Extracellular vesicles:

Biogenesis, Co-evolution and Insights from Parasitology.

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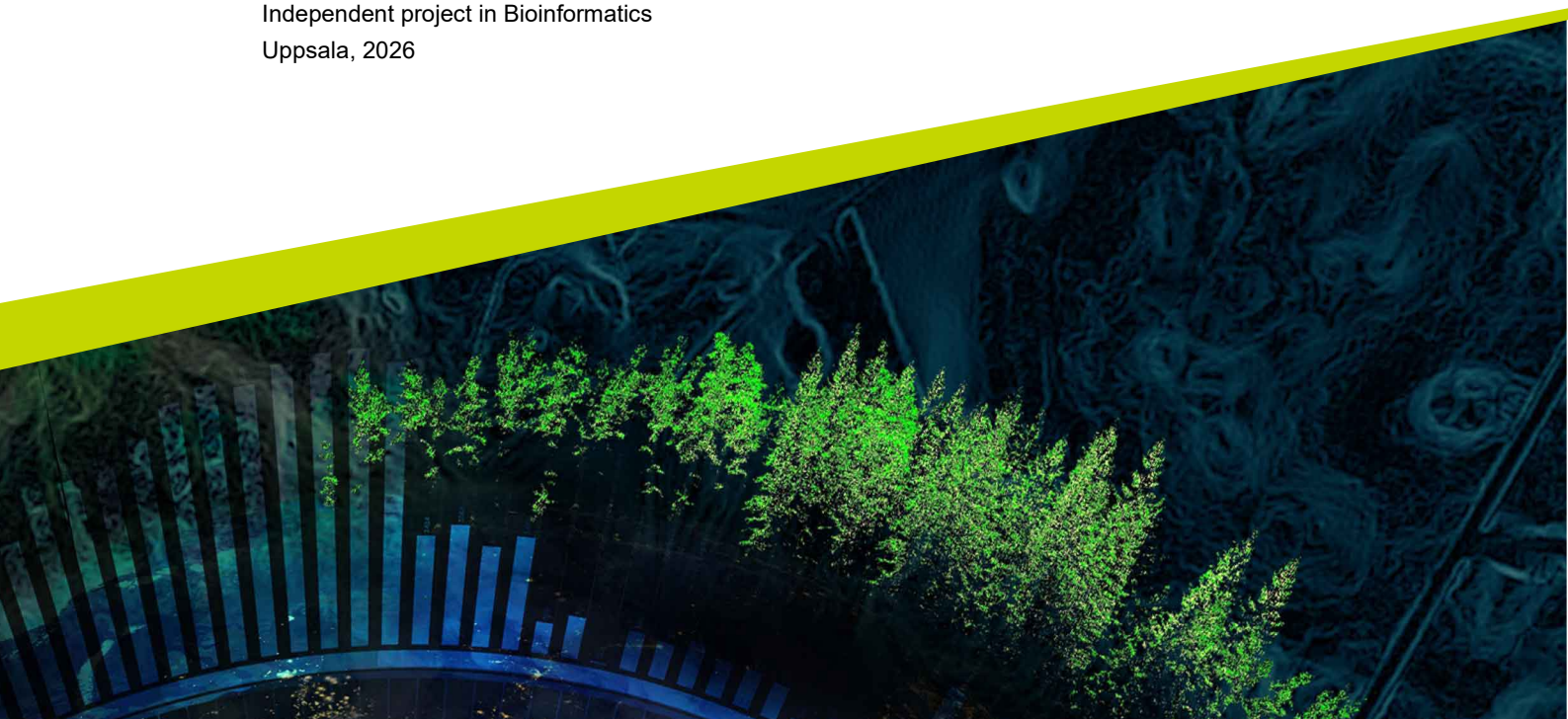

Extracellular vesicles: Biogenesis, Co-evolution and Insights from Parasitology.

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Abstract

This thesis proposes a holistic view of extracellular vesicle cargo as a reflection of integrated and dynamically regulated cellular networks in moment of time of operation, as a Multi-Machinery Model of Cellular Communication, conceptualized from a system level perspective of extracellular vesicle cargo formation integrating theoretical analysis of nature of extracellular vesicles with the practical application of bioinformatics, aiming to contribute to the development of an anthelmintic vaccine. By employing Python-based computational methods and leveraging data from the NCBI database on extracellular vesicle contents, this work facilitates peptide and epitope structural candidate analysis to further vaccine design.

Keywords: Extracellular vesicles, Multi-Machinery Model, In Silico Vaccine Design, *Schistosoma mansoni*, Molecular Docking, Anthelmintic Resistance, Computational Biophysics

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I would like to extend my deepest gratitude to my feline co-investigators, Kanda and Loki, for their consistent role as the primary catalysts for my inspiration throughout this degree project.

Special recognition is due to Loki for his paws-on contribution to the conceptual framework of this study; through his rigorous methodology of napping on and chewing on primary literature, he ensured a unique level of engagement with the material. It is undeniable that the exploration of *Lokiarchaeota* and its pivotal role as the evolutionary junction between prokaryotic and eukaryotic membrane trafficking would not have reached its current depth, without his persistent interest.

This work is dedicated to the memory of Loki, who suddenly and unexpectedly succumbed to large cell leukemia after sixteen years of close to optimal feline health.

This thesis highlights the critical work being done at the Department of Animal Breeding and Genetics at SLU and the University Animal Hospital (Universitetsdjursjukhuset, UDS in the field of veterinary oncology. Loki's passing underscores the urgent clinical need to resolve the challenges addressed in this work: specifically, the significant biological variability in extracellular vesicle release rates between individuals and the current lack of a "neutral baseline" for liquid biopsy diagnostics in both animals and humans.

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1. Introduction

Extracellular vesicles (EVs) have emerged as mediators of intracellular communication in cell biology, transporting molecular cargo - proteins, lipids and nucleic acids between cells. Their role in physiological processes and disease states has made them a topic of research in molecular biology. Comprehension of the biogenesis, membrane composition and cargo regulation mechanisms of EVs is fundamental for analysis of cargo as well as the understanding of functional diversity and biological significance.

2. Extracellular Vesicles

Extracellular vesicles (EVs) are nanoscale lipid bilayer – enclosed particles secreted by nearly all cell types. They inhabit functions in intercellular communication, facilitating the exchange of biomolecules such as proteins, lipids, DNA and RNA. EVs are natural nanocarriers, exhibiting high biocompatibility making them promising tools in drug delivery and medicine. Their diversity is still not fully known, but different EVs show links to processes with genetic influence on the cell, involvement in the pathogen-host interactions, immune modulation, as well as inhabiting a key role in antibiotic and anthelmintic resistance mechanisms and cellular homeostasis (Du et al., 2023).

2.1 Membrane Composition of EVs

Extracellular vesicles are enclosed by a lipid bilayer membrane; their membrane composition is highly specialized, differing significantly from the membrane of their parent cell. The lipid profile of EVs is enriched in sphingomyelin, cholesterol, and di-saturated lipids, contributing to a greater degree of membrane rigidity compared to the cellular plasma membrane (Haraszti et al., 2016). They incorporate a diverse array of lipid molecules, including steroids, sphingolipids, and glycerophospholipids. The distribution of these lipids within the bilayer is asymmetric, with phosphatidylserine predominantly localized to the inner leaflet, while the outer leaflet is characterized by the presence of very long-chain sphingolipids and phosphatidylcholine (Simpson et al., 2008; Doyle & Wang, 2019). Lipid geometry inhabits a critical role in shaping membrane curvature, with cylindrical lipids such as phosphatidylcholine forming flat monolayers, conical lipids like phosphatidylethanolamine inducing negative curvature, and lipids with large head groups, such as lysophosphatidylcholine, promoting positive curvature (Larsen et al., 2020; Graham & Kozlov, 2013; Petelska et al., 2011).

In addition to lipids, EV membranes contain a diverse array of proteins which reflect their cellular origin and functional specialization. (Abels & Breakefield, 2021). Among the most commonly identified EV-associated proteins are tetraspanins, including CD9, CD63, and CD81, which play a role in vesicle biogenesis and cargo sorting (Mizenko et al.,2021). Other key proteins include components of the ESCRT pathway, such as TSG101 and ALIX (Kumar et al., 2024) as well as heat shock proteins HSP70 (Komarova et al., 2021) and HSP90 (Sager et al., 2022) which contribute to protein stabilization and stress responses.

Furthermore, the proteomic profiles of EVs reveal tissue-specific markers with distinct protein signatures for respective tissues. Several membrane-associated proteins such as Rab GTPases, annexins, and flotillin (Raposo & Stoorvogel, 2013) further define EV composition, influencing vesicle trafficking and membrane dynamics (Kumar et al., 2024).

2.2 EV Function

Extracellular vesicles are a dynamic intercellular communication tool and function as carriers of biomarkers, acting as nanoscale shuttles which transport a diverse array of biomolecules, including proteins, lipids, nucleic acids (mRNAs, miRNAs, DNA fragments), and even signaling molecules, between cells. This process transcends simple waste disposal, rather, it is a highly regulated mechanism for targeted delivery of functional cargo, thereby influencing the recipient cell's phenotype and behavior. EVs are integral components of complex biological systems, impacting processes ranging from immune modulation and tissue homeostasis to disease progression (Doyle & Wang, 2019).

Cells strategically leverage EVs to remodel their microenvironment, influence distant tissues, and coordinate multicellular responses. For example, in regard to cancer, tumor-derived EVs can promote angiogenesis (Ateeq et al., 2024), suppress immune responses (Robbins & Morelli, 2014), and facilitate metastasis by delivering oncogenic proteins and miRNAs to recipient cells (Becker et al., 2016). Conversely, in regenerative medicine, EVs derived from stem cells can promote tissue repair and reduce inflammation by delivering growth factors and immunomodulatory molecules to damaged tissues (Wang & Pan, 2023).

Extracellular vesicles function as units of biological information transfer, facilitating molecular delivery across proximal and distal signaling axes. Within the immediate environment, EVs are secreted into the extracellular matrix to coordinate cellular communities through paracrine and juxtracrine interactions. At transition to systematic communication, in higher pressure environments such as bloodstream, liquor or urine, the vesicle requires remodeling and protection. At entrance into a biofluid a dynamic biomolecular corona is gathered, as vesicles are surrounded by host proteins and metabolites that interact with the vesicle surface (Esmaeili et al., 2025). The study of EVs, therefore, offers a promising avenue for developing novel diagnostic biomarkers and therapeutic strategies. The potential is derived from the encapsulated cargo content, tissue specific membrane proteins reflective of cell of origin or the dynamically acquired biomolecular corona proteins. To this end, understanding the complexities of EV biology is critical for advancing our knowledge of intercellular and environmental organism communication across domains.

2.3 Biogenesis of EVs

EVs show variation in size and biogenesis when observed in nature, thus, to distinguish within the variety, the vesicles are grouped into three large groups.

The exosomes are 40-100 nm (Simpson, Jensen, & Lim, 2008) and are communication and delivery tools of the cell originate from the inward budding of endosomal membranes. Microvesicles, or ectosomes, appear to carry similar functions to the exosomes but are larger 100 -1000nm (Du et al., 2023) in size and arise through the outward budding of the plasma membrane. The final group, apoptotic bodies, are the giants at 500 - 5000 nm (Du et al., 2023), formed as one of the steps of programmed cell death and contains various cellular debris.

Exosomes are the most extensively studied class of extracellular vesicles. They are found *in vivo* in body fluids such as blood, urine, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, and breast milk (Simpson, Jensen, & Lim, 2008). They originate from multivesicular bodies (MVBs), specialized endosomal compartments formed during endosome maturation, which give rise to exosome upon fusion with the plasma membrane (Du et al., 2023).

Endosomes are organelles which serve as sorting stations within the cell. Maturation of MVBs involves a coordination of molecular switches and lipid modifications, the Rab GTPase is a switch which changes RAB5 into RAB7, signaling transition of early to late endosome (Xu et al., 2023). Another change which happens is the change of the membrane lipid composition, with a decrease of expression of phosphatidylinositol 3-phosphate (PtdIns3P) and an increase in phosphatidylinositol 3,5-bisphosphate ($PtdIns(3,5)P_2$). These molecular changes facilitate sorting of cargo proteins and cargo organization by process called fission, mediated by the Eps15 homology domain-containing (EHD) proteins, evolutionarily conserved group of ATP- binding proteins (Wallroth & Haucke, 2018).

2.4 Endosomal System and Multivesicular Bodies

Understanding mechanisms of endosomes provides valuable insight into sorting and cargo loading regulatory mechanisms occurring within the MVBs of the EVs cargo (Huotari & Helenius, 2011).

Endosome organelles are characterized by their life cycle stages; the early ones are marked with RAB5a (Nagano et al., 2019), RAB4 (Nag et al., 2018), transferrin receptors (Sheff et al., 2002) and EEA1 (Wilson et al., 2000). Late endosomes go through membrane metamorphosis, containing intraluminal vesicles and are marked by the RAB7(Feng & Wandinger-Ness**,** 1995), RAB9 (Ganley et al., 2004) and mannose 6-phosphate receptors (Nishida et al., 2024). Later stage endosomes are marked by RAB11, and are involved in intracellular recycling processes (Zulkefli et al., 2019).

Several sorting mechanisms are involved in the formation of multivesicular bodies and extracellular vesicles. A key role is occupied by the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, which consists of multiprotein complexes (ESCRT-0, -I, -II, -III) and the VPS4 complex. These components are essential for sculpting and severing cellular membranes. The ESCRT machinery is an ancient, evolutionarily conserved membrane remodeling system found across all domains of life (Du et al., 2023).

2.5 ESCRT Machinery across domains

The ESCRT machinery is a group of protein complexes which work together to reshape cell membranes and create small vesicles inside cells. The process can be broken down into recognition of marked proteins by ESCRT-0 which scouts and recognizes proteins marked with the ubiquitin molecule. Specificity of the ubiquitin is determined by E3 ubiquitin ligases, which recognizes signal features such as degrons, phosphorylation sites, and post-translational modifications, thereby directing proteins into distinct signaling and sorting pathways (Abels et al., 2021).

The future vesicle starts to form when ESCRT-I and ESCRT-II build a bridge, gathering ubiquitin marked proteins into one area. The bridge bends the cell membrane inwards, creating a small pocket around the scouted proteins. The vesicle forms when ESCR-III constructs a spiral filament around the neck of the developed membrane pocket; the filaments become shorter, forming a neck and at the end, separate the pocket into a distinct vesicle (Henne et al., 2011). However,

efficient membrane scission and recycling of the ESCRP machinery require the adaptor protein ALIX, which bridges ESCRT-III to the ATPase VPS4, enabling final vesicle release and disassembly of the ESCRT complex (McCulloughet al., 2018).

In eukaryotic cells, the ESCRT machinery is highly complex. It mediates the sorting of ubiquitinated proteins into intraluminal vesicles (ILVs) within MVBs for further processing by lysosomes or secretion as EVs (Meldolesi, 2018).

In plants, the ESCRT machinery is encoded by a large number of isoforms, including ESCRT-I, -II, -III, and VPS4/SKD1-LIP5 complex but lacks the ESCRT- 0 complex found in eukaryotes. Instead TOM1-like (TOL) proteins partially substitute by initiating cargo recognition and upstream machinery (Richardson et al., 2011). Plant-specific components like FREE1 regulate MVB biogenesis participating in plant specific phase separation mechanisms needed for membrane vesiculation (Gao et al., 2015). The ESCRT-III polymers form spirals filaments in plants dynamically remodeled by VPS4/SKD1 to constrict vesicle necks modification of the membrane. Unlike in other organisms, these necks in plants often remain stable rather than undergoing full scission leading to membranes separating into internal vesicles. This mechanism might be evolutionary related to ESCRT mechanisms which the plant uses for formation of luminal vesicles, the plant vacuoles, which are multifunctional organelles full of water that the cell uses for stability and hydration (Weiner et al., 2025).

In bacteria, there is no homolog to VPS4. ESCRT-like systems are simplified compared to eukaryotes. Bacteria are lacking a VPS4 homolog but are instead utilizing proteins such as Vipp1 and PspA which are structurally homologous to ESCRT-III for membrane remodeling under stress (Liu et al., 2021).

Extracellular vesicle contents are conserved across bacterial domains, containing proteins, nucleic acids and lipids as well as mediators of virulence, communication and environmental adaptation. Gram-negative cargo sorting is often passive; the vesicle bud is formed from outer membrane and driven by the lipopolysaccharide (LPS)-peptidoglycan crosslinking or VacJ/Yrb ABC transporter mutations (Schwechheimer & Kuehn, 2015). Gram positive bacteria show selective packaging processes and regulatory mechanisms. Streptococcus aureus has selective and stress inducible packaging of the β-lactamase (BlaZ) by the sublethal β-lactam, enabling horizontal transfer of antibiotic resistance. BlaZ loaded EVs show survival advantages to ampicillin-susceptible strains. (Bose et al., 2020).

Archaea possess sophisticated ESCRT which predate eukaryotic complexity, integrating VPS4-like ATPases and ubiquitin-like tagging for cargo organization. These ancestral systems demonstrate that core membrane remodeling and cargo sorting mechanisms existed long before the emergence of compartmentalized eukaryotic cells.

Archea *Asgard superphylum* is the evolutionary junction link between prokaryotic and eukaryotic membrane trafficking. Structurally, *Asgard* ESCRT-III assemblies resemble bacterial PspA/Vipp1 systems in their outward-facing α5 "spikes" but share conserved intersubunit interfaces with eukaryotic CHMP1B/IST1 complexes. Despite originating from archaea, *Lokiarchaeota* (*Loki*) exhibits the ability to interact with eukaryotic like phospholipids, highlighting its role as an evolutionary ancestral link between domains (Melnikov et al., 2025).

Archaeal ubiquitin-like proteins (SAMPs) often serve dual roles as sulfur carriers and protein modifiers. Gene duplication and diversification of ESCRT-III/VPS4 in archaea likely laid the groundwork for eukaryotic innovations, such as multivesicular body formation (Maupin-Furlow, 2013).

2.6 ESCRT independent Pathways

Cells utilize several ESCRT-independent pathways for extracellular vesicle biogenesis. Certain lipids can spontaneously induce membrane curvature by forming microdomains within the plasma membrane. A notable example is the ceramide-dependent pathway, where ceramide metabolism regulates membrane bending and budding, leading to vesicle release. The GTPase RAB31 promotes EV formation by inhibiting multivesicular endosome (MVE) degradation, effectively upregulating exosome secretion (Wei et al., 2021).

Some proteins possess self-sorting structural properties, allowing them to induce vesicle budding and cargo packaging. For instance, Pmel17, a protein involved in melanosome biogenesis, can self-assemble and trigger vesicle formation (Hoashi et al., 2006). Tetraspanins CD63 and CD9, enriched in extracellular vesicle membranes, are hypothesized to regulate EV uptake and cargo delivery due to their roles in membrane dynamics and viral entry. However, in recent years, CD63 analysis displayed reduced EV production yet did not impair delivery efficiency, and CD9 was recognized to have no impact on macropinocytosis-driven uptake or pH-dependent endosomal cargo release. The role of tetraspanins

is theorized to not be part of the regulatory communication involvement but active instead in the biogenesis or membrane organization of EVs (Tognoli et al., 2023).

The tetraspanins such as CD9, CD63 and CD81 organize specialized tetraspanins enriched microdomains (TEMs) within cellular membranes, serving as areas of protein clustering and facilitating vesicle biogenesis. These microdomains influence the membrane curvature in the ESCRT independent mechanisms, and the TEMS modulate curvature, supporting vesicle budding and fusion from the parent membrane. The Syndecan-Syntenin-Alix pathway exemplifies this alternative mechanism in which syndecans interact with syntenin and ALIX, stimulating intraluminal budding within endosomes. This process selectively enriches exosomes with cargo such as syntenin-1, syndecan and CD63, while leaving markers CD9 and CD81 unaffected (Roucourt et al., 2015).

3. Cargo Regulation of Extracellular Vesicles

Extracellular vesicles (EVs) serve as essential mediators of intercellular communication, transferring bioactive molecules such as proteins, lipids, and genetic material between cells. The cargo composition of EVs is precisely regulated to ensure that specific molecular messages are conveyed in response to environmental stimuli. This regulation is orchestrated by a complex network of signaling pathways which influence EV biogenesis, cargo sorting, and secretion dynamics (Marquant et al., 2025).

3.1 Mechanisms

Due to the interconnected nature of protein activation and cellular responses, regulatory mechanisms can be examined as individual pathways and in the broader context of cellular signaling, as responses to different stimuli. Several pathways are involved in modulating EV production and cargo selection, each with specific genetic components which contribute to functional diversity.

3.1.1 Hypoxia-inducible factor 1-alpha (HIF-1α)

Hypoxia-inducible factor 1-alpha (HIF-1α), encoded by the HIF1A gene, is a transcriptional regulator of cellular responses to low oxygen conditions. Under normoxia HIF-1a is hydroxylated by prolyl hydroxylases (PHDs) and targeted for proteasomal degradation by the von Hippel-Lindau (VHL) protein. When oxygen tension drops below 2%, PHD activity is inhibited, resulting in nuclear translocation of HIF-1a where it dimerizes with HIF- β and activated hypoxia responsive genes such as GLUT1, PDK1, VEGF, and EPO, which enhance glycolytic metabolism, promote angiogenesis, and alter EV cargo composition to adapt to low oxygen environments. Mutations in HIF1A or VHL deletions can result in constitutive HIF-1α activation, potentially increasing EV production and influencing pathological processes like cancer, where it affects vascularization, energy metabolism, and tumor progression (Muñiz-García et al., 2022).

3.1.2 Transforming Growth Factor-Beta (TGF-β)

TGF-β signaling modulates extracellular vesicle production and cargo, thereby influencing intercellular communication and various physiological processes, including immune regulation and tissue remodeling (Rodrigues-Junior et al., 2022).

The process begins with transforming growth factor-beta, TGF-β, existing primarily in latent non active form, and requires activation. Once activated the latent TGF-β ligand binds to the TGFBR2 receptor on the cell surface, initiating a signaling cascade where TGFBR2 recruits and phosphorylates TGFBR1 starting a signaling cascade. Phosphorylated TGFBR1 activates SMAD proteins (e.g.,SMAD 2/3) which form complexes with SMAD4 and translocate to the nucleus. These formed complexes act as transcription factors regulating genes critical for biogenesis of EVs (Rodrigues-Junior et al., 2022). One key target is gene RAB27B, which, when upregulated, enhances exosome secretion by modulating vesicle trafficking. By quantitative proteomics, it was discovered that TGF-β increases quantity of protein cargo and changes composition of EVs by downregulating expression of the RAB27B (Li at al., 2024).

This pathway also modulates extracellular vesicle cargo sorting through the involvement of a positive regulatory mechanism, specifically the direct interaction between TGF-β and syntenin. Syntenin, which also interacts with syndecans, directs the selective incorporation of molecular cargo into EVs. The interaction between TGF-β and syntenin prevents degradation of TβR1 and enhances SMAD signaling activation. Functioning as an adaptor and scaffold protein, syntenin facilitates selective sorting of biomolecules into exosomes in cargo sorting. This syntenin-mediated process is dependent on the two tandem PDZ domains, which serve as structural basis in mediating these cellular processes (Lee et al., 2023).

