Ixodes ricinus*) using alkyne derivatives of fucose.
2021 – 2023 Bc. Zuzana Dvorníková – Identification of glycosylated tick proteins containing immunogenic epitopes

Students (supervisor)

2023 – 2025 Matěj Doškář – Purification of newly synthesized tick glycoproteins
2025 – Sidney Robiczek – Comparative blocking efficiency of synthetic polymer vs animal-derived reagents in immunoassays
2025 – Hana Cábová – Use of immobilized growth factors for differentiation of primary osteoblasts

Other work for the Faculty and the Department of Chemistry

2014 SGA projects reviewer
2020 opponent of bachelor theses – František Jiřík – The effect of selected pharmaceuticals on growth, biomass and activity of soil microorganisms
2021 Den s Přírodovědou
2023 Supervisor of Petra Kozojedová during the internship through Centrum Radius – The use of immobilized factors for differentiation of primary osteoblasts
Vzdělání a řemeslo, Researcher's Night
opponent of the bachelor thesis of Manuel Stammmler – alpha-gal immunity in fish and its potential for modulating the effect of parasitic infections
2024 opponent of the bachelor thesis of Daniel Eicher – Optimizing growth conditions of new methane oxidizing and nitrifying bacteria
SGA projects reviewer
Týden s PřF, Open Day at the Faculty of Science, suburban camp in the Faculty of Science
2025 Týden s PřF, Open Day at the Faculty of Science, Researcher's night, suburban camp in Faculty of Science
SGA projects reviewer
2026 Týden s PřF, Open Day at the Faculty of Science

RESEARCH AND DEVELOPMENT ACTIVITIES

Research Focus: Glycobiology of ticks and tick-borne pathogens, allergenic glycans of arthropods, mass spectrometry, bioorthogonal chemistry, differentiation of primary cells

Practical skills: Biochemical analysis of proteins, glycoproteins, and protein-carbohydrate interactions. Labelling of proteins and glycans using bioorthogonal chemistry. In vitro tick feeding. Localization of proteins in tick tissues using immunofluorescence and electron microscopy. Research on tick lectins and glycoproteins, their characterisation, and isolation. Preparation of glycans for mass spectrometry, measurements using a MALDI TOF/TOF mass spectrometer, analysis of MALDI spectra. Work with tissue cultures, basic methods of molecular biology, aseptic work in a BSL-2 facility.

Involvement in R&D projects:

2011 – 2013 Antimicrobial proteins in immune system of competent and incompetent vectors of *Borrelia burgdorferi* spirochetes: the search for borreliacidal factor (Czech Science Foundation; P302/11/1901). Role: Member of the research team.

2018 – 2020 Interaction of genomic and subgenomic RNA flaviviruses with host and viral proteins (Czech Science Foundation, GA18-27204S), Role: Member of the research team.

2019 – 2020 Development of technology for early detection of tick-borne encephalitis based on changes in gene expression and protein production in antigen-presenting cells (MEYS INTER-ACTION LTARF18021). Role: Member of the research team

2020 – 2021 LTAUSA Interactions of flaviviruses with hosts at the transcriptional and translational level (MEYS, LTAUSA18040). Role: Member of the research team.

2024 – 2025 Specific interactions of adhesins of European spirochetes of Lyme disease with glycosaminoglycans with a well-defined structure at atomic resolution (Czech Science Foundation, 23-06525J). Role: Member of the research team.

2024 – 2026 OP-TAK - Development of diagnostic multiplex tests relevant to tick-borne emergent infections (OP-TAK CZ.01.01.01/01/22_002/0000957). Role: Member of the research team.

2025 – Tick-borne flaviviral sfRNA as an important factor of viral infection pathogenesis in the human host and tick vector (Czech Science Foundation; 25-20074L). Role: Member of the research team.

Active participation and presentations at conferences:

Sterbova J., Ondrus J., Fussy Z., Sterba J., Vechtova P., Grubhoffer L. Production of sialylated glycoproteins in *Ixodes ricinus* ticks and tick cell lines. XXIII Setkání biochemiků a molekulárních biologů. XXIV 11. a 12. 11. 2025, Brno (poster)

Sterbova J., Kozojedova P., Grubhoffer L., Sterba J. The use of immobilized growth factors for differentiation of primary osteoblasts. XXIII setkání biochemiků a molekulárních biologů. 11.11.2024 až 13.11.2024, Brno (poster)

Sterbova J., Ondrus J., Sterba J., Grubhoffer L. Detection of metabolically created sialic acid in *Ixodes ricinus* ticks and tick cell line using Click chemistry. XXII Setkání biochemiků a molekulárních biologů. 14-15.11 2023, Brno (poster)

Sterbova J., Kocova P., Grubhoffer L., Sterba J., BONCAT and Click-on-membrane reaction combined with 2D-electrophoresis in identification of differently expressed proteins in TBEV infected human neural cells, XXVI Biochemical Congress, 29.8.-1.9.2021, Ceske Budejovice, CR (poster)