TGF-β is also playing a role in the immune homeostasis and regulation of balance between immunosuppressive regulatory T cells and pro-inflammatory T helper17 cells. Activated macrophages secrete the TGF-β predominantly in the latent form, where it is non-covalently associated with the latency-associated peptide and complexed with latent TGF-β binding protein in the extracellular matrix (Wang et al., 2023). This duality positions TGF-β signaling as a potential target for development of extracellular vesicles based immune stimulants and immune regulatory particles, which could therapeutically recalibrate immune responses in the host.

Dysregulations of TGF-β is linked to genetic mutations in key components such as gene encoding TGF- β1 and receptors TGFBR1 and TGFBR2 as wells as the

intracellular SMAD2, SMAD3 and SMAD4 regulators (Bertrand-Chapel et al., 2022). Mutations in TGFBR1 are observed in a wide range of malignant cancer types (Moore-Smith & Pasche, 2011).

The influence of the TGF-β has been studied in tumor microenvironments. In breast cancer cells, a hyperactivation of TGF-β leads to a marked increase in the secretion of small extracellular vesicles (sEVs) which in turn amplify TGF-β signaling beyond what is achieved by control group with soluble TGF-β, indicating a secondary activator role. These sEVs act as pro-metastatic signals to neighboring cells and may play a role in regulation of less aggressive cancer cells into invasive metastatic phenotypes. These mechanisms could be disrupted by dimethyl amiloride leading to reduction of signaling, furthering the argument for the need of a therapeutic tool to battle trafficking inhibitors beside receptor blockers in cancer treatment (Teixeira et al., 2023).

3.1.3 Wnt / β- Catenin

The Wnt/ β-catenin signaling pathway is a cellular communication mechanism which regulates formation of extracellular vesicles, which have been implicated in cancer metastasis. The pathway is initiated by binding of the Wnt proteins, a family of secreted signaling glycoproteins to Frizzled receptors, a family of seven-transmembrane receptors, and their co-receptors, such as LRP5/6, on the cell surface. This ligand-receptor interaction triggers a cascade of events inside the cell which affects gene expression, ultimately benefiting the cell by regulating processes like cell growth, differentiation, and migration (Pascual et al. 2025).

The pathway is broadly divided into canonical (β-catenin dependent) and non-canonical (β-catenin independent). The canonical Wnt pathway is distinctly linked to EV regulation. In this pathway, Dishevelled (Dvl) protein is recruited to the Wnt-Frizzled-LRP5/6 complex, which prevents the β-catenin destruction complex composed of Axin, APC, GSK-3β, and CK1 from phosphorylating β-catenin. Normally, this complex phosphorylates β-catenin, targeting it for ubiquitination and proteasomal degradation. This stabilization of cytoplasmic β-catenin allows it to translocate to the nucleus and upregulate Wnt target genes, including those involved in EV production such as Caveolin-1(Cav1) (Song et al., 2024).

Cav-1 is a regulatory gatekeeper and is a key protein component of caveolae, which are small, flask-shaped invaginations of the plasma membrane. These structures are involved in various cellular processes, including endocytosis, signal transduction, and lipid regulation. Cav-1 facilitates the selective loading of specific proteins, lipids, and nucleic acids (including miRNAs) into EVs. Cav-1

interacts with RNA-binding proteins like hnRNPA2B1, which then bind to specific miRNAs. This complex facilitates transcription of target genes involved in cell proliferation, differentiation, and survival (Ni et al., 2020; Lee et al., 2019; Lecarpentier et al., 2019).

In contrast, CD9 expression has been shown to downregulate the Wnt/ β-catenin signaling pathway, providing a negative regulatory control on this EV associated signaling axis (Chairoungdua et al., 2010).

3.1.4 Sphindolipid signaling

In previous research, the ceramide rich vesicles were identified as triggers for exosome budding before the importance of extracellular vesicles was fully understood (Trajkovic et al., 2008). It is currently recognized that the formation of EVs is actively induced by the sphingolipid ceramide and sphingolipid signaling mechanisms. Sphingolipids are a class of lipids found in eukaryotic cell membranes, particularly abundant in lipid rafts. Beside being structural components, sphingolipids are bioactive molecules involved in apoptosis, cell migration, inflammation and EV transport (Horbay et al., 2022).

Ceramide, a bioactive sphingolipid with signaling properties that induces negative membrane curvature due to its molecular geometry and long hydrophobic tails in aqueous environment. By that process, ceramide promotes EV formation by driving membrine budding and formation of intraluminal vesicles within multivesicular bodies, as well as other vesicles that require membrane curvature for their biogenesis (Ghadami & Dellinger, 2023).

The enzyme neutral sphingomyelinase 2 (nSMase2, also known as SMPD3) hydrolyses sphingomyelin to generate ceramide, increasing local ceramide concentrations in the membrane and promoting the formation of ceramide rich microdomains associated with enhanced EV production. SMPD3 activity is upregulated by cellular oxidative stress (Xi et al., 2024).

The sphingolipid composition and biogenesis of EVs have been linked to the ESCRT-dependent pathway through the regulation of ceramide concentration by CERT-Tsg101 formation. In experiments, overexpression of CERT upregulated the secretion of EVs, while downregulation reduced EV formation and ceramide and sphingomyelin concentration in EVs (Crivelli et al., 2022).

3.1.5 ESCRT Regulation

Ubiquitination of cargo proteins serves as a primary signal for ESCRT-mediated sorting (Henne et al., 2011). Attachment of ubiquitin molecules to target specific proteins directs them to ESCRPT-0 complex that facilitates reaction with HRS and STAM1. Regulation of ubiquitination process is influenced by activation of alpha V integrins (αV integrin), while TGF-β signaling network contributes to the coordinated sorting of proteins into extracellular vesicles. The mechanism also has adaptors such as ARRDC and ubiquitin ligase NEDD4 that show selective cargo sorting characteristics within this pathway (Ju et al., 2025).

3.1.6 Tetraspanin-Enriched Microdomains

Proteins such as CD9, CD81, and CD63, integral components of the tetraspanin family, are frequently employed as markers for the identification and study of extracellular vesicles. These transmembrane proteins were historically posited to orchestrate the formation of microdomains at the cell surface, thereby playing a central role in EV biogenesis and cargo selection (Kowal et al., 2016).

Recent investigations have challenged this paradigm; a study utilizing CRISPR-Cas9 technology to generate MCF7 breast cancer cell lines lacking CD9, CD81, and CD63, both individually and in combination, revealed minimal influence on the overall protein composition of EVs. This research also explored the hypothesis that EV functionality is contingent upon their cellular origin, with plasma membrane-derived EVs and endosome-derived EVs exhibiting distinct protein profiles. Data suggests that CD9-positive EVs may originate predominantly from the plasma membrane, while CD63-positive EVs are more frequently associated with endosomal compartments, aligning with their exosomal classification (Fan et al., 2023).

The same study highlighted structural and functional similarities within EV subpopulations, particularly between those expressing CD9 and CD81. While individual knockout of CD9 or CD81 did not significantly impact EWI-2 levels, simultaneous ablation of both tetraspanins resulted in a marked reduction in CD9P-1 and EWI-2 protein abundance within EVs, corroborating a direct association between these molecules (Fan et al., 2023).

The EWI-2 protein, localized via interactions with tetraspanins, functions as an immunoglobulin-like domain protein implicated in cell-cell adhesion processes. It is involved in the PI3K/AKT signaling pathway which suggests a role in cell

proliferation and survival mechanisms (Kummer et al., 2020). Expression level of EWI-2 has been correlated with both favorable and unfavorable prognoses in various metastatic cancers, indicating a complex and context-dependent function (Fan et al., 2023). EWI-2 is also expressed on immune cells, particularly on the B cells (Le Naour et al., 2004).

3.1.7 Rab GTPase Signaling

Rab GTPases are switch regulators which are synthesized in an inactive form in the cell and are activated by the Rab guanylate exchange factor. The active form of Rab GTPases are monomeric GTP-binding proteins which ensure that EV cargos are delivered to their destinations within endocytic trafficking (Langemeyer et al., 2014).

The Rab protein family exhibits remarkable functional diversity, with nearly 70 membranes, making it the largest branch of the Ras small GTPase superfamily. Several members are associated with regulation of intracellular trafficking. Once activated, the Rab GTPases orchestrate the delivery of cargo proteins to their cellular compartments, supporting extracellular vesicle formation with cargo (Raghavan et al., 2022).

Rab4 is primarily involved in mediating rapid recycling of endocytosed material from early endosomes back to the plasma membrane, thereby facilitating efficient membrane turnover and receptor recycling (Wilson et al., 2023). Rab5 is essential for the early stages of endocytosis, where it promotes the fusion of clathrin-coated vesicles and the formation of early endosomes (Saitoh et al., 2017). As endocytic cargo progresses through the pathway, Rab7 assumes a central role in directing the transport from early to late endosomes and ultimately to lysosomes for degradation (Mulligan & Winckler, 2023). Rab9 contributes to intracellular trafficking by regulating the movement of cargo from late endosomes to the trans-Golgi network, which is important for the recycling of specific proteins and lipids (Barbero et al., 2002). Rab10 orchestrates sorting events during endosome recycling, ensuring the proper distribution of membrane components (Etoh & Fukuda, 2019). Rab11 is recognized for its function in controlling slow recycling processes, particularly through the perinuclear recycling compartment, thereby maintaining cellular homeostasis (Ferro et al., 2021). Rab35 is implicated in the regulation of fast recycling pathways and also participates in cytokinesis, underscoring the diverse and critical functions of Rab GTPases in cellular trafficking dynamics (Koutanti et al., 2006).

3.1.8 Tp53 Tumor Suppressor

The TP53 gene encodes the p53 protein, an evolutionary conserved tumor suppressor which maintains genomic integrity and cellular stress responses. Upon activation on signals such as DNA damage, oncogene activation or hypoxia the p53 acts as a transcription factor, modulating expression of genes involved in cell cycle. In the context of EV biosynthesis, the p53 protein adapts a key role in apoptosis, indirectly participating in the signaling pathway for EV formation (Aubrey et al., 2016).

Mutations in the TP53 gene are the most common genetic alterations in human cancers. Mesenchymal stem cell (MSC)-derived exosomes are a type of extracellular vesicles enriched with bioactive molecules that mediate many of the immunomodulatory effects attributed to MSCs. In hematological malignancies, these exosomes can induce cell cycle arrest in leukemia cells via the p53 pathway and as such, reduce chemoresistance (Saadh et al., 2025).

Extracellular vesicles are increasingly recognized as important mediators in cancer development and progression, owing to their ability to encapsulate and transport mutant p53 proteins and other oncogenic cargo to neighboring cells (Aubrey et al., 2016).

4. Cellular communication

The cargo composition of extracellular vesicles reflects the physiological and pathological state of the cell. Under conditions of cellular stress, molecular composition of EVs is altered. These changes show dynamic characteristics of cellular adaptability to signaling from outside of the cell (Abramowicz et al., 2019).

Cellular communication is a process by which cells exchange information to coordinate their activities, maintaining homeostasis, and responding to environmental changes. Previously, cellular signaling was understood as an exchange of discrete, soluble signaling molecules. These molecules traverse the extracellular space either bound to carrier protein, if hydrophobic, or as freely diffusing entities when hydrophilic, ultimately interacting with specific transmembrane receptors on target cells (Su et al., 2024).

This communication relies on the transmission of signals which can be classified according to the distance over which they act and are delivered. Traditionally, the three models of signaling recognized are autocrine, paracrine and endocrine.

With the current understanding that extracellular vesicles are capable of protecting the cargo from degradation as well as travel across large distances between the cells to the target tissue, the complexity of signaling processes might involve multiple types of stimuli transfer pathways (Seger & De Keukenaer, 2021).

Autocrine communication is when the cell communicates with its own receptors, regulating responses with feedback loops and fine tuning of cellular and DNA activity. During infection, the mechanics of cargo loading and release of extracellular vesicles can be hijacked, forcing the cell to reabsorb a loaded vesicle to promote cellular proliferation. Examples of this have been observed in oncological studies; the mechanism is pervasive across many cancer types with some common genetic signatures such as exhibiting loops where the tumor secretes factors which act as cellular receptors involving growth factors (TGF-β, EGF, VEGF) and cytokines (IL-6 and TNF-α) and promoting proliferation, survival and migration.

Another autocrine mechanism is Wnt signaling in gynecological, breast and colorectal cancers leading to β-catenin activation and tumor progression (Schauer et al., 2025).

Paracrine signaling is a secretion of signaling molecules over short distances to the cells within the microbiome. Extracellular vesicles are the mediators of these processes, carrying cargo to the recipient cells. Membrane structure of the EVs can display proteins and antigens, interacting with immune cells to stimulate and suppress local immune responses. This mechanism carries potential for vaccine development. Mesenchymal stem cells derived EVs have demonstrated a role in enhanced angiogenesis, fibroblast and re-epithelialization in wound models, carrying potential for regenerative medicine. This form of signaling is also known as horizontal gene transfer, in the context of antibiotic resistance, where EVs transfer genetic material with resistance genes to other bacteria (Amabebe et al., 2024).

Endocrine signaling is a communication tool of complex organisms. It occurs when a signaling molecule is released into the system, blood or intracellular space, to travel for longer distances and longer periods of time to reach the target cells in different tissues or organs. Circulating EVs have been identified as carriers of pathogenic proteins in neurodegenerative diseases and shown ability to cross the blood-brain barrier (Kumar et al., 2024).

4.1 Stress

Cells are continuously exposed to an array of stimuli, these stimuli can be classified into physiological and pathological categories. In response to such stimuli, cells initiate signaling cascades. Stress mediators can be categorized as part of well characterized signaling pathways (Sarapultsev et al., 2023; Yaribeygi et al., 2017).

4.1.1 Hypoxia

Cells depend on oxygen for essential metabolic and biosynthetic processes. Under conditions of low oxygen availability, or hypoxia, they activate adaptive mechanisms to preserve homeostasis and ensure survival. A key regulator of this response is the hypoxia-inducible factor 1-alpha (HIF-1α) pathway, which modulates gene expression to support metabolic adaptation, intracellular trafficking, and increases secretion of extracellular vesicles (EVs) triggered by hypoxic stress (Fleshner et al., 2017; Sarapultsev et al., 2023).

Under normoxic conditions, HIF-1α is continuously synthesized but rapidly degraded to prevent unnecessary activation of hypoxia-responsive genes. This degradation is mediated by prolyl hydroxylase domain enzymes, which act as oxygen sensors by catalyzing the hydroxylation of specific proline residues on HIF-1α (Pro402 and Pro564 in humans). Hydroxylation serves as a recognition signal for the von Hippel-Lindau protein, a crucial component of the E3 ubiquitin ligase complex, which tags HIF-1α with ubiquitin, targeting it for proteasomal degradation. This oxygen-dependent regulation maintains low HIF-1α levels when oxygen is abundant (Sarapultsev et al., 2023).

During hypoxia, intracellular oxygen levels fall, leading to PHD inhibition. Without hydroxylation, HIF-1α evades degradation and accumulates in both the cytoplasm and nucleus. Once in the nucleus, it heterodimerizes with HIF-1β, forming the HIF-1 transcriptional complex which binds hypoxia response elements in gene promoters. This transcriptional activation reprograms cellular metabolism by upregulating glycolytic enzymes and glucose transporters (Fleshner et al., 2017). HIF-1α downregulates ATP6V1A, a component of V-ATPase on lysosomal surfaces, impairing lysosomal function and homeostasis. Impaired lysosomes reduce fusion of multivesicular bodies with lysosomes, resulting in increased secretion of intraluminal vesicles, i.e., EVs. In response to oxidative stress like hyperoxia, Caveolin-1 (Cav-1) is upregulated and facilitates the transport of hnRNPA2B1 and associated miRNAs into EVs (Sarapultsev et al., 2023).

EVs secreted under hypoxia exhibit distinct molecular signatures reflecting HIF-1α-driven transcriptional changes. There is upregulation of glycolytic enzymes such as lactate dehydrogenase A (LDHA) and enolase 1 (ENO1) at the start of anaerobic glycolysis, and pro-angiogenic factors like vascular endothelial growth factor (VEGF), which promotes blood vessel formation and physiological adaptation to stress. Hypoxia associated microRNAs such as miR-210 important for mitochondrial function and long non-coding RNAs (lncRNAs) involved in cellular stress responses, are enriched in hypoxic EVs. These EVs can also alter the extracellular environment by contributing to localized acidosis (Fleshner et al., 2017; Sarapultsev et al., 2023).

4.1.2 Metabolic Stress

Metabolic stress arises when a cell's energy demand exceeds supply, disrupting homeostasis and challenging survival. It can result from nutrient deprivation, altered glucose or lipid metabolism, hypoxia, or mitochondrial dysfunction, all conditions which are common in tumors, ischemic tissues, and degenerating

neurons. Cells adapt through transcriptional, post-transcriptional, and translational remodeling, in which extracellular vesicles inhabit a central role by redistributing metabolic cues and signaling molecules (Yaribeygi et al., 2017).

One key metabolic stress sensor is AMP-activated protein kinase (AMPK), a serine/threonine kinase activated by increased AMP/ATP ratio. AMPK promotes catabolic pathways to restore ATP and modulates EV biogenesis. AMPK activation enhances autophagy and endolysosomal trafficking, pathways intersecting with exosome secretion machinery, allowing cells under metabolic duress to offload damaged proteins and organelles via EVs, supporting proteostasis (Fleshner et al., 2017).

Metabolic stress also reprograms EV cargo. For example, glucose deprivation increases secretion of EVs enriched in glycolytic enzymes, metabolic intermediates, and stress-responsive miRNAs such as miR-210 and miR-155. These vesicles influence recipient cell metabolism by promoting glycolytic flux, angiogenesis, and immune modulation. In cancer, this adaptation of the tumor microenvironment fosters proliferation and invasion (Sarapultsev et al., 2023).

Mitochondrial dysfunction, a hallmark of metabolic stress, contributes to altered EV secretion. Damaged mitochondria release mitochondrial DNA (mtDNA), cytochrome c, and cardiolipin-enriched vesicles via EVs. These mitochondrial EVs (mitoEVs) act as damage signals, activate innate immunity (e.g., via toll-like receptor 9, TLR9), and mediate metabolic reprogramming (Fleshner et al., 2017). Hypoxia stabilizes hypoxia-inducible factors (HIFs), reprogramming gene expression for anaerobic metabolism. HIF-1α upregulates glycolytic and angiogenic genes, many enriched in EV cargo; hypoxic cells increase EV output carrying VEGF, carbonic anhydrase IX (CAIX), and lactate dehydrogenase (LDH), contributing to extracellular acidification and vascular remodeling (Sarapultsev et al., 2023).

Lipid metabolism influences EV biology. Metabolic stress alters membrane lipid composition; ceramide generated via neutral sphingomyelinase 2 (nSMase2) regulates exosome budding. Increased ceramide during stress enhances EV production, correlating with export of stress and inflammatory signals. EVs convey protective or pathological functions depending on their context; in skeletal muscles, exercise-induced EVs carry myokines and regulators like PGC-1α, promoting metabolic health, while adipocyte-derived EVs in obesity disseminate inflammatory miRNAs (miR-29, miR-34a) exacerbating insulin resistance (Fleshner et al., 2017).

Moreover, EVs modulate stemness and plasticity. Nutrient-poor tumor microenvironments stimulate EV release from cancer-associated fibroblasts (CAFs) and senescent cells, carrying metabolites, survival miRNAs, and drug resistance proteins, aiding tumor cell dedifferentiation and chemotherapy resistance. Thus, EVs are versatile messengers in metabolic stress adaptation, with emerging potential in biomarker discovery and therapy development in metabolic diseases and cancer (Sarapultsev et al., 2023).

4.1.3 Thermal Stress

Thermal stress from hyperthermia or hypothermia impacts protein folding, membrane fluidity, cytoskeletal integrity, and enzyme kinetics. Cells activate a heat shock response (HSR) primarily via heat shock factor 1 (HSF1). Upon thermal insult, HSF1 trimerizes, translocates to the nucleus, and upregulates heat shock proteins (HSPs) like HSP70, HSP90, and HSP27. These chaperones prevent protein aggregation and assist proteostasis restoration. HSPs are secreted via EVs in heat stress, acting as extracellular danger signals, activating immune and survival pathways in recipient cells (Fleshner et al., 2017).

Heat-induced EVs display altered size and increased secretion, enriched with HSPs, oxidized proteins, proteasome subunits, and heat-responsive miRNAs such as miR-21 and miR-146a, which modulate inflammation and apoptosis. This cargo reprogramming reflects donor cell proteostasis and inflammatory state, informing intercellular communication during fever, heat stroke, or hyperthermia therapy (Sarapultsev et al., 2023).

At the membrane, thermal stress affects lipid rafts and curvature, promoting vesicle budding and inclusion of stress proteins in EVs. This may expel damaged proteins, reducing cytotoxic burden. In cancer therapy, hyperthermia sensitizes tumors to radiation or chemotherapy; EVs released under such stress carry HSPs enhancing antigen presentation and immune recruitment, serving as stress markers and immunological adjuvants. Tumor-derived EVs from hyperthermia can also promote angiogenesis and tumor survival, underscoring complex thermal adaptation-tumor progression interplay (Fleshner et al., 2017).

Hypothermia induces cytoskeletal changes and metabolic suppression. Cold stress leads to EVs differing in cargo from heat shock EVs, carrying cold shock proteins (CSPs) with RNA-binding motifs stabilizing survival transcripts. These EVs contribute to neural function preservation in therapeutic hypothermia models for ischemic injury (Sarapultsev et al., 2023).

Thermal-stress effects on EV communication are conserved evolutionarily. For example, in thermotolerant plants and fungi, EVs carry RNA-binding proteins and thermoprotective metabolites facilitating systemic stress adaptation. Overall, EVs are critical to cellular thermal stress responses, exporting chaperones, damaged proteins, and regulatory RNAs to reduce toxicity and coordinate multicellular responses. Their dual nature, protective yet potentially pathogenic, makes them relevant biomarkers and therapeutic targets (Fleshner et al., 2017).

4.1.4 Oxidative Stress

Oxidative stress results from imbalance between reactive oxygen species (ROS) production and antioxidant defenses, damaging proteins, lipids, and nucleic acids, compromising cellular functions. It is implicated in neurodegeneration, cancer, cardiovascular diseases, and aging. Extracellular vesicles occupy key roles in propagating and mitigating oxidative stress responses (Pizzino et al., 2017).

Oxidative stress activates redox-sensitive pathways including nuclear factor erythroid 2–related factor 2, MAPK cascades, and NF-κB signaling, regulating antioxidant genes, inflammation, and apoptosis. ROS induces ceramide synthesis via nSMase2, enhancing exosome formation. EVs secreted under oxidative stress are enriched in oxidized proteins, lipid peroxidation products, and miRNAs such as miR-21, miR-144, and miR-34a. They carry antioxidant enzymes (peroxiredoxins, glutathione-S-transferases, catalase) which protect recipient cells by enhancing redox resilience (Pizzino et al., 2017). EVs from severely oxidatively stressed or dying cells may propagate damage. Mitochondria-derived vesicles (mitoEVs) containing mitochondrial DNA and oxidized cardiolipin act as damage-associated molecular patterns, activating innate immunity via pattern recognition receptors like TLR9, triggering inflammation and further oxidative damage (Fleshner et al., 2017).

In cardiovascular systems, endothelial cells under oxidative stress release EVs enriched in adhesion molecules, cytokines, and pro-thrombotic factors, contributing to vascular inflammation and atherosclerosis. In the nervous system, neuron and glia-derived EVs modulate neuroinflammation, synaptic plasticity, and cell survival during oxidative stress (Pizzino et al., 2017). In cancer, oxidative stress exhibits dual roles: promoting tumorigenesis and inducing cell death. Tumor-derived EVs under oxidative stress carry pro-survival and antioxidant components, facilitating chemoresistance and immune evasion. Therapeutic strategies raising ROS, like radiotherapy, alter EV profiles, contributing to therapy-induced senescence (Fleshner et al., 2017). Oxidative stress modifies EV

lipid composition; lipid peroxidation changes membrane curvature, fluidity, and vesicle budding dynamics. Lipid mediators (prostaglandins, eicosanoids, oxidized phospholipids) trafficked via EVs influence inflammation and vascular tone (Pizzino et al., 2017).

Chronic oxidative stress induces senescence-associated secretory phenotype (SASP), with EVs releasing IL-6, IL-8, matrix metalloproteinases (MMPs), and redox enzymes, propagating senescence and inflammation contributing to aging tissue decline (Fleshner et al., 2017). Clinically, EVs might serve as oxidative stress biomarkers, their cargo reflecting donor tissue oxidative status, detectable in biofluids like blood, urine, and cerebrospinal fluid. Therapeutic EVs loaded with antioxidants are emerging strategies to mitigate ischemia-reperfusion injury, neurodegeneration, and inflammation (Pizzino et al., 2017).

4.1.5 Acidic Stress

Acidic stress denotes cellular exposure to low extracellular or intracellular pH, from lactic acidosis, tumor microenvironments, ischemia, chronic inflammation, or ion homeostasis disruption. Cellular acidification alters enzyme activity, ion channels, protein folding, and metabolism. Adaptive responses restore pH homeostasis or trigger programmed cell death if the stress is unresolved. Extracellular vesicles inhabit key roles in adaptation and intercellular communication under acidic stress (Sarapultsev et al., 2023).

Acidic stress modifies EV biogenesis by altering intracellular trafficking and sorting. Reduced endosomal pH shifts MVB fusion preference from lysosomes to the plasma membrane, enhancing exosome secretion. Acidic conditions also enhance plasma membrane microvesicle budding by altering lipid raft composition and cytoskeletal dynamics (Fleshner et al., 2017).

EVs secreted under acidic stress carry metabolic reprogramming cargos such as glycolytic enzymes (LDH-A, PKM2), monocarboxylate transporters (MCTs), and stress-responsive miRNAs. In cancer, acidic stress upregulates EVs with pro-invasive proteins (MMPs), carbonic anhydrase IX (CAIX), and oncogenic lncRNAs. These facilitate extracellular matrix remodeling, angiogenesis, immune evasion, and metastasis (Sarapultsev et al., 2023).

Tumor acidic select aggressive cancer cells by eliminating acid sensitive cells while favoring those that adapt through increased glycolysis and acid resistant metabolism. There glycolytically adapted aggressive phenotypes gain survival, invasivity and immune evasive advantages diving for a more malignant tumor population. Acidic EVs further support tumor growth by influencing stromal,

immune, and endothelial cells by inhibition of T-cell proliferation, promotion of M2 macrophage polarization, and increasing endothelial permeability (Fleshner et al., 2017).

Acidic pH alters EV physical properties including membrane lipid bilayer composition, vesicle stiffness, fusion ability, and uptake. Acidification induces conformational changes in membrane proteins and tetraspanins (CD9, CD63), influencing targeting specificity (Sarapultsev et al., 2023).

Mechanistically, acidic stress activates pathways intersecting with EV release, e.g., AMPK and HIF-1α signaling, upregulating glycolysis, pH regulation, and vesicle trafficking genes. HIF-1α target genes (VEGF, GLUT1, BNIP3) are enriched in acidic EV transcriptomes, indicating coordinated adaptation (Fleshner et al., 2017).

Beyond cancer, acidic EVs promote angiogenesis and survival in ischemic tissues and contribute to cartilage degradation and inflammation in osteoarthritis (Sarapultsev et al., 2023).

Clinically, acidic stress induced EVs are promising diagnostic and prognostic cancer biomarkers, their acidification-specific cargos detectable in plasma or urine. Therapeutically, pH-responsive EV engineering exploits acidic tumor microenvironments for targeted drug or RNA delivery (Fleshner et al., 2017).

4.1.6 Radiation

Radiation stress from ionizing radiation (X-rays, gamma rays, particle radiation) induces DNA damage, oxidative stress, and senescence. It activates DNA damage response (DDR) pathways (ATM/ATR, p53), generates reactive oxygen/nitrogen species (ROS/RNS), and alters cell cycle dynamics. Radiation also elicits intercellular communication via extracellular vesicles mediating radiation-induced bystander effects (RIBE) (Sarapultsev et al., 2023).

Ionizing radiation modulates EV biogenesis by activating DDR pathways enhancing exosome and microvesicle release. EVs from irradiated cells contain DNA repair proteins, damage-associated molecular patterns (DAMPs), and cytokines (IL-6, TGF-β). They also carry p53-regulated transcripts, γ-H2AX, and apoptosis-related miRNAs (miR-34a, miR-21) (Fleshner et al., 2017).

Radiation-induced EVs (RI-EVs) transmit genotoxic stress signals to non-irradiated neighboring cells, causing DNA damage, chromosomal instability, and

gene expression changes—challenging the paradigm that radiation effects are cell-autonomous (Sarapultsev et al., 2023).

RI-EVs modulate immune responses by presenting neoantigens and activating innate immunity through DAMPs; they may induce immunosuppression or enhance anti-tumor immunity depending on context (Fleshner et al., 2017).

In cancer therapy, radiation-elicited EVs promote tumor repopulation, angiogenesis, and metastasis by transferring pro-survival factors and remodeling the tumor microenvironment. Glioblastoma-derived EVs post-irradiation confer resistance signals (EGFRvIII, HSP70, miR-221). Conversely, RI-EVs may sensitize tumors to immune clearance or act as biomarkers of therapy response (Sarapultsev et al., 2023).

Radiation alters EV membrane and protein profiles, affecting vesicle integrity, fusion potential, targeting, and biodistribution (Fleshner et al., 2017).

Clinically, RI-EVs serve as minimally invasive biomarkers for radiation exposure, monitoring, and toxicity prediction. Engineered EVs are explored as vehicles for radiosensitizer and RNA delivery (Sarapultsev et al., 2023).

4.1.7 Electromagnetism

Electromagnetic fields (EMF), including low-frequency non-ionizing and radiofrequency radiation, modulate cellular redox balance, calcium signaling, and gene expression. EMF exposure alters EV release in a cell type- and dose-dependent manner by influencing endosomal sorting complexes (ESCRT), ceramide production, and calcium-regulated exocytosis. Reactive oxygen species (ROS) generation, mitochondrial activity, and transcription factors NF-κB and HSF1 are implicated in these effects (Fleshner et al., 2017).

EMF induced calcium influx via voltage-gated or TRP channels modulate MVBs formation and microvesicle budding, altering EV output. EMFs also reprogram EV cargos via redox-sensitive transcriptomic and proteomic changes (Sarapultsev et al., 2023).

EVs from EMF-exposed cells carry stress proteins (HSP70, SOD1), mtDNA fragments, and regulatory miRNAs (miR-21, miR-155, let-7 family), mediating

protective or harmful responses depending on the exposure context (Fleshner et al., 2017).

Such EVs mediate electromagnetic bystander effects (EM-BE), propagating stress signals to non-exposed cells leading to DNA damage and altered gene expression, analogous to radiation-induced bystander effects (Sarapultsev et al., 2023).

Functionally, EMF-modified EVs affect nervous and immune systems by altering synaptic activity, neuroinflammation, cytokine secretion, and macrophage polarization. Prolonged EMF exposure may cause chronic inflammation via EV-mediated signaling with implications for metabolic syndrome, neurodegeneration, and cancer (Fleshner et al., 2017).

Clinical research on EMF-EVs is emerging with interest in biomarkers for exposure and EVs as therapeutic delivery vehicles or modulators of bioelectrical signaling in regenerative medicine (Sarapultsev et al., 2023).

4.1.8 Cell Death

Programmed cell death (PCD) ensures removal of damaged, infected, or unnecessary cells, essential for development, immune defense, and homeostasis. Major PCD forms include apoptosis, pyroptosis, necroptosis, and ferroptosis, each with distinct molecular pathways. Cells undergoing death release extracellular vesicles which reflect death progression and modulate immune responses, tissue remodeling, and intercellular signaling (Fleshner et al., 2017).

Apoptosis, initiated by intrinsic or extrinsic stimuli, involves caspases (-3, -8, -9), Bcl-2 proteins, and apoptosome complexes. During apoptosis, membranes bleb, nuclei condense, and cellular contents are packaged into apoptotic bodies (ABs) (500–5000 nm). ABs, a subtype of EVs, form via cytoskeletal dynamics regulated by Rho-associated kinases (ROCKs) and actomyosin contractility. They carry intact organelles, nuclear fragments, and bioactive molecules, as well as express signals like phosphatidylserine which promote phagocytosis. ABs transfer DNA, miRNAs, and signaling proteins, influencing immune regulation and regeneration (Sarapultsev et al., 2023).

Cells also release microvesicles and exosomes during early death stages. Caspase-driven cleavage of Rho GTPases and cytoskeleton facilitates

microvesicle shedding. ER stress and mitochondrial damage enhance exosome release through ceramide-dependent or ESCRT pathways (Fleshner et al., 2017).

Necroptosis (mediated by RIPK1/3, MLKL) releases EVs carrying DAMPs like HMGB1 and mtDNA, promoting inflammation and immunity. Pyroptosis (gasdermin-D pore formation and caspase-1 activation) releases EVs containing inflammasome components and IL-1β (Sarapultsev et al., 2023).

EV release during cell death is regulated by ROS, calcium influx, ATP depletion, and lipid peroxidation. Key regulators include p53 (upregulating TSAP6 to promote exosomes), ceramide (from nSMase2 facilitating MVB vesicle formation), caspases (altering budding dynamics), and calcium (activating scramblases and calpain for microvesicle shedding) (Fleshner et al., 2017).

Death-associated EVs have dual roles: apoptotic bodies induce immune tolerance by promoting anti-inflammatory cytokines, while necrotic EVs may exacerbate inflammation, causing tissue damage or autoimmunity. They also promote tissue regeneration, e.g., endothelial apoptotic bodies transfer VEGF and miR-126 enhancing angiogenesis. Dying tumor cells release EVs priming antigen-presenting cells or supporting tumor repopulation via growth factors. EVs serve as biomarkers of cell death, with circulating EVs bearing apoptosis markers reflecting tissue injury or therapeutic response. They are investigated for real-time tumor burden and immune activation monitoring during radiotherapy and chemotherapy (Sarapultsev et al., 2023).

Understanding EV regulation during cell death offers diagnostic and therapeutic opportunities in pathology, oncology, and regenerative medicine (Fleshner et al., 2017).

5. Tree of Life

Extracellular vesicles are now recognized as ancient, conserved vehicles of intercellular communication which span the entire tree of life. From the simplest unicellular organisms to complex multicellular eukaryotes, EVs have been adapted as modular systems for transporting molecular information across cellular boundaries. Their structure, biogenesis, and function show both remarkable conservation and lineage-specific adaptations, reflecting evolutionary pressures and ecological niches. This section explores the diversity of EV production in bacteria, archaea, parasites, and eukaryotes, highlighting evolutionary trajectories and mechanistic distinctions (Brakhage et al., 2021; Gill et al., 2019).

The earliest evidence of EV production arises from prokaryotes, where both Gram-negative and Gram-positive bacteria secrete vesicles into their surrounding environment. In Gram-negative species, outer membrane vesicles (OMVs) are derived from the outer membrane and typically range from 20–250 nm. OMVs carry lipopolysaccharides (LPS), outer membrane proteins, toxins, nucleic acids, and even quorum sensing molecules. Their release is promoted by alterations in peptidoglycan crosslinking, accumulation of misfolded proteins in the periplasm, and envelope stress (Schwechheimer & Kuehn, 2015).

Gram-positive bacteria, lacking an outer membrane, were once thought incapable of vesicle secretion. However, they produce cytoplasmic membrane-derived vesicles (CMVs) through mechanisms involving localized cell wall remodeling or autolysin activity. CMVs are enriched in lipoteichoic acids, virulence factors, and signaling peptides, and contribute to biofilm formation, antibiotic resistance, and horizontal gene transfer (Schwechheimer & Kuehn, 2015).

Archaea, representing a distinct domain of life, have a membrane bound extracellular vesicle form (Liu et al., 2021) and also release membrane vesicles (MVs), especially in extreme environments (Baquro et al., 2025). *Sulfolobus islandicus* produces EVs containing CRISPR-Cas components and chromosomal DNA fragments. Archaea often utilize an ESCRT-III-like complex, evolutionarily related to eukaryotic MVB machinery, for EV budding (Liu et al., 2021). This suggests an ancient evolutionary link between archaeal and eukaryotic vesicle systems, further strengthened by structural homologies in their membrane sculpting proteins.

Parasitic EVs communication and cargo transportation have been found in both inter species and across species of parasite-parasite and parasite-host communication. *Fasciola hepatica* secreted EVs vesicles which have been analyzed show distinct subpopulations enriched in potentially stress-related proteins such as Hsp70. These EVs originated from endosomal compartments and carried ESCRT pathway proteins involved in vesicle formation. This suggests a conserved mechanism of EV biogenesis linked to stress and cellular homeostasis (Qadeer et al., 2024; Cwiklinski et al., 2015).

In protozoan parasites such as *Trypanosoma cruzi*, *Leishmania spp.* and *Plasmodium falciparum*, EVs function in immune evasion, nutrient acquisition, and inter-parasite signaling (Kwaku et al., 2024). These EVs are often enriched in trans-sialidases, glycosylphosphatidylinositol (GPI)-anchored proteins, and RNA cargos which modulate host immune signaling (Torrecilhas et al., 2020).

Plasmodium spp. infected red blood cells release microvesicles which contain parasite proteins and host-derived molecules, influencing macrophage activation, endothelial adhesion, and disease severity in malaria (Dey et al., 2024). In *Leishmania spp.* EVs containing GP63 metalloproteases interfere with host macrophage signaling and antigen presentation, altering immune polarization (Silverman et al., 2010). Helminths such as *Schistosoma mansoni* and *Fasciola hepatica* produce EVs from both adult worms and eggs. These EVs carry immunomodulatory proteins, miRNAs, and tetraspanins which suppress pro-inflammatory responses and promote regulatory T-cell phenotypes in the host. EVs thus serve as stealth mechanisms to facilitate chronic infections (Roig et al., 2018).

In multicellular eukaryotes, EV production is intricately linked to cellular differentiation, tissue specialization, and systemic communication. EVs in eukaryotes are primarily categorized into exosomes, microvesicles, and apoptotic bodies, as previously defined, and arise from complex organellar systems including endosomes, lysosomes, and the plasma membrane (Yáñez-Mó et al., 2015).

In plants, EVs are increasingly recognized for their roles in development and defense. Plant EVs, particularly from the apoplast and root exudates, contain defense-related RNAs and proteins. These vesicles can transfer small RNAs (sRNAs) to fungal pathogens, leading to gene silencing via a process termed cross-kingdom RNA interference (ckRNAi). Additionally, plant EVs may play a role in nutrient mobilization and root microbiome modulation (Rutter et al., 2018).

In fungi, including yeasts and filamentous fungi like *Candida albicans* and *Cryptococcus neoformans*, EVs mediate stress responses, virulence, and interspecies communication. Fungal EVs can pass through rigid cell walls, possibly using specialized vesicle transport routes or local enzymatic degradation. They carry bioactive lipids, ergosterol, mRNAs, and allergens which influence host immune responses and fungal pathogenesis (Rizzo et al., 2020).

In invertebrates such as *Drosophila melanogaster*, EVs participate in development, neuronal patterning, and immunity. Exosomal release in *Drosophila* can be Rab11-dependent, and these vesicles may transport Wnt/Wg signaling components (Koles et al., 2012). In marine invertebrates, EVs mediate symbiosis and cellular recognition (Zhang et al., 2024).

Vertebrates exhibit highly regulated EV production across all tissues and fluids. In mammals, EVs are found in blood, cerebrospinal fluid, urine, saliva, and breast milk. They are central to immune modulation, coagulation, neural communication, and tissue repair. In the nervous system, neuron-derived exosomes facilitate synaptic plasticity and glial signaling, while EVs in the immune system mediate antigen presentation, cytokine signaling, and pathogen clearance (Raposo & Stoorvogel, 2013; Liegertová & Janoušková, 2023).

6. Biotechnological Applications

Nanomedicine leverages nanoscale carriers such as liposomes, micelles, dendrimers, and polymeric nanoparticles for targeted drug delivery and diagnostics, improving bioavailability and reducing off-target effects in both human and veterinary medicine. Despite advances, challenges persist, including limited tissue specificity, suboptimal pharmacokinetics, toxicity, scalability, and difficulty crossing biological barriers like the blood-brain barrier (Wang et al., 2025).

Extracellular vesicles have emerged as promising nanocarriers. Their biocompatibility, innate ability to cross biological barriers, and capacity to deliver diverse therapeutic cargo (proteins, RNAs, small molecules) offer significant advantages over synthetic nanoparticles. EVs can be engineered for targeted delivery, reducing immunogenicity and enhancing therapeutic precision (Du et al., 2023).

The best practices for developing EV based drug delivery platforms are still evolving. A promising functional and scalable methodology has been suggested, using mesenchymal stem cells cultured in bioreactors, where stimuli such as hypoxic stress are applied to enhance EV yield for preclinical applications. The problem of separation of vesicles from complex culture media is classically solved by ultracentrifugation combined with density gradient methods, but that methodology has limited scalability and methodology by tangential flow filtration and ultrafiltration that have performed well in preprocessing when followed with size exclusion chromatography. Through this, one can recover a purified concentrate of EV fraction suitable for research.

There are two main strategies for loading cargo into manufactured extracellular vesicles; the endogenous loading production introduced or stimulated during internal cellular biogenesis of EVs, where the EV membrane remain intact and maintain biological activity, and exogenous loading when membranes are disturbed and engineered to encapsulate artificially introduced drugs with strategies to handle reduced stability and risk of aggregation of the hybrid vesicle. (Brezgin et al., 2024).

The production of EVs unfortunately faces hurdles in yield, consistency, and scalability. While stem cell-derived EVs show regenerative and immunomodulatory potential, concerns include tumorigenicity, ethical issues, and high production costs (Dauphin et al., 2025). However, parasites and bacteria represent alternative EV sources. Parasites, in particular, naturally produce

abundant EVs with immunomodulatory properties, offering cost-effective production, though safety and immunogenicity remain concerns (Dauphin et al., 2025). Bacterial EVs are scalable but may carry endotoxins (Toyofuku et al., 2019).

Extracellular vesicles (EVs) have undergone a remarkable transformation in scientific perception, evolving from being dismissed as cellular debris to gaining recognition as biological mediators. In particular, their natural biocompatibility, ability to traverse biological barriers, and inherent targeting properties, have catalyzed interest in their application across therapeutic delivery, diagnostics, and regenerative medicine (Yáñez-Mó et al., 2015).

The historical trajectory of EV research can be traced to the mid-20th century, with Peter Wolf's initial observation of "cellular dust" in blood plasma. For several decades, these vesicles were largely regarded as inconsequential byproducts of cellular turnover. It was not until the 1980s, following the discovery of exosomes as specialized vesicles involved in transferrin receptor disposal in reticulocytes, that the field began to appreciate the regulated and purposeful nature of EV biogenesis. The subsequent decades witnessed a paradigm shift, particularly when exosomes were implicated in immune modulation, such as the presentation of MHC class II-peptide complexes by B cell-derived vesicles to T cells (Yáñez-Mó et al., 2015).

Advances in isolation techniques, including ultracentrifugation and nanoparticle tracking analysis, alongside the establishment of standardized nomenclature, have since solidified EVs as a legitimate and rapidly advancing domain within molecular biology (Zhang et al., 2025).

One of the most compelling avenues of EV research lies in their utility as precision delivery vehicles. Unlike synthetic nanoparticles, EVs possess a lipid bilayer and surface proteins that confer prolonged circulation, immune evasion, and the remarkable ability to cross restrictive barriers such as the blood-brain barrier. For instance, mesenchymal stem cell-derived EVs have been utilized to deliver anti-inflammatory agents or gene-silencing RNAs, while dendritic cell-derived vesicles have shown promise in cancer immunotherapy by presenting tumor antigens to cytotoxic T cells (Chen et al., 2025).

Research explores the possibilities of loading EVs with exogenous therapeutic cargoes, carrying potential for encapsulation of small RNAs and carrying smaller chemotherapeutic drugs such as doxorubicin. Primarily, the goal is to find solutions to the cellular penetration problem within the densely packed

extracellular matrix and dense variety of tumors where low yield of penetration is possible with synthetic nanoparticle systems (Rankin-Turner et al., 2021).

Recent advances show that engineering protein association with lipid membranes, particularly through lipid anchors or targeting sequences, can significantly enhance the efficiency of protein loading into EVs. The critical parameters influencing this process include incubation temperature, cargo concentration and the structure of lipid anchors as well as the cellular origins of the EVs (Marquant et al., 2025).

EV shows targeting ability through modulation of the membrane composition. Engineering of EV membranes via genetic or chemical modification is an area of research which hopes to further deepening the understanding of protein to protein interaction in cellular communication, and molecular basis of targeting specificity (Liu et al., 2023).

There are some challenges in the isolation technique of EVs. The small size, low density, and wide distribution of EVs in the complex bodily fluid environment make it quite challenging for researchers to obtain high-purity EVs (Du et al., 2023; Ragni, 2025).

EV isolation methods face significant limitations due to the inherent heterogeneity of EV subgroups. While exosomes and microvesicles can be distinguished at the proteome level, no universal, cell-type-protein markers exist to definitively separate these vesicle types. For example, commonly used exosomal markers like tetraspanins are enriched in MVs as well, complicating purification workflows with overlaps (Haraszti et al., 2016; Shami-shah et al., 2023).

Exogenous cargo loading, especially for large or hydrophobic molecules, remains technically challenging, with current methods achieving limited efficiency (Rankin-Turner et al., 2021).

Future directions in the field are likely to focus on the development of next-generation “smart vesicles” with programmable targeting, controlled release, and multiplexed cargo capabilities. These are hybrid systems which integrate synthetic and natural vesicle components that may offer enhanced consistency and tunability. Advances in artificial intelligence and modular engineering are poised to accelerate the optimization of EVs for personalized medicine, while the advent of EV-based biosensors could revolutionize real-time disease monitoring.