Šterba J., Vancová M., Štěrbová J., Malinovská L., Wimmerová M., Grubhoffer L. Origin of Sialic Acid in Adult Tick Females (*Ixodes ricinus*), 12th international Congress of Cell Biology 21-25.7.2016, Prague, CR (poster)

Sterbova J., Ondrus J., Sterba J., Vancová M., Konik P., Kotas P., Grubhoffer L., Localization and identification of fucosylated glycoproteins in *Ixodes ricinus* ticks, 16. Škola hmotnostní spektrometrie, 13.-18.9. 2015 Frymburk, CR (poster)

Sterbova J., Sterba J., Vancova M., Zavadilova H., Grubhoffer L., Fucosylation in *Ixodes ricinus* ticks: localization and purification of fucosylated glycoproteins using bioorthogonal chemistry, 13th Bratislava Symposium on Saccharides, 22-26.6. 2014, Smolenice, Slovakia (poster)

Dupejova J., Sterba J., Tykalova H., Vancova M., Strnad M., Grubhoffer L., Fucosylation in *Ixodes ricinus* ticks: localization and purification of fucosylated glycoproteins using bioorthogonal chemistry, 17th European Carbohydrate Symposium (EuroCarb 17), 7-11.7. 2013, Tel-Aviv, Israel (poster)

Dupejova J., Molekulárnický retreat, Lesovna Svatý Tomáš, Šumava, Detection of glycans in *Ixodes ricinus* ticks using bioorthogonal chemistry. (presentation)

Dupejova J., Sterba J., Tykalova H., Vancova M., Zavadilova H., Strnad M., Bell-Sakyi L., Grubhoffer L., All that glitters is not gold ... or glycan. (Detection of glycans in *Ixodes ricinus* tick cell cultures and tissues using bioorthogonal chemistry), Biochemický sjezd, 26-29.8. 2012, Brno, CR (poster)

Dupejova J., Sterba J., Vancova M., Grubhoffer L., Characterization of the hemelipoglycoprotein from the tick, *Dermacentor marginatus*, Ticks and Tick-borne Pathogens Conference 7, 22.8.-2.9.2011 Zaragoza, Spain (poster)

Publications:

1. **Dupejova J.**, Sterba J., Vancova M., Grubhoffer L.: Hemelipoglycoprotein from the ornate sheep tick, *Dermacentor marginatus*: structural and functional characterisation. *Parasites&Vectors*, 2011, 4:4. IF 2,9
2. Sterba J., **Dupejova J.**, Fiser M., Vancova M., Grubhoffer L.: Fibrinogen-related proteins in ixodid ticks. *Parasites&Vectors*, 2011, 4:127. IF 2,9
3. Vancova M., Sterba J., **Dupejova J.**, Simonova Z., Nebesarova J., Novotny M.V., Grubhoffer L.: Uptake and incorporation of sialic acid by the tick *Ixodes ricinus*. *Journal of Insect Physiology*, 2012, 58:1277-1287. IF 2,37
4. Sterba J., Vancova M., **Sterbova J.**, Bell-Sakyi L., Grubhoffer L.: The majority of sialylated glycoproteins in adult *Ixodes ricinus* ticks originate in the host, not the tick. *Carbohydr Res*, 2014, 389:93-99. IF 1,9
5. Hönig V, Svec P, Halas P, Vavruskova Z, Tykalova H, Kilian P, Vetiskova V, Dornakova V, **Sterbova J**, Simonova Z, Erhart J, Sterba J, Golovchenko M, Rudenko N, Grubhoffer L.: Ticks and tick-borne pathogens in South Bohemia (Czech Republic) – Spatial variability in *Ixodes ricinus* abundance, *Borrelia burgdorferi* and tick-borne encephalitis virus prevalence. *Tick Ticks Borne Dis*, 2015, 6:559-67. IF 2,7
6. Vechtova P, **Sterbova J**, Sterba J, Vancova M, Rego R.O.M, Selinger M., Strnad M., Golovchenko M., Rudenko N, Grubhoffer L.: A bite so sweet: the glycobiology interface of tick-host-pathogen interactions. *Parasites & Vectors*, 2018, 11:594. IF 3
7. **Sterbova J.**, Kocova P., Pekarek L., Selinger M., Ondrus J., Grubhoffer L., Sterba J.: Click-on-membrane for detection of metabolically labelled proteins and RNA. *CHEMICKE LISTY*, 2021, 115:662-668. IF 0,35
8. **Sterbova J**, Grubhoffer L, Sterba J. Immunogenic and cross-reactive carbohydrate determinants in ticks and other arthropods. *J Appl Biomed*, 2026, 24:63-71. IF 2.0
9. **Sterbova J**, Ondrus J, Fussy Z, Vechtova P, Grubhoffer L, Sterba J. Click chemistry reveals sialylated glycoprotein biosynthesis in *Ixodes ricinus* ticks and tick-derived cells. *Int J Biol Macromol*. 2026, 365:152305. IF 8.5