7. Extracellular Vesicles in Antimicrobial and Antiparasitic Strategies

The global rise of antimicrobial and antiparasitic resistance poses a significant challenge to public health. Traditional therapies are increasingly compromised by the evolution of resistance mechanisms in both bacteria and parasites. Extracellular vesicles secreted by pathogens are critical mediators of intercellular communication, immune modulation, and horizontal gene transfer, thus occupying a role in mechanics of resistance.

Bacterial EVs are central to microbial community dynamics, facilitating communication, genetic exchange, and environmental adaptation. These vesicles carry diverse cargo-proteins, lipids, RNAs, and DNA, which contribute to quorum sensing, nutrient acquisition, and virulence. EVs can manipulate host immune responses, suppress macrophage or dendritic cell activation and promote chronic infection. Conversely, EVs from probiotic bacteria can enhance host immunity, highlighting their dual role in pathogenesis and immune defense (Hosseini-Giv et al., 2022).

The clinically pathogenic function of bacterial EVs is mediating horizontal gene transfer, including the transfer of antibiotic resistance genes such as those encoding β-lactamases. This process accelerates the spread of multidrug resistance among bacterial populations. EVs may also deliver virulence factors, compounding the challenge of treating resistant infections (Salehi Moghaddam et al., 2025). Targeting EV-mediated gene transfer, by inhibiting vesicle secretion or uptake, or neutralizing their cargo-represents a promising strategy for curbing resistance dissemination (De Langhe et al., 2024).

Bacterial resistance mechanisms include efflux pumps, enzymatic drug degradation, target modification, reduced permeability, and biofilm formation. These adaptations, coupled with the overuse of antibiotics and slow development of new drugs, have exacerbated resistance. Novel approaches, such as EV-based interventions, are needed to address these entrenched mechanisms (Toyofuku et al., 2019).

EV focused strategies against antibiotic resistance include inhibition of secretion and uptake pathways, preventing resistance gene transfer. By engineering the EVs, one can deliver antagonists and antibiotics as cargo directly to the infection sites and thus bypass degradation. As proteins on the EV surface carry potential of

direct interaction with immune cells, there is a possibility of engineered immune modulation by boost or downregulation of immune responses (Jiang et al., 2024).

Anthelmintic resistance in helminths mirrors bacterial resistance, involving target site mutations, altered drug metabolism, reduced uptake, and secretion of detoxifying enzymes-often via EVs. Helminth-derived EVs modulate host immunity and can carry factors which facilitate survival under drug pressure.

Potential strategies to handle these issues include direct approach with targeted delivery of anthelmintics via EVs which have the structural fluidity to overcome resistance barriers as well as direct inhibition of EV secretion of a parasite, as blocking the EV release may reduce the ability of the parasite to adapt to the environment, and thus decrease survival and resistance to drugs (Qadeer et al., 2024).

8. Parasitic Infections

Parasitic organisms utilize extracellular vesicle mediated RNA transfer as a mechanism to modulate host pathogen interactions. The genetic material identified within EVs during parasitic infections is notably diverse, including messenger RNA, microRNA, small interfering RNA, and long non-coding RNA. While the precise mechanisms by which these RNA species influence gene expression and cellular behavior in both parasite and host remain incompletely understood, accumulating evidence highlights their functional significance (Schemiko Almeida et al., 2024).

Following release into the extracellular environment, parasite-derived EVs deliver their molecular cargo to target host cells. The RNA content within these vesicles can be remarkably heterogeneous. For instance, a study on *Leishmania donovani* identified over 100 distinct miRNAs in EVs during infection, raising questions about whether EVs serve as storage reservoirs within the parasite or whether such diversity is an evolved strategy to overwhelm and suppress the host immune system through a broad spectrum of miRNA-mediated effects (Ganguly et al., 2021). In contrast, other parasites display specificity in their RNA cargo; for example, *Trypanosoma cruzi* EVs are enriched in tRNA-derived fragments which modulate host cell translation (Garcia-Silva et al., 2014). Similarly, *Plasmodium falciparum* infected erythrocytes release EVs containing both mRNA and miRNA, which have been shown to downregulate macrophage activity and contribute to immune evasion (Khammanee et al., 2025). A study of *Trypanosama cruzi* discovered that infective trypomastigotes trigger a higher release of vesicles from THP-1 monocytes to enhance parasite entry into host cells, finding virulence factors within the vesicles (Ramirez et al., 2017).

Upon uptake by host cells, the genetic impact of parasite EVs is highly variable and appears to be determined by both the nature of the cargo and the parasite species, suggesting that parasites may have evolved specialized strategies to manipulate host gene expression and immune responses. For example, *Fasciola hepatica* EVs modulate the expression of host genes involved in apoptosis, while *Toxoplasma gondii* EVs deliver mRNAs encoding virulence factors which are subsequently translated within host cells. *Leishmania* EVs are known to transfer miRNAs that inhibit NF-κB signaling pathways, thereby facilitating immune evasion (Schemiko Almeida et al., 2024).

These findings indicate that parasites are not passive invaders, they are highly skilled and evolutionary adjusted to manipulating eukaryotic host biology to survive and thrive by using extracellular vesicles designed for functionality that

benefit the parasite. However, it is important to note that few parasites employ the entire spectrum of these mechanisms; instead, many species exhibit specialization, deploying distinct survival strategies that best suit their ecological niche and life cycle (Schemiko Almeida et al., 2024).

One widely observed mechanism is the downregulation of pro-inflammatory pathways, particularly those involving key cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6. Parasite-derived EVs frequently contain microRNAs and other regulatory RNAs that target the mRNAs encoding these cytokines, effectively reducing their expression. This suppression impairs the host's ability to mount an effective inflammatory response, thereby facilitating prolonged parasite persistence within the host (Schemiko Almeida et al., 2024; Khosravi et al., 2020).

In addition to suppressing pro-inflammatory signals, some parasites actively promote anti-inflammatory pathways. *Fasciola hepatica* EVs have been shown to induce the expression of interleukin-10 in host immune cells, shifting the immune response toward a regulatory and anti-inflammatory phenotype. This upregulation of IL-10 skews the immune environment toward tolerance and immunosuppression, creating a biological milieu that is conducive to parasite survival and replication (Schemiko Almeida et al., 2024; Murphy et al., 2020).

Disruption of antigen presentation represents another critical immune evasion strategy. Parasite EVs may deliver small interfering RNAs or proteins that interfere with the host's major histocompatibility complex machinery, thereby reducing the efficiency of antigen processing and presentation to T cells. *Trypanosoma cruzi* EVs are enriched in tRNA-derived small RNAs that can modulate host cell translation and potentially impact antigen processing pathways (Schemiko Almeida et al., 2024; Bayer-Santos et al., 2014).

Parasites can also modulate the functional phenotype of macrophages through EV-mediated signaling. By delivering specific miRNAs or proteins, parasites can induce a shift in macrophage polarization from the classically activated, pro-inflammatory M1phenotype to the alternatively activated, anti-inflammatory M2 phenotype. M2 macrophages are less effective at pathogen clearance and are associated with tissue repair and immune regulation, which ultimately benefits the parasite by reducing the likelihood of its destruction. *Schistosoma mansoni* EVs have been shown to contain miRNAs that promote M2 polarization, thereby supporting parasite persistence (Schemiko Almeida et al., 2024; Yuan et al., 2021).

Additional mechanisms of host manipulation include the inhibition of apoptosis in infected cells, the induction of regulatory T cells which further suppress immune responses, and the delivery of circular RNAs which act as molecular sponges to sequester host miRNAs and disrupt normal gene regulation. The cargo profile of parasite EVs is dynamic and can adapt in response to environmental pressures such as drug exposure or host immune activity, reflecting a remarkable degree of evolutionary plasticity (Schemiko Almeida et al., 2024; Wu et al., 2019).

The biogenesis of extracellular vesicles differs among organisms, but EVs consistently play a significant role as mediators of intercellular communication, including cross-species interactions between hosts and parasites. This communication mechanism is increasingly recognized as central to the dynamic interplay occurring during parasitic infections (Cruz Camacho et al., 2023).

When selecting model organisms to investigate complex signaling networks and environmental adaptations, research increasingly favors parasites over bacteria, especially in the context of extracellular vesicle biology. Unlike bacteria, parasites possess intricate mechanisms for long-term survival within their hosts, and EVs have emerged as critical mediators in this process. Parasite-derived EVs facilitate bidirectional communication, enabling the transfer of molecular signals which can manipulate host cellular responses, promote immune evasion, and enhance the survival and replication of said parasite. These vesicles can be internalized by host cells, triggering changes in gene expression and cellular behavior which are essential for establishing and maintaining infection.

Parasites typically exhibit multifaceted life cycles involving multiple developmental stages and transitions between different host environments. The composition of their EVs-including proteins, lipids, glycans, and nucleic acids-varies dynamically according to the parasite's life stage and the specific host niche occupied. This plasticity provides a unique opportunity to study how EV cargo adapts to changing environmental pressures and host immune landscapes, offering insights into the molecular strategies parasites use to sense, respond to, and manipulate their surroundings. In contrast, bacteria, while capable of producing EVs, generally display less developmental complexity and environmental adaptability at the multicellular level (Manikantan et al., 2024; Kochanowsky et al., 2024; Khosravi et al., 2020).

Parasites occupy a unique position as models for studying extracellular vesicles mediated cellular mechanisms. Parasites, as eukaryotic organisms, share a greater degree of cellular complexity and regulatory sophistication with their hosts compared to bacteria. This evolutionary proximity enables parasites to exploit and

manipulate host immune pathways with a level of nuance that is generally absent in prokaryotic pathogens. In contrast, bacterial EVs, the OMVS from gram negative bacteria, primarily deliver toxins, enzymes, and a limited set of small RNAs. While bacterial OMVs can influence host responses, their mechanisms are generally less sophisticated, relying on broad cytotoxic effects or immune evasion rather than the cell type specific modulation observed in parasites. Thus, parasites potentially serve as superior models for dissecting the complexity of intercellular communication and immune modulation via EVs, providing insights which are more translatable to higher eukaryotic systems. Yet, current research on the topic is limited.

One of the challenges is that helminth EVs lack validated protein markers comparable to those used in mammalian or bacterial systems. Another challenge is the complexity of quality control of parasites, because these control specimens need to be matched to helminth species and life stage. The development of bioreactors might be needed to handle complexity of higher cellular complexity models such as helminths (Kink et al., 2024; White et al.,2023).

Another challenge in extracellular vesicle research is that observation of mechanisms such as immune modulation is more challenging in complex eukaryotic cells compared to simpler eukaryotic parasites. Complex eukaryotes possess highly redundant signaling networks and compensatory pathways. Disentangling the details of specialized survival tactics and the effects of a single EV cargo molecule is complicated by the presence of multiple, overlapping regulatory mechanisms. This makes it challenging to attribute effects to individual EV cargo molecules within the system (Qadeer et al., 2024; Wu et al., 2019).

8.1 Immunoregulatory studies

At a homeostatic neutral level, extracellular vesicles support immune regulation contributing to molecular clearance and immune maturation by transportation of communication molecules and cytokines. In pregnancy, vesicles are involved in mediation of immunosuppression by transport of checkpoint molecules (PDL1, FASL, CD39/CD73) and miRNAs. When under threat, the cellular communication patterns shift dynamically serving a protective and modulatory purpose of innate immune and adaptive responses (Buzas, 2024).

The encounter between host and pathogen is also a crash of extracellular vesicles of different origins. Parasitic immunoregulation strategies is a new emerging field of parasitology and immunology. In humans, infection with *Necator americanus* leads to upregulation of anti-inflammatory cytokine IL-10, which can be linked

with suppression of immune hyper responsiveness by release of secretion och immunomodulatory proteins such as hookworm derived Na-AIP-1 protein which in preclinical studies modulated immune responses by reduced inflammation (Loukas et al., 2016; Ferrira et al., 2017).

Immunological regulation in parasiotology, immunology and EV research is often investigated using well characterized parasites distinguished by their ability to survive or persist within the host. A study in pigs infected with *Ascaris suum* led to the observation of multiple immune modulatory molecules and antigens within parasite derived EVs that interacted with host cells (Hansen et al., 2019). In horses, EVs from *Parascaris univalens* roundworm larval cultures also suggest involvement in immune signaling pathways. Both of these organisms and EVs are vaccine candidates for farm animals (Tufvesson., 2022; Manikantan et al., 2024).

Helminths, a type of parasitic worms, are an interesting group of organisms; in research of immunomodulation during parasitic infections, they secrete extracellular vesicles and complex groups of immunomodulatory molecules, potentially including mechanisms of cytokine mimicry and cytokine inhibitors, which alter the host immune responses towards tolerance or suppression (Drurey & Maizels, 2021).

Trichuris muris, the excretory/secretory products of helminth worms, are thought to contain protease inhibitors, which can be involved in immunomodulation of innate immune responses. This mechanism has been studied directly and indirectly by eliciting immunoregulatory EVs from macrophages (Bancroft & Grencis., 2021). Extracellular vesicles from the *Trichuris muris* have been used in the vaccination studies in C57BL/6 mice, the immunisation resulted in a statistically significant reduction in worm burden compared to control. The mice produced higher levels of IgG1 antibodies and high levels of anti-parasitic infection specific IgG2a/c. However, some of the mice developed chronic infection, which indicates a variable protection from extracellular vesicle developed vaccines in the current state of vaccine design (Shears et al., 2018).

Toxoplasma gondii is a very successful parasite predator, attributed to atypically located organelles-micronemes, rhoptries and dense granules as well as distinct cytoskeletal structures. These adaptations allow *Toxoplasma gondii* to attach to host cells and secrete invasion factors. The invasion process is orchestrated through the microneme proteins and rhoptry neck proteins which together form a moving junction, which anchors and facilitates translocation of effector molecules through EV secretion at the entry within the host cell (Najm et al., 2023; Portes et al., 2020).

Molecular diversity of parasites and biological adaptations to different environment and infection methods lead to a development of specificity on parasite-derived EVs, making them a promising candidate for vaccine development (Deurey et al., 2020).

Parasites, such as *Leishmania*, *Trypanosoma*, and *Plasmodium*, naturally secrete large quantities of EVs, and these vesicles can be harvested and utilized for drug delivery purposes. The advantage of using parasites for EV production lies in their ability to generate high yields of EVs without the need for extensive cell culture or genetic manipulation. Additionally, parasite-derived EVs often contain bioactive molecules which can modulate the immune system, making them attractive for immunotherapy applications (Vidal et al., 2024; Khosravi et al., 2020).

Extracellular vesicles from *Anisakis pegreffii* on healthy intestinal tissue allowed observation of mechanics of infection by harvesting the cargo from the EVs, subsequent analysis revealed the samples to contain biomolecules which downregulated genes associated with cell division and apoptosis. Further discoveries were an altering gene expression and involvement in signaling pathways related to cytokine activity (Cavallero et al., 2022; Stryiński et al., 2025).

8.2 Anthelmintic resistance

Anthelmintic resistance in helminths, particularly gastrointestinal nematodes, has emerged as a pressing challenge affecting human health, veterinary medicine, and global agriculture. The problem is no longer confined to high-prevalence regions; recent surveillance has detected spread of subspecies and reduced drug efficacy even in low-prevalence areas and non-traditional hosts, underscoring its global scope (D'Ambrosio et al., 2025; Brachmann et al., 2025). Resistance jeopardizes food security, livestock welfare, and economic stability, making it both a scientific and policy priority (Kapo et al., 2025; Mavundela et al., 2025).

The repeated and often prophylactic administration of anthelmintics exerts forceful selective pressure, accelerating the emergence of resistant populations. Farmer practices such as inaccurate dosing, reliance on a narrow range of drug classes, and suboptimal administration methods have been documented in diverse contexts, from communal farms in South Africa to commercial operations in Ethiopia and Bosnia and Herzegovina (Solomon et al., 2024; Kapo et al., 2025; Mavundela et al., 2025). Environmental persistence of resistant stages, combined

with inadequate biosecurity, amplifies the problem, allowing resistant genotypes to persist and spread.

Essential for timely intervention is accurate detection. The current strategies integrate in vivo fecal egg count reduction tests with *in vitro* assays such as egg hatch assays, alongside molecular methods including nemabiome (community of nematode species identified using DNA sequencing) analysis for species composition (Brachmann et al., 2025; D'Ambrosio et al., 2025). These approaches not only quantify treatment efficacy but also reveal shifts in parasite community structure post-treatment, offering insights into selective survival of resistant species.

Efforts to slow resistance evolution employ both chemical and non-chemical measures. Chemical measures include; rotational use of different drug classes, strategic combination therapies, and precision dosing based on accurate bodyweight. Non-chemical methods include selective breeding for parasite tolerance, improved pasture management, and novel biological controls. Yet nonetheless, assessment remains the cornerstones of control. Microbiome-targeted approaches are emerging, leveraging host-gut bacteria interactions to modulate parasite burdens (Rooney et al., 2023). Plant-derived compounds such as tannins are also being explored for their capacity to interact synergistically with commercial anthelmintics, potentially enhancing efficacy and reducing selection pressure (Sillanpää et al., 2025).

Molecular level resistance often arises from specific point mutations which alter the drug target sites or enhance drug efflux. For example, substitutions in βtubulin are well characterized in helminths parallel findings in fungi, where novel mutations like E198T confer high level benzimidazole resistance (Peng et al., 2025). Other mechanisms include overexpression of xenobiotic metabolizing enzymes and altered membrane permeability, contributing to multidrug resistance phenotypes. In *Fasciola hepatica*, exposure to triclabendazole increased EV production and led to measurable sequestration of triclabendazole and its active metabolites, suggesting a mechanism for drug clearance and detoxification (Davis et al., 2020).

9. Pre-Clinical Challenges

The primary hurdle in EV diagnostics is the high degree of biological variability and the inherent low concentration of EVs in human biofluids. Circulating EV counts vary significantly between healthy individuals, and these baseline levels are further shifted by inflammatory states or parasite infections. The selection of the biological matrix for EV isolation remains non-standardized and is typically dictated by organism and research objectives. The choice of biological matrix should be tailored to the organism and research question: plasma, serum, urine, or tissue for human and animal studies; culture supernatant for viral and bacterial EVs; and axenic culture supernatant at defined developmental stages for parasite development to ensure standardization and reproducibility in EV research (Shami-Shahet al., 2023 & Jamaly et al., 2018).Plasma and serum are commonly used due to their accessibility and established protocols, but the choice of anticoagulant can affect EV stability and composition. Urine and saliva offer non-invasive collection options, making them attractive for biomarker studies and population screening (Suresh & Zhang, 2025; Couch et al., 2021).

Blood collection should be performed using sterile, RNase/DNase-free tubes such as BD Vacutainer® SST™ to prevent nucleic acid degradation and exogenous contamination (Diehl et al., 2023; Szatanek et al., 2015).

Phlebotomy represents a primary source of experimental variance in extracellular vesicle (EV) research. Given that EV composition fluctuates dynamically with systemic inflammation, rigorous collection protocols are required to capture an accurate physiological snapshot.

Mechanical shear stress from small-gauge needles (<21G) or high-speed centrifugation can rupture these membranes, liberate sensitive cargo and thus render the biopsy invalid. Clinical best practice necessitates the use of large-bore needles (≥21G) to minimize hemolytic shear stress. When choosing a collection tube, K2-EDTA or citrate are the preferred anticoagulants, as heparin may destabilize vesicular membranes and inhibit downstream polymerase chain reaction (PCR) assays. Following collection, tubes must be handled with gentle inversion, avoiding agitation to prevent platelet activation. To mitigate stress-induced vesiculation from leukocytes, primary processing must be finalized within 1–2 hours of draw (Buntsma et al., 2022; Lucien et al., 2024; Konoshenko et al., 2018; Szatanek et al., 2015). Alternatively, allowing blood to clot upright at room temperature for 30–60 minutes ensures optimal separation of cellular components without inducing platelet activation or artificial vesicle release (Diehl et al., 2023).

The thermodynamic stability of the EV lipid bilayer is best preserved at 4°C, a temperature that suppresses both metabolic activity and the enzymatic degradation of luminal cargo. For long-term preservation, samples must be stored at -80°C. Storage at -20°C is contraindicated because ice crystal formation can physically rupture vesicular membranes. Repeated freeze-thaw cycles lead to the loss of miRNA integrity and the formation of irreversible aggregates driven by van der Waals forces. Samples should be aliquoted for single-use to ensure they are never refrozen post-thawing (Suresh & Zhang, 2025).

The selection of a resuspension buffer is critical for yield recovery, particularly when accounting for the "sticky," hydrophobic nature of the EV protein corona. While Phosphate-Buffered Saline (PBS) is standard, it must be filtered through a 0.22 μm membrane to eliminate background artifacts and particulates that may be erroneously quantified as EVs during Nanoparticle Tracking Analysis (NTA). The addition of cryoprotectants, such as human serum albumin (HSA) or trehalose, can further prevent vesicular adhesion to tube walls. To minimize adsorptive loss, researchers must use low-protein-binding polypropylene microcentrifuge tubes, as standard plastics significantly reduce final yields (Diehl et al., 2023).

Preanalytical isolation typically relies on differential centrifugation, utilizing sequential increases in centrifugal force to fractionate particles by density and size. The workflow begins at 300 ×g to pellet intact cells, followed by 2,000 ×g to remove platelets, and 10,000 ×g to eliminate larger apoptotic bodies (Szatanek et al., 2015; Aliakbari et al., 2024). Ultracentrifugation at 100,000 ×g for 70 minutes at 4°C using a swinging-bucket rotor is the gold standard for pelleting small EVs, as it ensures even pelleting and minimizes aggregation (Szatanek et al., 2015; Konoshenko et al., 2018; Shami-Shah et al., 2023). Washing the EV pellet with sterile, 0.22 μm-filtered PBS and repeating ultracentrifugation further removes protein contaminants and increases sample purity (Aliakbari et al., 2024; Diehl et al., 2023; Szatanek et al., 2015). Principal advantage of UC lies in its accessibility and its capacity to enrich vesicles without the need for specialized reagents. A significant limitation is the co-isolation of protein aggregates and other contaminants, which can compromise the integrity of downstream analyses, the high g-forces employed in UC may damage vesicle structure and function (Chhoy et al., 2021). To address some of these limitations, Density Gradient Ultracentrifugation employs a medium such as sucrose or iodixanol to separate EVs based on their buoyant density. This technique offers improved purity by allowing EVs to be distinguished from similarly sized but denser or lighter contaminants. Density gradient methods remain labor-intensive and time-consuming, and may still result in overlap with lipoprotein fractions, thereby limiting their utility in certain contexts (Konoshenko et al., 2018).

Size-Exclusion Chromatography (SEC) is increasingly favored in clinical settings. SEC utilizes a stationary phase of porous beads to elute larger vesicles first, effectively separating them from smaller, soluble protein aggregates. This "gentle" approach is superior for preserving the structural integrity and the biological corona of the vesicles. Instrument settings (column pore size, flow rate, and fraction collection) are critical, as excessive flow or inappropriate matrix can cause vesicle loss or co-elution with protein aggregates. Alternative polymer precipitation methods, while rapid and scalable, often co-isolate non-vesicular components, reflecting a trade-off between yield and specificity (Suresh & Zhang, 2025; Couch et al., 2021). This method often requires large sample volumes and multiple runs to achieve a sufficient yield, and some overlap with similarly sized particles may still occur (Sidhom, Obi & Saleem, 2020). Filtration and ultrafiltration methods utilize membrane filters with defined pore sizes to concentrate and partially purify EVs based on size. While these approaches are scalable and straightforward, they are prone to filter clogging and the potential loss or deformation of vesicles. Additionally, smaller vesicle populations may be lost during the filtration process (Shami-Shah et al., 2023). Polymer precipitation, using agents such as polyethylene glycol (PEG), is a rapid technique that does not require specialized equipment. However, it carries a high risk of co-precipitating non-EV proteins and particles, which can interfere with subsequent analyses (Lobb et al., 2015; Shami-shah et al., 2023).

The International Society for Extracellular Vesicles (ISEV) mandates a tripartite validation framework to confirm the purity and identity of isolated fractions. Verification of lipid bilayers via Transmission Electron Microscopy (TEM) or Cryo-EM that provides direct evidence of vesicular integrity and confirms presence of a lipid bilayer, which distinguishes EVs from protein aggregates or other nanoparticles (Bağcı, 2022).

Determination of size distribution and concentration using NTA or Tunable Resistive Pulse Sensing (TRPS). Validation of endosomal origin markers (e.g., tetraspanins) via Western blotting or high-resolution flow cytometry (Welsh et al., 2017; Yim et al., 2023; Spectradyne, n.d).

Common cargo molecules of research interest are miRNA, DNA and proteins. For the analysis of internal miRNA cargo, traditional phenol-chloroform (TRIzol) extractions are often bypassed due to suboptimal recovery and contaminant carryover. Instead, silica-column-based methods (e.g., miRNeasy) are preferred. These protocols employ specialized lysis buffers to disrupt the EV membrane and columns that selectively bind small RNA species, ensuring the high-purity yield required for Next-Generation Sequencing or qPCR. For DNA extraction, silica-based columns or magnetic beads are again optimal, especially for low abundance as they minimize loss and contamination. Sensitive quantification is achieved using fluorometric assays (e.g., Qubit), while integrity is checked by PCR or

electrophoresis (Kozhevnikova, Chernyshev & Yashchenok, 2023; De Sousa et al., 2023). Protein extraction from EVs typically employs buffers containing non-ionic or ionic detergents and protease inhibitors to solubilize both membrane and luminal proteins (Kowal et al., 2016). After lysis and clarification by centrifugation, protein quantification is performed using colorimetric or fluorometric assays. For comprehensive profiling, SDS-PAGE, Western blot, ELISA, or mass spectrometry are standard (Mosby et al., 2023). Comparative studies demonstrate that silica column- or bead-based extraction kits consistently outperform phenol-chloroform protocols for RNA and DNA yield and purity, especially for small RNA species (Elzanowska et al., 2022). For protein, detergent-based lysis followed by mass spectrometry or immunoassays is recommended for quantitative and qualitative profiling (Askeland et al., 2020). For high quality molecular analysis, the literature supports combining SEC or density gradient ultracentrifugation for isolation, followed by silica-based kits for nucleic acids and detergent-based buffers for protein extraction. Quality controls and negative controls are included for data integrity (Théry et al., 2018; Doyle & Wang, 2019).

In the context of pre-clinical challenges of pharmacological applications extracellular vesicles are increasingly viewed not as direct competitors to traditional synthetic lipid nanoparticles or liposomes, but as specialists occupying a niche for the transport of low-molecular-weight compounds. Representing a state of evolutionary biological optimization, natural EVs possess a functional architecture that synthetic pharmacology has yet to replicate, characterized by superior environmental plasticity and structural resilience that ensure cargo stabilization without the leakage common in more rigid, engineered spheres (Van der Meel et al., 2014; Al-Jipouri et al., 2023; Wang et al., 2025; Wang et al., 2026; Li et al., 2026).

The clinical utility of EVs is inextricably linked to their complex, heterogeneous membrane proteome, which extends beyond identification tetraspanins markers to include tissue-specific targeting ligands, environmentally sensitive integrins and MHC-antigen-presenting proteins that are protected from enzymatic degradation by their association with the vesicular bilayer and vesicle corona (Zeng & Morelli, 2018; Ciobanasu & Le Clainche, 2025).

10. Vaccine development

Conventional vaccine development relies on presenting antigens from inactivated or attenuated pathogens, recombinant proteins, or nucleic acids to the host immune system in order to trigger an immune response without causing disease (Plotkin, 2014).

The process begins with the selection of an appropriate antigen, usually a protein or peptide from the pathogen known to elicit an immune response. The antigen must be abundantly and consistently expressed, capable of inducing a strong immune response in the host, and specifically recognized by B-cell or T-cell receptors through unique epitopes not commonly found in host proteins, to avoid autoimmunity (Wu, Sun & Qi, 2024; Fan et al., 2023). The antigen must not provoke excessive reactogenicity or resemble host molecules, as these factors could trigger autoimmunity (Guimarães et al., 2015).

Highly variable antigens should be avoided; instead, the antigen should be conserved among different strains or isolates of the pathogen to ensure broad protection (Vita et al., 2019). For T cell-based vaccines, the antigen must be efficiently processed and presented by antigen-presenting cells. This requires efficient cleavage into peptides that bind with high affinity to major histocompatibility complex MHC molecules (Miller et al., 2015).

An important consideration is the recognition of the peptide MHC complex by T-cell receptors (Szeto et al., 2020). The stability of the antigen after purification is also a key parameter for large scale production in expression systems such as bacteria, yeast, or mammalian cells (Plotkin et al., 2017).

Despite these advances, conventional vaccines have demonstrated limited efficacy against parasitic diseases. Parasites possess complex life cycles, extensive antigenic variation, and sophisticated immune evasion mechanisms, which hinder the development of long-term sterile immunity both in natural infection and following vaccination (Lopes et al., 2024).

The effectiveness of vaccination fundamentally depends on the process of antigen presentation. Antigen presentation alerts the adaptive immune system, allowing it to specifically recognize, remember, and help eliminate pathogens (Joffre et al., 2012).

Antigenic material presented to the immune system during vaccination can be derived from various sources. Traditionally, vaccine antigens have been obtained

from intracellular components produced within infected host cells, typically encountered in attenuated or inactivated infections (Plotkin, 2014). More recently, recombinant antigens laboratory engineered proteins, peptides, or nucleic acids designed to mimic pathogen structures developing a synthetic strategy for eliciting targeted immunity (Viana Invenção, 2021).

Further innovations in vaccine design include the use of genetic material, such as plasmid DNA, RNA, or messenger RNA, as the basis for antigen delivery. In DNA-based vaccines, the introduced DNA enters the cell nucleus, where it is transcribed into mRNA. Messenger RNA vaccines deliver engineered mRNA directly to the cytoplasm, bypassing the need for nuclear entry. In both strategies, cytoplasmic ribosomes synthesize the encoded antigenic proteins endogenously within the host cell, mimicking the intracellular production of antigen during natural infection. The synthesized antigens are presented on the cell surface by major histocompatibility complex class I (MHC I) molecules. Presentation via MHC I activates cytotoxic CD8+ T lymphocytes and, through cross-presentation pathways, stimulates CD4+ T helper cell responses, generating immunity (Sahin et al., 2014).

Peptide-based vaccine mechanisms further advance this strategy by designing amino acid sequences to mimic specific epitopes, parts of a protein recognized by the immune system. Selection of these peptides is guided by their immunogenic potential, their ability to target desired immune pathways, and their capacity for efficient uptake by APCs such as dendritic cells, macrophages, and B lymphocytes (Sette & Rappuoli, 2010).

Upon administration, peptides are taken up by APCs through endocytosis or pinocytosis, the fate of the peptide then depends on its length and composition (Wang et al., 2022).

Peptides are grouped into short peptides (typically fewer than 12 amino acids), synthetic long peptides (SLPs, greater than 20 amino acids), and multi-epitope peptides. Short peptides are directly loaded onto MHC I molecules in the cytosol. This can occur via peptide exchange on recycling MHC I molecules at the cell surface or through the classical endogenous pathway involving proteasomal degradation and translocation by the transporter associated with antigen processing into the endoplasmic reticulum (Hewill, 2003).

Longer peptides, upon uptake, are degraded by proteases within endosomes or lysosomes, resulting in smaller peptide fragments that are loaded onto MHC class II molecules for presentation to CD4+ helper T cells or, via cross-presentation pathways, onto MHC I molecules for recognition by CD8+ cytotoxic T cells

(Joffre et al., 2012). Multi-epitope peptides incorporate several epitopes, thus broadening immune coverage (Li et al., 2025).

The advantages of peptide-based vaccines include customizable specificity and the capacity for adjustment to target closely related pathogens. This method has generated robust and long-lasting immunity with effective antibody production. Peptide vaccines are considered safer than other alternatives because no infectious agents are introduced, and the small peptide length reduces the risk of cross-reactive reactions, autoimmunity, and allergies (Skwarczynski & Toth, 2016).

Synthetic peptides are scalable and stable for industrial production. The challenges with peptide vaccination are that peptides degrade quickly, have a short shelf life, and may possess lower immunogenicity compared to other vaccination methods. As a result, they often require carrier proteins or delivery enhancements, such as cell-penetrating peptides or encapsulation within vesicles, to reach their target cells (Hans et al., 2006; Buonaguro & Tagliamonte, 2023).

The criteria for a theoretical ideal peptide vaccine candidate include several key physicochemical and immunological properties. First, the peptide should be of appropriate length between 8 and 20 amino acids to ensure compatibility with the major histocompatibility complex MHC molecules for effective antigen presentation (Abdulhameed Odhar et al., 2023; Kumar et al., 2023).

High aqueous solubility is essential to prevent peptide aggregation and precipitation, which can reduce stability, bioavailability, and immunogenicity. In general, peptides exhibiting solubility greater than 1 mg/mL in aqueous buffers at physiological pH are considered suitable, with typical desirable ranges extending up to 10 mg/mL. Peptides with solubility below this threshold are prone to forming aggregates and may require chemical modifications such as cyclization and encapsulation within nanoparticle delivery systems to enhance their stability (Skwarczynski & Toth, 2016; Xiao et al., 2025).

Another critical property is the peptide's net charge, ideally ranging from 0 to +2 at physiological pH. This moderate positive charge promotes favorable electrostatic interactions with the negatively charged membranes of antigen-presenting cells facilitating efficient cellular uptake without inducing cytotoxicity or unwanted aggregation (Ramirez-Acosta et al., 2022).

Alongside solubility and charge, the calculated instability index from *in silico* prediction tools such as ProtParam is used to assess the peptide's probability for degradation in vitro, with lower values indicating greater stability (Guruprasad et al., 1990; Gasteiger et al., 2005; Terziyski et al., 2023).

Immunologically, the peptide sequence should be evolutionary conserved across pathogen strains to offer broader vaccine coverage and efficacy (Vita et al., 2019). Strong predicted binding affinity to MHC molecules is evaluated via tools like NetMHCpan, this bioinformatic tool is used to predict robust activation of T-cell response (Peters & Sette, 2005).

Several bioinformatic platforms facilitate structure guided peptide properties evaluation. PepCalc, is an online tool that estimates peptide parameters (molecular weight, isoelectric point, net charge, hydrophobicity, and aqueous solubility) based on amino acid sequence. ProtParam is often used in complement with PepCalc, calculating the instability index of peptides and grand average of hydropathicity (GRAVY) score as well as aliphatic index, which collectively inform predictions of peptide stability and manufacturability (Gasteiger et al., 2005; Hossain et al., 2024; ProtParam documentation).

Computational approach allows for the prioritization of peptides with favorable characteristics and strong immunogenic potential, thereby evaluating vaccine candidates (Mamun et al., 2025).

Another approach is the epitope-based vaccines, focusing on the antigen and epitope selection as the foundation of vaccine efficacy. By using genetic and proteomic data for the organism, one can isolate conserved and immunodominant regions to maximise cross-strain protection. Computational methods are then used to predict T-call and B-cell epitopes by *in silico* screening and feedback guidance for in vitro immune assays design to confirm immunogenicity, followed by in vivo studies in animal models (Wei et al. 2025).

11. Bioinformatic pipeline

The foundation for this work was established through an extensive literature review, which was used to identify critical knowledge gaps which could be addressed through bioinformatic analyses, within the field of vaccine development. This work was the overarching long-term objective of a series of theses and research initiatives under the Swedish University of Agricultural Sciences (SLU) program aiming to develop an extracellular vesicle cargo based parasite vaccine. Two molecular classes have emerged as particularly promising: peptides and miRNAs.

To support this ambition, a pipeline was developed to support the identification and prioritization of peptide-based vaccine candidates from extracellular vesicle cargo proteins of parasitic organisms. The system integrates automated data retrieval, *in silico* proteolytic digestion, physicochemical characterization, epitope prediction, candidate scoring, and visualization into a reproducible, modular workflow implemented in Python with a Streamlit-based interface.

The code begins with necessary imports, including Streamlit for the web app interface, bioinformatics libraries (Biopython), data processing tools (Pandas, NumPy), and visualization frameworks (Matplotlib, Plotly).

This code uses the Biopython Entrez API to query the NCBI protein database by organism and protein keyword.

The "Epitope Prediction Threshold" and "Sliding Window Size" parameters in the code influence the sensitivity, specificity, and resolution of predicted B-cell linear epitopes in protein sequences. Lower threshold at 0,5 leads to a generation of more epitopes with higher sensitivity but carries the risk of finding false positives. A higher threshold at 2,0 finds fewer epitopes but with higher specificity and fewer false positives. A small sliding window of up to 3 residues detects fine local variations but can generate too much data while a higher sliding window at 15 residues gives a broader epitope region with less precision. Adjusting these parameters allows the user to tailor epitope finds and focus on high confidence candidates.

Fetched FASTA for the protein is trimmed by removal of 10 residues from N and C terminal to analyze antigenic regions. Trimmed Fasta is then pre-processed by trimming by *in silico* enzymatic digestion simulation (trypsin digestion) to split the protein sequence at lysine (K) or arginine (R) sites not followed by proline (P), mimicking biological proteolysis.

Shorter fragments are potential antigens; they are displayed in a table with calculated biochemical properties using Biopython and PepCalc documentation formula of calculation of molecular weight, instability, GRAVY score, and aliphatic index. The hydrophobicity score is calculated after gathering data based on the Parker scale, thus reflecting peptide surface accessibility and solubility characteristics.

Users can analyze individual finds and click select, thus generating a theoretical epitope by using a BepiPred-like sliding window algorithm scoring each residue based on average hydrophilicity values, as such predicting linear B-cell epitopes. Matplotlib is used for epitope score profile calculation.

12. Results

The following results present the biophysical foundation written in defense of the Multi-Machinery Model establishing a quantitative framework for analyzing the complexities of extracellular vesicle communication. Central to this presentation are the biophysical formulas developed to simulate and predict vesicle secretion rates under specific environmental stressors. These formulas are designed to serve as computational filter for subtraction of background environment noise from observed experimental data. The formulas represent environmental determinants identified in literature during wet lab observation: hypoxia, thermal variation and hydrogen ion concentration (pH). Each equation utilizes sensitivity constants and saturation parameters to reflect the nonlinear behavior of biological regulatory machinery.

To bridge the divide between theoretical modeling and experimental application, this thesis addresses methodological barriers in the analysis of extracellular vesicle cargo and clinical translation of cargo composition. This thesis introduces a bioinformatic pipeline designed to optimize acquisition and computational processing of proteomic databases, by identification of stable immunogenic peptides for the rational evaluation of vaccine candidates by epitope analysis using the *Schistosoma mansoni* organism, the Sm-TSP-2 antigen and HLA-DR1 (MHC-II) receptor.

12.1 Multi Machinery Model Formulas

The nonlinear relationship of how hypoxia affects extracellular vesicle rate production is illustrated in Figure 1, The formula addresses declining oxygen tension and kinetic flux, characterized by Hill type sigmoidal response. The formulation allows for the isolation of hypoxia specific signaling by establishing a predictive baseline for cellular output under varying oxygen concentrations.

$$r(t) = r_T \cdot q_S(S) \cdot g_W(W) \cdot \lambda_{hyp}(x)$$

While

$$\lambda_{hyp}(x) = \frac{K^n + x_0^n}{K^n + x^n}$$

Figure 1. Multiplicative phenomenological model for impact of hypoxia on extracellular vesicles rate release.

Impact of thermal variation accounting for cold and hot is defined in Figure 2, using Arrhenius inspired scaling factor to account for metabolic velocity and membrane fluidity. The formula is a temperature dependent variable.

$$r(T,\tau) = r^* \cdot \exp\left[-\frac{E_a}{R}\left(\frac{1}{T_K} - \frac{1}{T_{0K}}\right)\right] \cdot \left(\frac{\eta(T_0)}{\eta(T)}\right)^{\beta} \cdot \exp\left[-\left(\frac{T - T_0}{\sigma_T}\right)^{p}\right] \cdot [1 + A_{stress}(T)]$$

$$A_{stress}(T) = A_+ \frac{(\max(0, T - T_0))^m}{T_{50,+}^m + (\max(0, T - T_0))^m} + A_- \frac{(\max(0, T_0 - T))^h}{T_{50,-}^h + (\max(0, T_0 - T))^h}$$

Figure 2. Multiplicative phenomenological model for impact of temperature on extracellular vesicles rate release.

Figure 3 presents phenomenological modulatory effect of pH changes on vesicle biogenesis. The formula captures the shift in cellular behavior at low pH, often associated with pathological stress and survival strategies of parasites and oncological cancer cells.

$$r(pH_c, pH_e) = r^* e^{-\frac{\Delta G(pH_c, pH_e)}{RT}} \left[1 + A_0 \frac{(H_e/H_0)^n}{K_{pH}^n + (H_e/H_0)^n}\right] e^{-\left(\frac{pH_c - pH_0}{\sigma_{up}}\right)^{p_{up}}} e^{-\left(\frac{pH_0 - pH_c}{\sigma_{low}}\right)^{p_{low}}}$$

Figure 3. Multiplicative phenomenological model for impact of pH on extracellular vesicles rate release.

12.2 Vaccine Candidate Evaluation

The selection of an optimal protein of interest is a critical decision in vaccine design, as the target must be both essential to the pathogen's life cycle and accessible to the host's immune system. By analyzing the biophysical profile of optimal proteins researchers can identify conserved, surface-exposed domains that are likely to elicit a protective humoral response across multiple pathogen variants. This screening process is particularly vital when dealing with large, complex proteomes commonly found in multicellular parasites, where identification of a single, stable, and immunogenic peptide. This bioinformatic pipeline facilitates the *in silico* identification of vaccine candidates by integrating remote data acquisition with physicochemical and immunogenetic profiling. Utilizing the Biopython mediated Entrez Utility Interface, the system retrieves validated GenBank records and FASTA sequences from the NCBI database based on user defined taxonomic and proteomic parameters. The workflow simulates physiological or analytical processing through a trypsin digestion algorithm, which identifies cleavage sites at Lysine (K) or Arginine (R) residues, accounting for the inhibitory effect of C-terminal Proline, generating a library of peptides representative of natural antigen processing.

In a living system immune system interacts with fragments of proteins processed by antigen presenting cells, large proteins are broken down into small peptides via enzymatic proteolysis that is simulated in the labs by trypsin digestion and in this pipeline by the trypsin algorithm. Practical laboratory production uses the mass spectrometry and trypsin as gold standard protein digestion enzyme based on its high specificity. These fragments are then filtered through a diagnostic suite to assess their suitability for laboratory validation, utilizing the Instability Index to predict half-life, the Aliphatic Index for thermal stability, and GRAVY scores to evaluate global hydropathicity. The pipelines primary utility centers on a sliding-window algorithm that employs Parker Hydrophilicity Scale to predict linear B cell epitopes, prioritizing residues with high surface accessibility potential.

A "good" vaccine candidate is characterized by a negative GRAVY score (<0), indicating high solubility, and an Instability Index below 40, ensuring structural integrity during experimental assays. A "poor" candidate is typically identified by high positive GRAVY scores and low Parker peaks, suggesting sequestered, hydrophobic regions that are likely to aggregate or remain "invisible" to the humoral immune response. By correlating these biophysical requirements with predictive immunogenicity, the pipeline connects raw genomic data to prioritized, viable synthetic peptides, significantly reducing the experimental bottleneck in vaccine development.

Streamlit:
https://vaccinecandidadeapp-ouaeoojbhr3emtfqqr9dnv.streamlit.app/
Github
https://github.com/evgeniatrudova/vaccine_candidade_app

The initial high-throughput screening of the *Schistosoma mansoni* proteome Sm-TSP-2 was conducted using a Streamlit-based processing as illustrated in Figure 4. To optimize the identification of immunogenic hotspots, the epitope prediction threshold was set to a stringent 1.00, ensuring only high-confidence sequences were prioritized for downstream structural analysis. A sliding window size of 7 was implemented to calculate the mean scores of neighboring residues, effectively smoothing the data to identify continuous topological peaks rather than isolated amino acids. This window size corresponds to the typical length of a linear B-cell epitope or an MHC-II binding core, maximizing the biological relevance of the predicted hits.

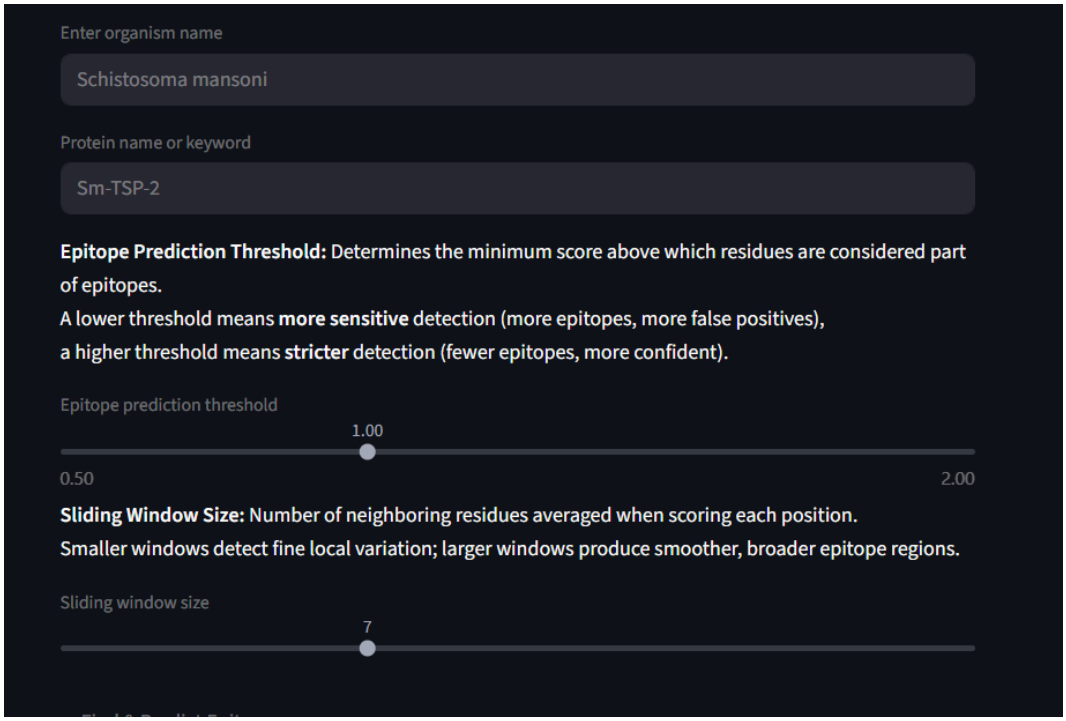


Figure 4. Bioinformatic Screening and Epitope Prediction Interface.

Structural and genomic data for the target antigen were retrieved from the NCBI Protein Database (PDB ID: 2M7S, Chain A), identified as a CD63-like tetraspanin (Sm-TSP-2) se Figure 5. In the bioinformatic pipeline, this protein is cross-referenced using two distinct identifiers to ensure data integrity: UniProt ID Q8ITD7, which provides the blueprint of the full-length sequence, and PDB ID 2M7S_A, which provides the high-resolution atomic coordinates for 3D modeling. The raw FASTA sequence was subjected to a strategic trimming protocol, removing 10 residues from both the N-terminus and C-terminus. This pre-processing step serves to isolate the core antigenic domains and eliminate disordered flanking regions or hydrophobic signal peptides that might otherwise introduce stochastic noise into the epitope prediction algorithm.
By refining the sequence to its structurally conserved core, the predictive accuracy for surface-accessible hotspots is significantly enhanced. This ensures that the downstream sliding-window analysis (utilizing a threshold of 1.00 and a window size of 7) focuses exclusively on the biologically relevant extracellular regions capable of forming a stable immunological synapse.

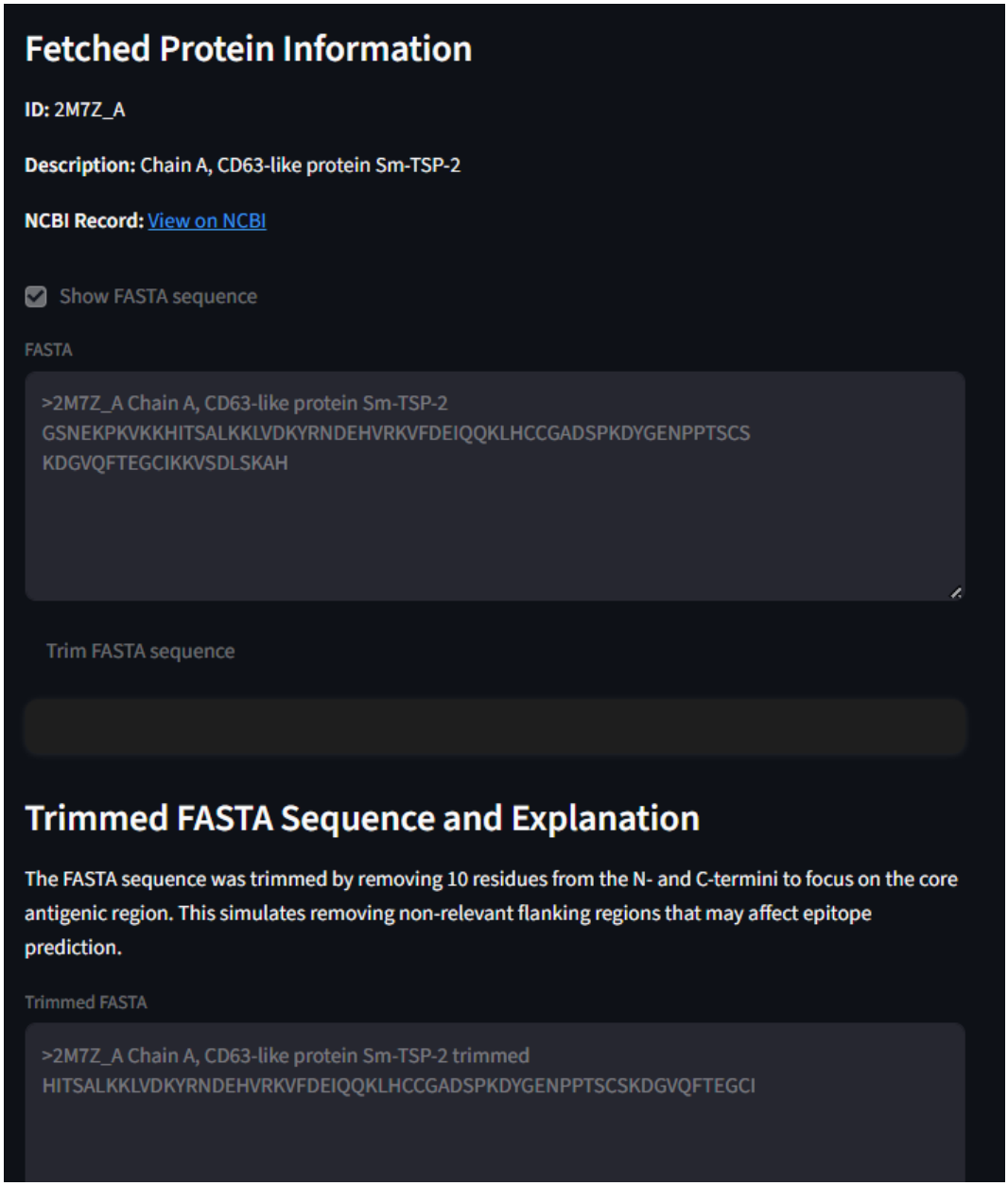


Figure 5. Sequence Retrieval, Nomenclature, and Pre-processing of the Sm-TSP-2 Antigen.

In the Figure 6 table is presented with the physicochemical profiling of tryptic peptides generated from the Sm-TSP-2 sequence. The top candidate, SNEKPK (derived from the peptide GSNEKPK), exhibits a profile optimized for surface exposure and immune recognition at length 7aa, that matches the core binding length required for many MHC-II molecules and B-cell epitopes, with molecular weight of 758.82 Da characteristic for lo weight, short peptide fragments suitable for synthesis. The instability index of -2.2 is negative, as long as value is below 40 the peptide is evaluated as biochemically stable and avoids rapid degradation. The Grand Average of Hydropathicity (GRAVY) is negative which combined with the high Parker Scale hydrophobicity score confirms the peptide is highly water soluble, this is desired for interaction with target. Aliphatic index is at a 0, which indicates an absence of aliphatic side chains (Alanine, Valine, Isoleucine, Leucine) this suggests that the epitope is not a part of a hydrophobic core. The second epitope suggestion EHVR has a Aliphatic Index (4833.33) which suggests that it is lipid associated and hydrophobic, this is characteristic of transmembrane domains and this epitope is rejected from further analysis.

Select	Predicted Epitopes	Peptide	Length	MW	Instability	GRAVY	Hydrophobicity	Aliphatic Index
☐	SNEKPK	GSNEKPK	7	758.82	-2.2	-2.514	1.357	0
☐	EHVR	NDEHVR	6	768.78	-4.23	-2.333	1.2	4833.33
☐	-	K	1	146.19	0	-3.9	3	0
☐	-	VK	2	245.32	-9.4	0.15	0.75	14500
☐	-	HITSALK	7	768.9	59.21	0.214	-0.243	12571.43
☐	-	K	1	146.19	0	-3.9	3	0
☐	-	LVDK	4	473.56	-51.3	0.15	0.675	17000
☐	-	YR	2	337.37	-79.55	-2.9	0.35	0
☐	-	K	1	146.19	0	-3.9	3	0
☐	-	VFDEIQQK	8	1006.11	72.33	-0.8	0.45	8500
☐	-	LHCCGADSPK	10	1030.18	55.22	-0.28	0.15	4900
☐	-	DYGENPPTSCSK	12	1297.35	36.63	-1.592	0.508	0
☐	-	DGVQFTEGCIK	11	1196.33	12.03	-0.173	0.182	6181.82
☐	-	K	1	146.19	0	-3.9	3	0
☐	-	VSDLSK	6	647.72	8.33	-0.167	0.55	11333.33
☐	-	AH	2	226.23	-37.45	-0.7	-0.5	5000

Figure 6. Analysis of Peptide Biochemical Properties and Epitope Selection.

Figure 7 presents visual interface provides a residue-by-residue comparative analysis between the predicted epitope and the parent peptide. The standardized color-coded scale visually represents match quality, facilitating the prioritization of high-fidelity candidates. This is a theoretical control that must be cross-referenced with the complete biochemical profile meant to be used as a priority list of epitope candidates.

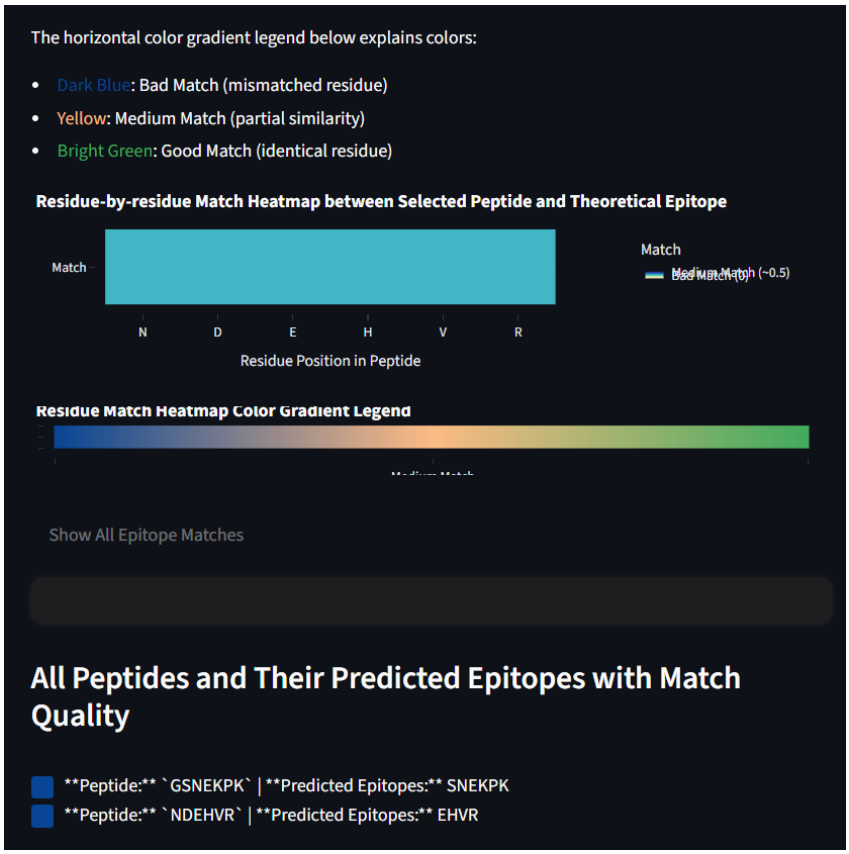


Figure 7. Heatmap-Based Epitope Match Quality Analysis

Figure 8 illustrates the structural interrogation of the *Schistosoma mansoni* tetraspanin-2 (Sm-TSP-2) candidate investigated within the UCSF ChimeraX environment. The visualization is adjusted to include B-factor coloring, where blue signifies high conformational stability, alongside a ball-and-stick rendering to evaluate the surface accessibility of the target epitope area. The file has been formatted and optimized for subsequent rigid-body docking simulations within the ClusPro platform.

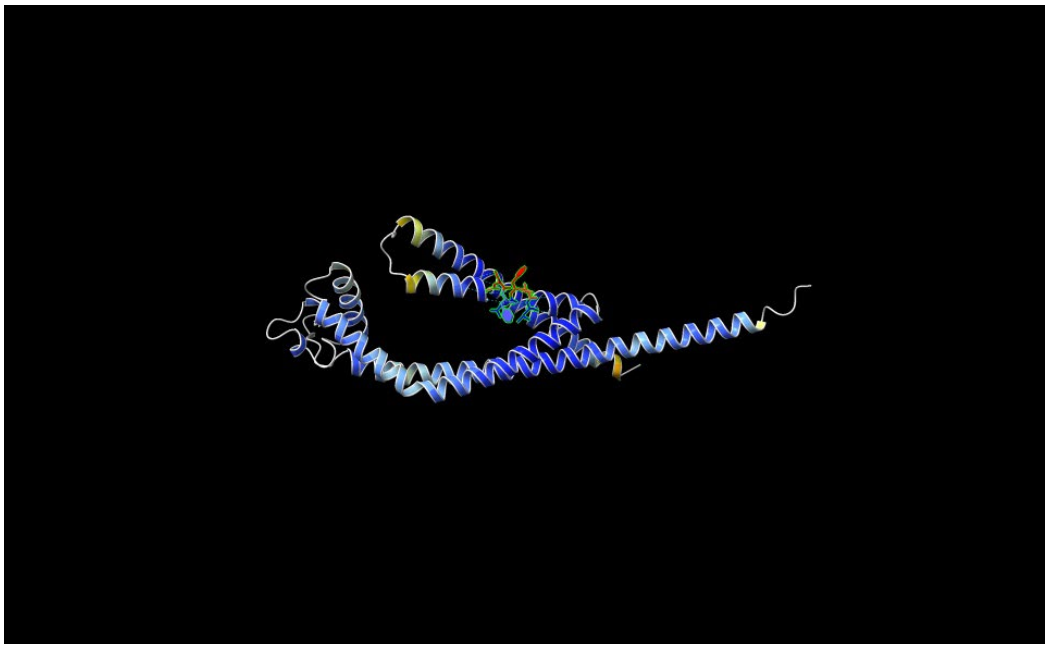


Figure 8. Structural Topography and Surface Accessibility of the Sm-TSP-2 Epitope.

Figure 9 displays the human immunological receptor HLA-DR1 (PDB ID: 1DLH), highlighting the Alpha chain (Chain A) in yellow and the Beta chain (Chain B) in magenta. The topography was analyzed using B-factor coloring to assess the stability of the binding cleft, utilizing ball-and-stick rendering to interrogate the specific residue interface intended for antigen presentation. This receptor model was prepared according to the requirements for ClusPro algorithmic processing to ensure accurate epitope sequestration analysis.

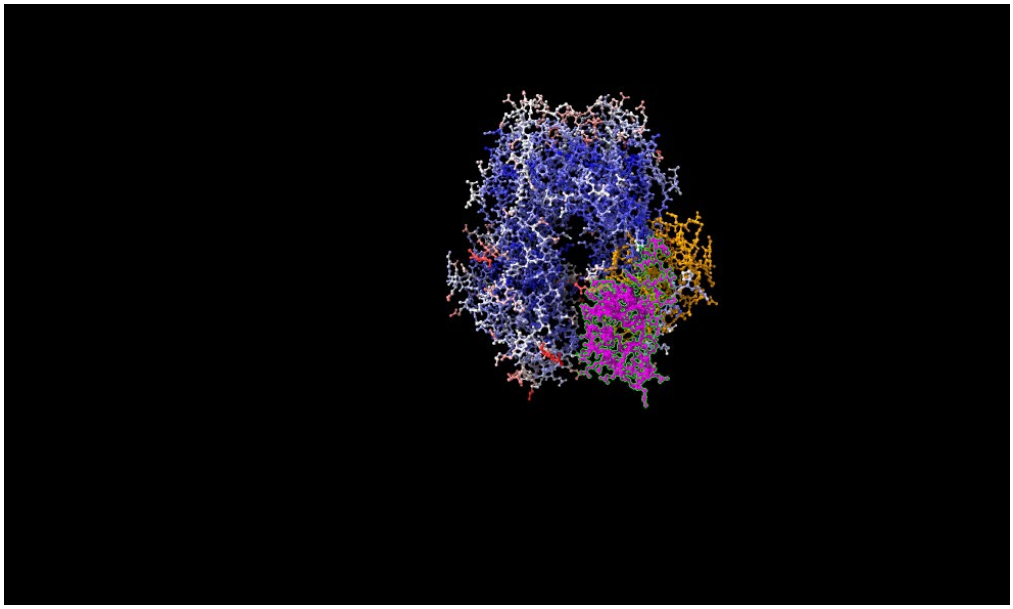


Figure 9. Structural Architecture of the Human HLA-DR1 (MHC-II) Receptor.

In the Figure 10, the balanced ClusPro score in presented, Cluster 0 is identified as the most statistically probable binding mode, containing 41 members. This indicates that the largest number of individual docking simulations converged upon this specific spatial orientation. The energy values represent the thermodynamic affinity of the interaction. A more negative score indicates a stronger spontaneous attraction. The Lowest Energy value of -1210.5 kcal/mol in Cluster 0 signifies a highly stable complex. The Center score represents the average energy of the cluster, while the Lowest Energy identifies the most optimized fit within that group. The minimal variance between these two values in Cluster 0 (a difference of ~11.2 kcal/mol) demonstrates a high level of structural consistency within the primary binding orientation.

Cluster	Members	Representative	Weighted Score
0	41	Center	-1199.3
		Lowest Energy	-1210.5
1	37	Center	-1092.9
		Lowest Energy	-1282.7
2	37	Center	-1215.3
		Lowest Energy	-1263.1

Figure 10. Statistical Convergence and Thermodynamic Stability of Molecular Docking Clusters for Balanced run using the ClusPro (PIPER) algorithm for Sm-TSP-2 Epitope (PDB ID: 2M7S, UniProt ID Q8ITD7) and HLA-DR1 MHC-II molecule (PDB ID: 1DLH).

Figure 11 shows favored docking results under the Electrostatically Favored scoring coefficient. Unlike the "Balanced" model, this scoring mode assigns higher weights to electrostatic attractions simulating the environment where long-range ionic interactions guide the parasite epitope into the MHC-II binding groove. Cluster 0 exhibits the highest statistical prevalence with 48 members, indicating that under electrostatic constraints, this specific orientation is the most frequent and favorable landing site for the ligand. Notably the 41 members appear in both tables showing cross-coefficient convergence, this provides a methodological robustness that number of orientations 41 is geometrically and chemically suitable for docking with the epitope.

Cluster	Members	Representative	Weighted Score
0	48	Center	-1105.7
		Lowest Energy	-1309.4
1	43	Center	-1169.1
		Lowest Energy	-1394.8
2	41	Center	-1133.0
		Lowest Energy	-1291.1

Figure 11. Electrostatically Favored Docking Analysis and Energy Landscapes run using the ClusPro (PIPER) algorithm for Sm-TSP-2 Epitope (PDB ID: 2M7S, UniProt ID Q8ITD7) and HLA-DR1 MHC-II molecule (PDB ID: 1DLH).

Figure 12 shows results of the molecular docking simulation under the Hydrophobic-Favored scoring coefficient. In this mode, the ClusPro algorithm prioritizes desolvation energy and non-polar attractions simulating the burial of hydrophobic side chains away from the aqueous solvent and into the non-polar pockets of the receptor. The energy scores in this model are significantly more negative than the previous Balanced or Electrostatic runs, with Cluster 0 reaching a Center energy of -2003.4 kcal/mol and a Lowest Energy of -2256.6 kcal/mol. This dramatic increase in stability suggests that the $SNEKPK$ epitope despite its overall hydrophilic nature possesses specific residues capable of forming powerful hydrophobic contacts within the MHC-II binding cleft.

Cluster	Members	Representative	Weighted Score
0	51	Center	-2003.4
		Lowest Energy	-2256.6
1	49	Center	-1798.7
		Lowest Energy	-2391.1
2	46	Center	-2046.7
		Lowest Energy	-2062.6

Figure 12. Hydrophobic Favored Docking Analysis Landscapes run using the ClusPro (PIPER) algorithm for Sm-TSP-2 Epitope (PDB ID: 2M7S, UniProt ID Q8ITD7) and HLA-DR1 MHC-II molecule (PDB ID: 1DLH).

Figure 13 illustrates the results of the docking simulation when the scoring function is restricted specifically to Van der Waals forces (shape complementarity) and Electrostatic interactions (charge complementarity). The weighted scores are lower (ranging from -213.2 to -286.0 kcal/mol) compared to the balanced or hydrophobic models. This is a common artifact of removing the heavily weighted desolvation/hydrophobic terms.

Cluster	Members	Representative	Weighted Score
0	69	Center	-245.1
		Lowest Energy	-255.7
1	65	Center	-224.6
		Lowest Energy	-253.1
2	61	Center	-213.2
		Lowest Energy	-286.0

Figure 13. Combined Van der Waals and Electrostatic (VdW + Elec) Docking Analysis run using the ClusPro (PIPER) algorithm for Sm-TSP-2 Epitope (PDB ID: 2M7S, UniProt ID Q8ITD7) and HLA-DR1 MHC-II molecule (PDB ID: 1DLH).

This visualization in Figure 14 and Video Figure 1 depicts the rigid-body docking of the *Schistosoma mansoni* tetraspanin-2 antigen (blue) into the binding groove of the human HLA-DR1 MHC-II receptor, processed via the ClusPro algorithm and rendered in UCSF ChimeraX. The ribbon diagram illustrates the secondary structural alignment, while the ball-and-stick model highlights the specific sequestration of the target peptide within the immunological synapse. The high degree of surface complementarity observed at this interface provides biophysical evidence of the epitope's stability and its potential for effective antigen presentation.

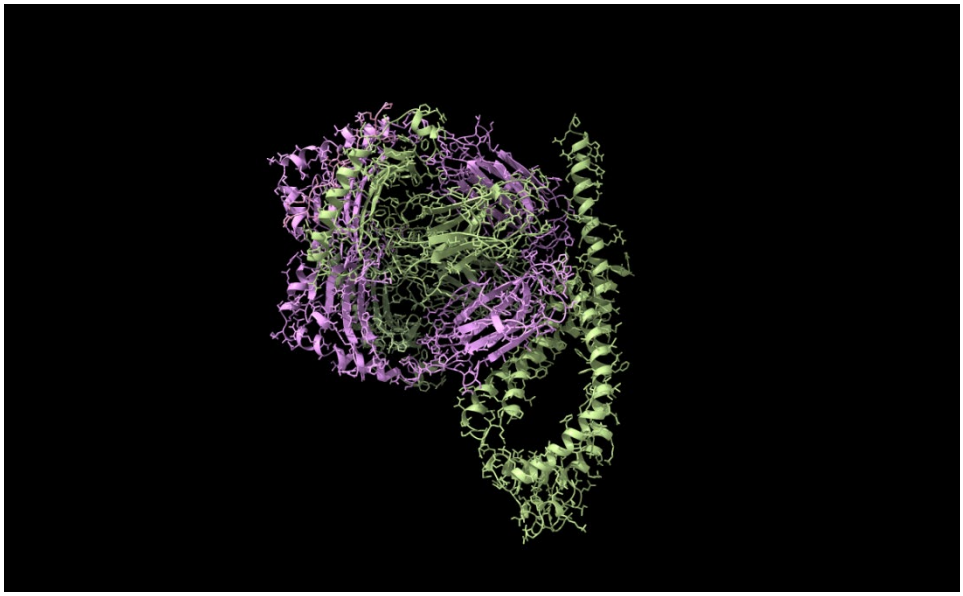
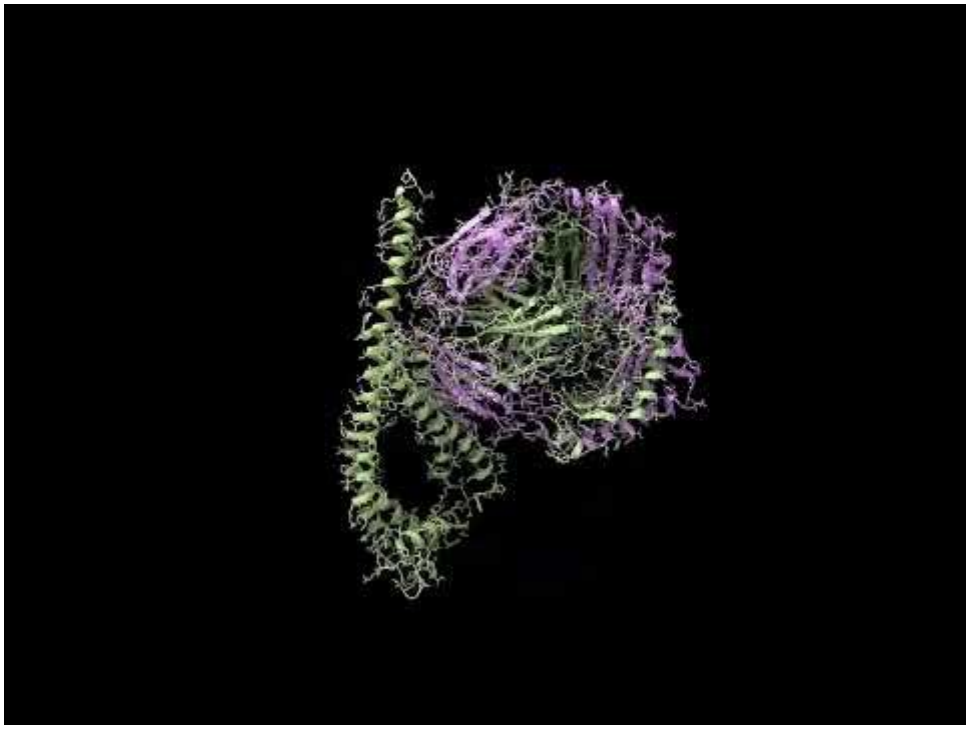


Figure 14. Visualization of the Immunological Synapse and Epitope Sequestration using ClusPro and visually interrogated within UCSF ChimeraX.



Video Figure 1. Dynamic Topographic Analysis and Binding Interface of the Sm-TSP-2/MHC-II Complex. This video provides a 360-degree rotational analysis of the docked complex, offering a comprehensive view of the spatial relationship between the parasite antigen and the human immune receptor using ClusPro and visually interrogated within UCSF ChimeraX.

13. Discussion

This thesis proposes a Multi-Machinery Model of Cellular Communication, conceptualized from a system-level perspective of extracellular vesicle cargo formation. In defense of the model, the thesis proposes a computational biology approach describing extracellular vesicles as a biophysical object governed under laws of physics and within a biological system, developing formulas that account for environmental stimuli such as hypoxia, thermal stress and pH fluctuations. This interdisciplinary investigation attempts to bridge the gap between molecular biology, mathematics and computational biology and biophysics needed for develop a strategy for development of robust baseline for extracellular vesicle rate formation. Central to this work is aimed to isolate meaningful biological signals from the environmental stimuli to improve diagnostic accuracy and understanding of extracellular vesicle cargo composition. Finally, the thesis discusses insights from parasitology, oncology and immunology and proposes a Python application for investigation of vaccine candidates and bioinformatic pipeline with molecular docking simulation.

13.1 Theoretical Framework of the Multi Machinery Model

The study of EV cargo has emerged as a promising avenue for uncovering the intricate and multilayered processes governing cellular communication. The current body of research, while expanding, often addresses EV biogenesis and cargo loading in smaller studies and in isolation, concentrating on individual signaling pathways or discrete cellular processes. This thesis proposes that the mechanisms underlying EV cargo formation involve the integration of multiple signaling pathways and cellular machineries, each responsive to a range of physiological and stress-related stimuli.

A comprehensive understanding of these interconnected processes holds significant potential for advancing disease modeling, elucidating mechanisms of pathogen resistance, and enhancing the mapping of intracellular signaling pathways. Extracellular vesicles are increasingly recognized as key mediators of intercellular communication, transporting a diverse array of proteins, nucleic acids, and lipids. The specific composition of EV cargo may provide direct insight into the regulatory networks and communication “language” which is operational within and between cells.

A critical barrier to progress in this field is the lack of standardized methodological approaches, particularly with respect to the application of consistent stress conditions and the establishment of reliable baseline controls. Analyzing EV production and cargo composition in pathological or perturbed states without a robust understanding of the corresponding neutral or healthy baseline limits the interpretability and comparability of possible discoveries. Establishing clear baseline conditions is essential for discerning the quantitative and qualitative changes in EV output analyzed by bioinformatics when specificity of cargo is analyzed. Without robust comparison, the data risks becoming false positive or false negative, thus risking the concealment of the possible existence of potential activation patterns of the underlying regulatory machinery.

The body of research on extracellular vesicles is still in its early research phase, but the potential of synthetically imitating naturally occurring extracellular vesicles as delivery vehicles in pharmacology has already been emphasized, making that a statistically significant focus within the field. However, current approaches tend to concentrate less on reproducing the natural processes underlying EV formation, such as the finely regulated expression of surface proteins and mechanisms of vesicle budding and cargo sorting. Instead, much of the research effort is directed toward the development of synthetic nanomaterials designed for cargo encapsulation and delivery.

Although these artificial systems are frequently engineered to replicate selected functional aspects of EV-mediated transport, they remain challenging to regulate within complex biological environments. Their design is predominantly cargo-centered, often neglecting the broader physiological context of the host or patient into which the delivery is intended. As a result, rather than functioning as accurate analogues of natural extracellular vesicles, such constructs carry the risk of acting as synthetic intercellular debris, useful for only single time usage. This fact naturally raises significant concerns regarding their long-term stability, safety, and overall biocompatibility within living systems.

Passive encapsulation of cargo methods results in heterogeneous liposome populations characterized by a broad size distribution, and formation of large multilamellar vesicles at 1 to 5 μm in diameter, which are approximately 10 to 100 times larger than exosomes, when the larger apoptotic bodies also classed as exosomes are excluded from size scale. Extracellular vesicles naturally produced by living cells exhibit a relatively homogeneous population with smaller diameter exosome populations. This uniformity pattern supports the research on active encapsulation of cargo with regulated biogenesis and budding processes.

Cargo centered design in pharmacological research often discovers and highlights challenges which evolution previously solved when developing extracellular vesicles. Delivery of therapeutic nucleic acids presents inherent challenges due to their negatively charged and hydrophilic nature, which hinders efficient passage across biological membranes. Simple encapsulation of nucleic acids within a lipid bilayer does improve the passage and provides protection of the cargo from nucleases, yet shows poor stability in vivo. This instability primarily arises from weak electrostatic interactions between the negatively charged nucleic acid and the largely neutral lipid bilayer. Evolutionary solution to this problem is transport of nucleic acids by protein bindings with the membrane. In laboratory studies this is measured as encapsulation efficiency and volume of leakage of cargo from the lipid bilayer particles.

This indicates that extracellular vesicles which show effective and targeted delivery in biological systems are likely to protect the cargo by strong and stable linkage between the nucleic acid cargo and the lipid bilayer, ensuring protection under pressure and during the time the cargo spends in the systemic circulation.

Alternatively, cargo might be sorted by internal cationic or ionizable domains which electrostatically attract the negatively charged cargo in direct electrostatic stabilization to the inner membrane of the extracellular vesicle.

The majority of extracellular cargo consists of nucleic acids, proteins and lipids. From a biochemical perspective, when these components coexist in a solution, the negatively charged nucleic acids are likely to exhibit strong binding affinity to the proteins present. This might indicate that the architecture of extracellular vesicles may include substructures such as smaller lipid assemblies or complexes which isolate and compartmentalize the cargo types, perhaps in easily digestible single bilayer membranes which have yet to be visualized using current imaging techniques.

The membrane of natural extracellular vesicles is enriched with proteins, some of which are known. For example, tetraspanins (CD9, CD63, CD81), Lamp2b, lactadherin, PTGFRN and TSPAN family proteins, which are collectively involved in regulation, budding and cargo loading. The natural extracellular vesicles are also protected by expression of CD47 which prolongs circulation and prevents phagocytosis, it/they display protein modulation with phosphatidylserine attachment in uptake process with the target recipient cell. Synthetically developed and membrane hybridized extracellular vesicles show no or badly controlled ability to deliver cargo to the intended tissue and further have no cellular protection from the intracellular environment. This challenge is faced by using known proteins on the surface of the membrane as anchors or sorts for

surface engineering, thus enabling fusion with targeting peptides, antibodies, signal motif modifications and designed adaptor proteins/ligands capable of pulling specific cargoes into the lumen thus ensuring safe intracellular delivery (Brezgin et al., 2024).

This thesis acknowledges solution-focused research and the pharmacological capacity to engineer highly complex cellular and vesicle-based delivery systems, including phi29 pRNA–based RNA nanoparticles (Shu et al., in Guo & Afonin, 2022), mesoporous silica nanoparticles for siRNA transport (Tarannum & Vivero-Escoto, in Guo & Afonin, 2022), RNA micelles (Yin, Shu & Guo, in Guo & Afonin, 2022), engineered EV hybrids (Ishiguro & Patel; Nordmeier, Hsiung & Portnoy, in Guo & Afonin, 2022), exosome-like nanoparticles for siRNA delivery (Lu & Huang, in Guo & Afonin, 2022), aptamer-guided RNA nanoparticle conjugation (Leonard et al., in Guo & Afonin, 2022), immunostimulatory RIG-I–activating nucleic acid nanoparticles (Johnson, in Guo & Afonin, 2022), and self-assembled RNAi nanostructures for multiplexed gene silencing (Ben, Salah & Rossi, in Guo & Afonin, 2022).

This thesis proposes a more holistic view of EV cargo as a reflection of integrated and dynamically regulated cellular networks at specific moments of biological operation. The increasing focus on task-specific synthetic nanomolecules for cargo delivery, often associated with higher toxicity or limited nucleic-acid specificity, highlights the importance of gaining deeper control over low-toxicity natural EV cargo management systems and foundational EV biogenesis before further expanding synthetic nanoparticle design. At present, synthetic systems cannot fully replicate the natural mechanisms by which extracellular vesicles dynamically adjust cargo composition and adjust membrane protein expression in response to stimuli, leaving a major gap in our fundamental understanding of cellular communication.

By adjusting laboratory methodology to combine both controlled and stress-induced and/or disease-induced environment stimuli, the quality of data can be expected to improve. Cargo loading and regulatory machinery might additionally carry cooperative or competitive characteristics where EV variety, travel destination, production volume and production timing can be reflective of interplay, with a threshold level of stimuli for activation of the respective machinery.

To illustrate this model, a farming practice rooted in folk traditions worldwide offers an intuitive example of stress-induced stimulation of EVs, namely red chili flakes. Red chili flakes containing capsaicin are added to the feed of farm animals as an empirical method for controlling bacterial and parasitic infections. At the

molecular level, many farm animals lack sensitivity to capsaicin's typical burn sensation, yet capsaicin effectively binds to transient receptor potential vanilloid 1 (TRPV1), receptors located in the gut (Rosca et al., 2020). TRPV1 is a polymodal, non-selective cation channel expressed widely across species and cell types, functioning as a versatile cellular sensor for environmental and stress signals such as heat, acidity, oxidative stress, and chemical irritants. Structurally, TRPV1 forms a tetrameric channel permeable to calcium ions, which activation triggers a calcium influx as a crucial second messenger in downstream signaling cascades (Rosca et al., 2020).

Functionally, TRPV1 serves as a danger detector on both immune and neuronal sensory cells. Upon capsaicin binding and activation, TRPV1 undergoes conformational changes which prompt rapid calcium entry, initiating signaling pathways involving cytokines and transcription factors which modulate gene expression and peptide secretion. A key downstream response tied to this signaling is the enhanced shedding of extracellular vesicles, accompanied by remodeling of EV cargo to include components which exert protective and defensive functions during inflammation or infection (Rosca et al., 2020).

In the context of the gut, capsaicin-induced TRPV1 activation mimics mild inflammatory signaling, resulting in increased mucus production and enhanced communication within the microbiome. These changes translate into strengthened antiparasitic and antibacterial defenses (Rosca et al., 2020). Moreover, TRPV1's expression on microglial cells in the brain further exemplifies its role in EV production, where activation in microglia promotes microvesicle release, facilitating neuroimmune communication during inflammatory processes (Marrone et al., 2017). This example underscores the existence of at least two cellular states: a neutral baseline and a stress induced activated state, each characterized by distinct EV production profiles and cargo composition.

Beyond the gut, EV mediated communication between cancer cells and healthy cells exemplifies the functional consequences of stress-induced EV cargo remodeling. For instance, extracellular vesicles derived from head and neck squamous carcinoma cells can induce nerve growth and activate transcriptional programs associated with injury response and regeneration in recipient healthy cells, mediated by EV cargo through the TRPV1 receptors (Inyang et al., 2022).

Complementing these findings, investigations into TRPV1 in the hippocampus region reveal stress-associated upregulation of this receptor, with pharmacological inhibition producing anxiolytic and antidepressant effects, further linking TRPV1 actuation, cellular stress pathways, and EV mediated intercellular communication.

Molecular understanding of extracellular vesicles is still young and lacking system level integration yet carries close to limitless potential in many science areas, among these, the potential ability to hijack the bodies own mechanisms to regulate and adjust processes of disease. This brings the potential to resolve many diseases with early diagnostics and new combo therapies where the disease itself is slowed down at a cellular and extracellular vesicle communication level.

Extracellular vesicles have historically been regarded as cellular waste disposal mechanisms. However, advancements in technology which enable the isolation and characterization of their cargo have prompted a paradigm shift in perspective. This raises a critical question: how can one develop a comprehensive and robust analytical framework for extracellular vesicles, given their ubiquitous presence across all cell types, cellular processes, and disease states?

Current research predominantly investigates EVs within the confines of specific diseases or isolated cellular mechanisms implicated in disease progression. Such an approach, however, fails to capture the dynamic and systemic nature of cellular communication. This thesis aims to delineate potentially significant regulation mechanisms and stress responses involved in extracellular communication, thereby providing a broader contextual understanding of function.

Another objective of this project is outlining insights derived from parasitology which offer considerable promise for advancing the study of extracellular vesicles. As eukaryotic organisms have co-evolved with higher organisms such as humans, parasites possessed more intricate extracellular regulatory systems than prokaryotes, utilizing proactive “hijacking” strategies. These strategies mirror carcinogenesis of some aggressive cancer microenvironments resolving fundamental challenges of resource extraction and immune evasion (Pawlowka et al., 2026) and influence the cell by signal manipulation to express new supportive roles and in some cases suppress hosts immune system responses (Morckhoven et al., 2023). However, despite these mechanical and extracellular vesicle cargo parallels the fields of parasitology, oncology and immunology operate as largely independent disciplines with need for interdisciplinary research and expanded funding.

Parasitology as a field, is refocused on anthelmintic resistance where extracellular vesicles play one of key roles. Parasitic helminths significantly undermine global agricultural productivity by infecting livestock, thereby contributing to substantial economic losses and exacerbating food insecurity. Widespread use of anthelmintics in livestock has driven the resistant parasite populations to increase in various regions, reducing the drugs' effectiveness.

This growing resistance crisis necessitates the exploration of innovative, sustainable control strategies which can operate independently or synergistically with traditional therapeutics. Development of a new methodology for vaccine development, where exploration of pathogen derived extracellular vesicle cargo, containing material such as peptides, microRNA and mRNA with immuno modulating properties can and should be explored further.

The theoretical development of a peptide-based vaccine would be initiated with the isolation of extracellular vesicles from the target parasite, employing a validated methodology such as that outlined in the laboratory science section of this work. Following isolation, the EV preparation would undergo comprehensive proteomic profiling to separate and characterize the mixed molecular cargo, with particular focus on confirming the purity, size distribution, and identity of constituent peptides.

The primary objective of proteomic data mining at this stage is to identify peptide sequences which are representative of the pathogen, and which correspond to structurally accessible epitopes located on the parasite's outer membrane. This requires an involved bioinformatic analysis, beginning with epitope prediction based on immunogenic potential.

Predictive modelling would consider a range of parameters, including molecular weight and peptide length, predicted binding affinity to host major histocompatibility complex alleles, sequence conservation across diverse parasite strains, hydrophilicity, surface accessibility, and conformational flexibility. The epitope prediction data would then guide the rational design of a candidate vaccination peptide, which could subsequently be matched to peptides physically recovered from EV isolates. In doing so, theoretical predictions and empirical laboratory findings are integrated to refine the selection process and identify the most promising immunogenic candidates.

Key selection criteria for optimal vaccine peptide–epitope pairing include molecular weight and structural size, high-affinity binding, broad population coverage and strong sequence conservation.

For industrial development of an EV derived peptide vaccine, the workflow would involve synthetic production of the selected peptide antigen, alongside formulation of an effective delivery system capable of targeting the appropriate host immune cells while preserving peptide stability and immunogenicity. Consideration would also be given to adjuvant selection, thermostability for field deployment, and manufacturing processes compliant with good manufacturing practice.

In cancer research, the EVs have been shown to carry not only cargo but also dynamic molecular fingerprints on the membrane; these are tissue specific signatures from lipids, proteins, RNAs and DNA fragments from their parent cells. Cancer types show variance in these membrane fingerprints, making it possible to identify cells of origin in control studies. A strong hypothesis is that the unique combination of fingerprints carry "gain-function" characteristics which are linked to the extracellular vesicle mediated cell communication which participates in the process of not only cargo drop off but also modulation of tumorigenesis and epigenetic remodeling of the cell (del Real Mata et al., 2025; Abhange et al., 2021).

The strategy of fingerprinting and identifying EVs, not only by cargo contents but also cell or organism of origin, holds potential for application in medicine. One of these areas is early cancer detection which can considerably reduce cancer surgery costs in healthcare, and late-stage mortality. Nonetheless, cancer is a complex area of research, and "liquid biopsy", as serum tests containing EVs are sometimes referred to, are difficult to scale up and make cost effective compared to current clinical diagnostics. Moreover, EV isolation is a heavy laboratory technique which needs to be engineered for instrument processes. Cancer might also show local EV distribution rather than a systematic one which reaches the blood volume, to exemplify a few of the challenges of effective diagnostics.

Another problem might be the currently limited information on extracellular vesicle communication of different cancer types, and at which stage in development the cell identifies with distinct fingerprinting of a malign cancer (Sullivan et al., 2017). This area is under global investigation. In Sweden, the collaboration platform SweSEV is established for EV research. In the time period 2023-2025, a group led by Rossella Crescitelli at Sahlgrenska is actively exploring intercellular communication within the tumor microenvironment across a variety of cancer types. While Susanne Gabrielsson's research team at the Karolinska Institutet is focused on elucidating the immunological roles of EVs in cancer progression and therapy (Crescitelli, 2025; Gabrielsson, 2024; SweSEV).

In a clinical setting, many benign growths, cell differentiations and polyps remain undiscovered and many never develop to malign uncontrolled invasion, metastasis of carcinoma (Davis et al., 2001). Furthermore, early diagnostics with sensitivity for benign growth would carry risks to diagnose statistically significant portion of population as "false malign positive" for common benign cell growth types like gastrointestinal polyps (Abdulqader, 2024; Harder et al., 2024).

Thus, the selection of cancer cell lines for biomarker development should be done in cooperation with current pathology diagnostics and strengthen the slower

and manually harder diagnose groups of tumors with distinct systematic EV biomarker distribution.

From a bioinformatics perspective, the future of extracellular vesicle research lies in developing a computational framework which can handle the large data volume and dynamic parameters observed in nature. There is a lot of potential in development of model simulation of EV communication as, intercellular signaling system with EVs as autonomous agents carrying cargo categorized into states such as neutral, stressed, or diseased.

By simulating these agents within a multi-agent environment, we can capture the dynamic nature of cellular communication. In this model, environmental stressors and pathological signals would serve as inputs which modify the internal states of EV agents. The simulation would represent a network in which EV agents are generated, travel through the system, and interact with target cells. Development of state transition and decision-making algorithms would be needed to reflect biological behavior. These algorithms would need to be based on laboratory examination of cellular mechanics. To realistically capture biological variability, the framework must incorporate stochastic and probabilistic elements, embracing the inherent noise and unpredictability of biological systems. Together, these features would offer insights into improvement of analysis of laboratory methods and guide research on unlocking cellular communication.

Advancing extracellular vesicle research through functionality and evolutionary simulations is a multidisciplinary pursuit, drawing not only on computational modeling but also powerful insights from laboratory experiments. Recent work demonstrated that EV complexity marked by compositional heterogeneity and function diversity can be approached by integration of bioinformatics and synthetic laboratory models.In the Katla, Lin and Pérez-Mercader (2025) study, a fully synthetic, abiotic system simulated micelle formation with core biological features of extracellular vesicles through a nonbiochemical process. The nonamphiphilic molecules were exposed to green light and polymerized into amphiphiles, which spontaneously self-assemble into vesicular structures. These vesicles reproduce themselves nonlinearly via the expulsion and reorganization of partially polymerized amphiphiles, thereby manifesting loose heritable variation across successive generations. This artificial mechanism mirrors the compositional diversity and inheritance observed in natural EV populations and might be a synthetic model of simple EV budding process.

Further supporting this paradigm, experimentation on peptides encapsulated in vesicles provided insights into evolutionary cycles with repetitive hydrothermal pressure changes which promoted selection and self-optimization among the

vesicle population. In the dynamic conditions which simulate the environment, the vesicles showed notable enhancements in stability, permeability, and size across generations (Mayer et al., 2018).

EV heterogeneity study methodology leaped forward with the integration of laboratory science, bioinformatics and evolutionary biology, combining techniques such as single-vesicle cryo-TEM imaging analyzed by machine learning segmentation to characterize vesicle morphologies, and identify subpopulations based on shape and size, in molecular biology evolutionary modeling (Kapoor et al., 2025).

While ongoing research seeks to reconstruct evolutionary processes within controlled environments, it is equally important to examine naturally evolved, resilient models across a range of organisms, including bacteria and parasites. Among these, parasites such as helminths and protozoa are of particular interest, as they exhibit dynamic mechanisms of extracellular vesicle cargo regulation which have co-evolved with their hosts. These mechanisms may functionally parallel those observed in higher organisms and show evolutionary survival strategies with sophisticated vesicular machinery linked to immune modulation, host invasion, and intercellular communication.

13.2 In Silico Validation and Biophysical Modeling of the Multi Machinery Model

From a computational and biophysical perspective, the Multi-Machinery Model formalizes the extracellular vesicle as a state-dependent autonomous agent operating within a biological multi-agent environment. This model acknowledges the profound, yet under-characterized, influence of exogenous environmental stimuli on the regulatory networks governing cellular communication. By establishing these mathematical foundations, the framework enables the construction of increasingly complex systems-level descriptions of intercellular signaling. Empirical observations derived from laboratory experimentation and biophysical calculations identify three primary physical determinants that modulate the kinetic state of these agents: hypoxic stress, thermal fluctuations and acidic stress.

13.2.1 Hypoxia

Hypoxia is reduction in oxygen tension, which significantly increased the rate of extracellular vesicle secretion. This has been observed in breast cancer cells (King et al., 2012 & Wang et al., 2014) and in pancreatic cancer cells (Patton et al., 2019) where not only number of vesicles increased but also cell size distribution

was noticed. In more modern studies the hypoxia has been linked to lysosomal homeostasis and HIF-1a (Wang et al., 2023).

Mathematical derivation follows the system biology modeling framework established by Smith and Fleck (2017). Adopting their approach to coarse-grained biological descriptions, the model simplifies the intricate, multi-step processes of EV biogenesis into a modular multiplicative rate equation. This methodological choice allows the complex interplay between internal cellular states and external metabolic factors to be subsumed into discrete, state-dependent variables that reflect the system's overall kinetic behavior.

By describing impact of hypoxia with a multiplicative phenomenological model of instantaneous rate of vesicle release as a product of state dependent and stimulus dependent factors, the following rate equation can be formulated.

$$r(t) = r_T \cdot q_S(S) \cdot g_W(W) \cdot \lambda_{hyp}(x)$$

While

$$\lambda_{hyp}(x) = \frac{K^n + x_0^n}{K^n + x^n}$$

r($\boldsymbol{t}$) : EVs released per minute
rT: baseline EV release in neutral state
qS(S): "internal state" of the cell (mechanisms active/inactive)
gW(W): metabolic activity factor
$\boldsymbol{\lambda}$hyp (x): effect of oxygen level
x : variable of interest
x_o : baseline value of variable
K : half sensitivity constant
n : steepness
Baseline: 100 vesicles/min
Baseline oxygen: 40mmHg
K = 15mmHg
n = 2
Gs = 1
Gw = 1
Hypoxia O_2: 10 mmHg
r(t) = 100 · 1 · 1 · (225 + 1600 / 225 +100) = 100 · 1 · 5.6154 = 561.54 vesicles/min

Result: Change from 100 vesicles at neutral state to theoretical rate of 561.54 vesicle/min at hypoxia 10 mmHg.

The hypoxia formula suggests to evaluate rate of extracellular vesicle secretion as r(t), that is not a fixed constant but a product of regulatory and environmental input where rT is the baseline at neutral state (e.g., 100 vesicles/min) while internal cellular states are described by dependent internal state and metabolic activity factors, set at 1 for unity and future development of separate intracellular and extracellular stress impacts on the cell.

13.2.2 Temperature

Extreme thermal fluctuations serve as a biophysical determinant for accelerated extracellular production (Baxter et al. 2019) this was proven in investigation of lytic cell death in THP-1 monocytes where the extracellular vesicles were observed under non apoptotic conditions using nanoparticle tracing analysis that confirmed increase in particle concentration compared to untreated control cells. Findings of this study are used to set a stress induced activation amplitude for the thermal flux formula at the A = 2.3 representing observed increase in EV production capacity.

The literature on the topic is limited, but primary findings suggest that the cellular reaction to temperature is more complex then reactivity to hypoxia, the cell shows a decreased EV rate in cold temperatures and slightly increased EV rates in the warm temperatures, when observed in the prime non apoptotic area that can be triggered by extreme temperature shifts and leads to drastic shedding and increase of extracellular vesicles before the cell dies.

From a biophysical perspective, the Arrhenius Law defines the temperature dependence of reaction rates. As thermal energy within the cellular environment rises, the increased kinetic energy heightens the frequency and probability of molecular interactions. This allows the system to overcome energy barriers, in this model set at 42.6 kJ/mol a standard required for physical transformation of the lipid bilayer. There transformations include the induction of membrane curvature, which is needed for EV formation and part of the ESCRT machinery mechanics for vesicle release.

However, the EV rate is state dependent secretion, which can be simulated by avoiding a linear progression and calculating a metabolic tolerance factor V(T) with Gaussian formula and activate it with stimuli sensitive Hill function. Theoretically this would account for the different reactivity in different temperatures as internal capability to produce vesicle is included in the calculations, the suppression noticed in lower temperatures would be due to loss of kinetic energy as per Arrhenius law and the slight increase in EV rate production in moderate heat would be per gain of kinetic energy as per Arrhenius

law that overrides the biological strain of tolerance factor that should be noted in both thermic changes as the cell is active in non-optimal conditions. Extreme stress shedding as observed in THP-1 monocytes (Bacter et al., 2019) likely happens as a sharp switch activation, this is nonlinear reaction as buffer limit is reached and is possible when modeled by Hill function in a biological model.

$$r(T,\tau) = r^* \cdot \exp\left[-\frac{E_a}{R}\left(\frac{1}{T_K} - \frac{1}{T_{0K}}\right)\right] \cdot \left(\frac{\eta(T_0)}{\eta(T)}\right)^{\beta} \cdot \exp\left[-\left(\frac{T - T_0}{\sigma_T}\right)^{p}\right] \cdot [1 + A_{stress}(T)]$$

$$A_{stress}(T) = A_+ \frac{(\max(0, T - T_0))^m}{T_{50,+}^m + (\max(0, T - T_0))^m} + A_- \frac{(\max(0, T_0 - T))^h}{T_{50,-}^h + (\max(0, T_0 - T))^h}$$

r* : baseline for EV release at the set point of 100 vesicles/min at 37ºC
E_a : Standard activation energy for biochemical reactions (0.44eV approximately 42.6 kJ/mol).
R : Universal gas constant (8.314J/(mol · K))
T_K,T_{0K} : Absolute temperature and baseline temperature in Kelvin (310.15 for 37ºC).
$\eta(T_0)/\eta(T)$: biophysical proxy for semi permeable lipid bilayer of extracellular vesicle membrane dynamics that is sensitive to physical resistance of the environmental changes in temperature, set at baseline temperature and current temperature
β: scaling exponent for viscosity impact, set at 0.2
σT : Tolerance width, defining temperature range for metabolic function set at 6ºC
p : Gaussian decay set at 4
A : Amplitude, A for strong heat is set at 2.3. If A+ = 0, no heat stress effect. If A+ > 0, EV rate changes according to cell type. If A- = < 0, slower metabolism in many cell types leads to systematic decrease of EV rate production (Huang et al., 2023). Exception is specialized cold exposure brown fat derived miR.378a-3p hepatic (BAT) cells that increase secretion of EVs (Xu et al., 2023)
T50 : Half activation temperature offsets for stress response.
m, h : Hill function steepness coefficients for the switch like activation of stress machinery.

Example set in a situation where temperature increases from the optimal 37ºC to 40ºC. The cell is assumed to have efficiency score with baseline of optimal temperature, as the temperature rises the Arrhenius law speeds the processes. A rate of V (t) at 1.0 would mean optimal function while any changes would decrease or increase the efficiency of the cell. By using Hill function for activation and Guassian function for tolerance, the biological conditions are described to include switch like functionality of stress activation stimuli and

metabolic buffer tolerance of stimuli to mimic the realistic response to the environmental changes in temperature.

r* : 100 vesicle/min
T+ = 40 ºC
ΔT+ = 40 – 37 = 3 ºC
A+ = 2.3
T50 = 2ºC, moderate heat
m = 2, slope
Δtemp = 1+ 2.3 · (9/ (4+9)) = 1 + 1.592 = 2.592
σT = 6ºC
Tnormalization = ΔT+ / σT = 3/6 = 0,5
p = 4
T normalization^p = 0,5 ^{4} = 0,0625
V (T) = exp [−(0.0625)] ≈ 0.939

Tolerance factor decreased from 1.0, which indicated that heat stress is likely to push the EV production to be faster while the internal machinery slows for to 93.9% at 40ºC.

$$\Phi_{Arr} = \exp\left[-\frac{E_a}{R}\left(\frac{1}{T_K}-\frac{1}{T_{0K}}\right)\right] = \exp\left[-\frac{42.6\ \text{kJ/mol}}{8.314\text{J/(mol}\cdot\text{K}}\left(\frac{1}{310+3}-\frac{1}{310}\right)\right] =$$

1.171

$$\Phi_{phys} = \left(\frac{\eta(T_0)}{\eta(T)}\right) = \left(\frac{0.691}{0.653}\right)^{0.2} = 1.011$$

$r \cdot \Phi_{\text{Arr}} \cdot \Phi_{\text{phys}} = 100 \cdot 1.171 \cdot 1.011 = 118.4$, increase with 18.4 vesicles/min which is 18.4%.

$V(T) \cdot 118.4 = 111.2$ vesicles/min at metabolic tolerance, this is the realistic increase that is lower then theoretical increase due to less efficient production rates at thermal strain.

When the temperature decreases moderately from the 37ºC to 34ºC, the tolerance factor remains symmetrical, the efficiency drop is the same at V (T) = exp [−(0.0625)] ≈ 0.939 however metabolic baseline and viscosity changes drastically with decreased temperature.

$$\Phi_{Arr} = \exp\left[-\frac{E_a}{R}\left(\frac{1}{T_K}-\frac{1}{T_{0K}}\right)\right] = \exp\left[-\frac{42.6\ \text{kJ/mol}}{8.314\text{J/(mol}\cdot\text{K}}\left(\frac{1}{310-3}-\frac{1}{310}\right)\right] = exp$$ -0.1614 = 0.851

$$\Phi_{phys} = \left(\frac{\eta(T_0)}{\eta(T)}\right) = \left(\frac{0.691}{0.734}\right)^{0.2} = 0.988$$

$r \cdot \Phi_{\text{Arr}} \cdot \Phi_{\text{phys}} = 100 \cdot 0.851 \cdot 0.988 = 100 \cdot 0.841 = 84.1$ vesicle /min which is realistic for EV rate decrease, when exposed to lower temperature.

13.2.3 pH

Acidification triggers EV shedding in melanoma at shift from neutral pH(7) to acidic pH (6.5) noted in the microenvironment of melanoma (Parolini et al., 2009). Mechanics of influence is not a good fit for linear variable influence, there is a need to separate physical energy from biological signaling responses, this would allow the formula to account for signaling to increase or decrease or eventually shut down from metabolic exhaustion and apoptosis. Because pH acts as both a thermodynamic barrier and a signaling trigger for the shedding flux this can be described as asymmetric tolerance model.

The pH-dependent flux formula is written in three layers that determine the extracellular vesicle secretion rate by decoupling fundamental bioenergetic constraints from regulated biological signaling. At its core, the formula uses Gibbs free energy to establish a thermodynamic barrier, which dictates the energetic cost of vesicle biogenesis. This physical foundation is modulated by a Hill activation function that governs the non-linear biological behaviors of the cell, allowing the system to transition between baseline secretion and high-output signaling states in response to environmental stimuli. The formula incorporates a Gaussian decay function to simulate the asymmetric cellular sensitivity to pH fluctuations to reflect the physiological limits of the system.

$$r(pH_c, pH_e) = r^* e^{-\frac{\Delta G(pH_c, pH_e)}{RT}} \left[1 + A_0 \frac{(H_e/H_0)^n}{K_{pH}^n + (H_e/H_0)^n}\right] e^{-\left(\frac{pH_c - pH_0}{\sigma_{up}}\right)^{p_{up}}} e^{-\left(\frac{pH_0 - pH_c}{\sigma_{low}}\right)^{p_{low}}}$$

r (pHc, pHe): rate of extracellular vesicle production/min vesicle/min
r : standard secretion rate at optimal homeostasis (37oC pH 7.2) set at 100 vesicle/min
pHc = Cytosolic pH (≈ 7.2) homeostasis
pHe = Endosomal pH (≈ 6.5)
Ao = Amplitude of the pH response, maximum potential strength of the pH induced response.
He/H_0= Proton concentraton/neutral state
K_{pH} = sensetivity to the pH changes
ΔG (pHc,pHe) = free energy difference across the membrane
R : Universal gas constant (8.314J/(mol · K))
T = temperature (K)
n = steepness of response

Example of asymmetric pH tolerance where cells show more sensitivity to acidic decrease in cytosolic pH.

R total = thermodynamic barries module x Hill function x tolerance module
r = 100 vesicles /min
RT = 8.314J/(mol·K) x 310K = 2577.34 J/mol
pHc = 7.2
pHe = 6.2
r * Exp (- ΔG /(pHc,pHe)) = r * e0 = 100 x 1 = 100
Ao = 2
He/H0 = 1
KpH = 1
n = 2
r total = 100 x 1 x 2 x 1 = 200 vesicles /min at neutral baseline

Formula suggests that at neutral baseline the combination of signaling and physical parameters lead to increase of EV rate production from baseline 100 vesicle/min to 200 vesicles/min, attempting to reflect the homeostasis to signaling active state.

Provided calculations demonstrate in silico validation of hypoxia, temperature and pH within the Multi-Machinery Model illustrating how environmental stressors are mathematically integrated to predict cellular behavior, by quantifying the transition from the neutral physiological state to a stressed state, describing dynamics of cellular communication.

13.3 In Silico Workflow for Proteomic Processing and Vaccine Candidate Evaluation

The practical application of this thesis is a code pipeline engineered for modular and reproducible experimental workflow to strengthen *in silico* proteomic processing and selection of cargo peptides for experimental trials in parasite studies. By automated protein sequence retrieval from NCBIs Entrex with Biopython the researcher can extract protein sequences from a wide range of organisms with real-time access to updated databases for optimal data mining. Preprocessing data with trypsin digestion generates peptide fragments which reflect potentially present fragments in simulation of antigen-processing in vivo. This enables identification of peptides suitable for immune recognition. Analysis of fragments by their physicochemical properties can support evaluation and selection of peptide vaccine candidates. Using a BebiPred-like algorithm and Parker hydrophobicity scale displays potential in per residue approach to peptide-epitope match, which might serve as B-cell epitopes in nature in an attempt to identify immunogenic hotspots. The app generates a peptide library that mirrors the products of natural protein degradation and laboratory mass spectrometry, this ensures that the identified epitopes are not theoretical but have characteristics of bioavailability that increases likelihood of presentation to B-cells.

Python and Streamlit were selected to bridge the gap between bioinformatics and clinical utility, the Biopython and Pandas libraries are robust and powerful tools for the task of sequence manipulation using FASTA files as input data files. Streamlit allows a Python script to be interactive web-based dashboard, enabling visualization of data without additional environmental setup and free of use permanent hosting of the application in public access. The code of the application is minimal and does not reflect full complexity of the epitope conformation analysis, designed as easy screening of candidates for laboratory processing by a single script/dashboard.

To expand the computational pipeline the user needs to view the application as preliminary selection of peptides designed for proteomic docking processing. The application generates a grading system of multiple epitopes to visualize biochemical robustness by color coded scale to represent match quality with dark blue signifying a poor match with mismatched residues, yellow to indicate moderate similarity and bright green to identify identical residues. This allows the researcher to prioritize peptides that are theoretically stable and suitable.

To bring the analysis from theory into robust in sillco analysis, the user needs to use the provided direct link to the NCBI for every retrieved sequence, this feature allows for manual verification of organism, protein and PDB identifier as well as

confirmation of the taxonomic parameters for the experiment. This data needs confirmation from the cross reference database UniPro where the protein name can be searched by Entry ID and search results provide a 3D visualization options, by choosing the result with the 3D visualization the user can visualize and dock the proteins of the researched organism against molecular fit, or docking into a suitable receptor protein. In the UniPro database, the user needs to follow Structure Feature Viewer in the navigation to find the preliminary 3D visualization and table with structural data categorized by determination method, including NMR, X-ray crystallography (PDB) and computational modeling (AlphaFold).

Following the identification of high priority, the AlphaFold Protein Structure Database is suitable for providing a macromolecular crystallographic information file mmCIF, to coordinate the target protein. Once the file is reprieved the user needs to choose a suitable receptor and download the pdb and mmCIF files for the receptor.

In this study, the protein of interest is a *Schistosoma mansoni* from the family of *Schistosomatidae*, a parasite that exists in water in Africa, Asia and South America and causes a fever disease with internal bleeding and chronic organ damage on humans and agricultural animals consuming unfiltered water. The disease is treated by a common anthelmintic agent prazikvantel (Vale et al., 2017, Park et al., 2019).

Selected example is from organism *Schistosoma mansoni the protein is Schistosoma mansoni tetraspanin-2 (*Sm-TSP-2 Q8ITD7). The receptor is a HLA-DR1 MHC-II (PDB ID 1DLH) human immunological molecule with antigen recognition functionality.

For the identification of vaccine candidates derived from extracellular vesicle cargo, best practices involve the characterization of the EV protein profile to establish a primary sequence database. This pipeline is usable in processing of the protein database, by cross-reference of sequences against *in silico* trypsin degradation patterns, ensuring that the identified peptides represent stable, bioavailable fragments. To reduce experimental bottlenecks identified peptides are filtered through a diagnostic suite to evaluate biochemical robustness and integrity. By choosing candidates with high Parker hydrophilicity scale scores via a sliding window algorithm (typically 7) to predict linear B cell epitopes. Solubility is evaluated based on negative GRAVY score (typically <0). Stability is evaluated by instability index being set at below 40. “Good” candidates exhibit high surface accessibility and low instability while “Poor” candidades can have

high GRAVY scores and low Parker peaks which is likely associated with hydrophobic regions at risk for aggregation.

The physicochemical profiling of the peptides identified SNEAKPK, derived from GSNEKPK as a top candidate with a length of 7aa matching the MCH-II binding cores and molecular weight of 758.82 DA and instability index of -2.2. The negative GRAVY score and high Parker score confirm the high-water solubility. Second identified candidate EHVR was rejected due to very high aliphatic index at 4833.33 suggesting that it is a transmembrane domain protein. The primary heatmap analysis scores both candidates in the same shade of blue, which shows that the score is just a primary evaluation, needing biochemical analysis of data.

The topographical analysis of the vaccine candidate is performed within the UCSF ChimeraX environment. Rather than manual file management, the software allows for direct database integration via the command-line interface. By executing *open Q8ITD7 from alphafold* and *open 1DLH from alphafold* commands, the researcher instantly retrieves the high-confidence computational model of the *S. mansoni* antigen and the experimental crystal structure of the human MHC-II receptor. To move beyond a simple ribbon diagram, two specific visualization techniques are employed. Ball and stick representation shows the protein backbone and renders individual atoms and side chain orientation; this is interesting information because the target epitopes risk being locked withing the protein. B factor coloring representation is a proxy for structural flexibility and confidence. The blue colored regions show low B factor and show stability while red and yellow regions indicate areas of high mobility and lower structural certainty, often located at ends of the protein, by selecting a stable region the area is evaluated for suitability for docking to receptor.

Another structural information retrieval from NCBI and UniProt are the structural information about the protein, the Sm-TSP-2 is a member of tetraspanins superfamily of proteins with architecture of dedicated area of host-parasite interaction located in the large extracellular loop, this area is a main target for vaccine design and is located in the 107-184 residues.

The epitope identified by the vaccine candidate evaluation app is located on the 61-67 residues, which is not on the large extracellular loop, but in a different structurally defined area located between first and second transmembran domain called small extracellular loop. This area is suitable for host interaction when confirmed for high surface accessibility by the B factor coloring stability, as blue in color. Even without optimal position in the “cone” of large loop tetraspanins

core this area remains accessible to the host immune machinery, classically viewed as secondary target area after large loop potential.

The structural and residue location information needs confirmation by return to NCBI protein database, where the conserved domain databases information is accessible as a table with confirming residue locations. And a cross reference with UniProt database where the same information is located in the subcellular location, with separated extracellular and cytoplasmic domains. This dual database approach ensures that the epitope is not a mathematical match, but a structurally sound surface exposed candidate.

Once the protein and receptor are structurally analyzed the files are saved as pdb files, and uploaded to the external ClusPro platform, that runs on the Kozakov-Vajda docking algorithm for rigid body protein-protein docking with protein of interest in this case Q8ITD7 file and receptor as 1DLH file. The algorithm provides a docking score, that is used for evaluation of vaccine candidates. ClusPro uses fast fourier transform PIPER methodology for the docking, where the receptor is fixed in space while the ligand is translated around it in billions of possible orientations, this is computationally possible because of the fast fourier transport algorithm that converts the 3D spatial data of the proteins into frequency domain, the system calculates interaction energy of multiple positions instead of checking each individual interaction docking. PIPER generates billions of results, and sorts it down to best fit matcher, where all values higher than -1000 energy weighted scores are not included in the results. The matches are compacted by balanced, electrostatically favored, hydrophobically favored and Van der Wall & electrostatically favored scores.

Results from the ClusPro scores are used to compare vaccine epitope candidates between each other in a way that the application script cannot do independently, the scores do reflect full complexity of the epitope conformation analysis. The docking output typically crashes without nucleotide compatibility, the score refrects on average energy of a cluster offering overview of energetic favorability. The “Lowest Energy” score pinpoints the absolute optimized fit, representing the most energetically favorable conformation.

As observed in image and video created by ChimeraX, the docking is performed in the central grove of the MHC-II receptor molecule with the surface complementarity with the antigen.

The structural modeling and molecular docking simulations, as illustrated in Figure 14 and Video Figure 1, demonstrate a high-fidelity interaction between the Sm-TSP-2 antigen and the binding groove. The observed surface complementarity and thermodynamic stability provide robust biophysical evidence that the

SNEKPT epitope is a viable candidate for effective antigen presentation and downstream vaccine development.

Beyond these specific results, the bioinformatic pipeline developed in this research serves as a framework for identifying vaccine candidates across a broad spectrum of pathogenic organisms. Its direct integration with the NCBI database enables the high throughput retrieval and processing of proteomic data from versatile parasites, facilitating the identification of immunogenic epitopes with high affinity for the MHC-II molecule. This capability is significant in addressing the escalating global crisis of anthelmintic resistance. By accelerating the in silico discovery by interdisciplinary analysis of data and discovery of laboratory stable surface exposed peptides the pipeline streamlines the preselection process of candidates often found at low volume in the extracellular vesicle cargo. The biochemical preselection by the application finishes in minutes and the full pipeline running time is 2-4 hours, mainly depending on the ClusPro productivity, however it does not require any payment requiring tools as software of UCSF ChimeraX is free to use.

13.4 In conclusion

While the Multi Machinery Model establishes a robust theoretical and computational foundation for interpreting extracellular vesicle dynamics, the immediate progression of this research necessitates rigorous empirical validation through "wet lab" experimentation to refine and calibrate the proposed biophysical formulas. This thesis wants to present parasitic organisms not merely as pathogens, but as sophisticated co-evolved entities that actively manipulate host microenvironments through targeted extracellular communication, a strategy that mirrors the complex signaling landscape observed in oncogenesis. Given that parasitic EV cargo likely harbors a diverse array of proteins with immunosuppressive properties, the MMModel offers a tool for navigating the challenges of detecting and processing the massive, multi-layered data streams inherent in cellular signaling. To ensure the model remains dynamic, ongoing sensitivity analysis will be required as it evolves alongside emerging wet-lab observations.

Reductionist pharmacology is the core of quality control and clinical application of applications of cellular biology. This approach prioritizes isolation of discrete molecular mechanisms to facilitate the development of highly controlled interventions using synthetic molecules. This strategy, centered on the precise modulation of a single causal factor, has proven effective for pathologies driven by isolated protein dysfunctions or monogenic mutations. But this framework falters when confronted with multifactorial etiologies and complex epigenetic

remodeling. Within the dense signaling architecture of eukaryotes, biological systems exhibit significant homeostatic plasticity. Control over interlinked, codependent feedback looped pathways with compensatory signaling is challenging for precise modulation. The middle ground is perhaps not in gaining dominance over functional mechanics of natural extracellular vesicles with influential synthetic molecules, but in rational design of biomimicking nanoparticles by imitation of natural cellular communication, understood by decoding influences of biological environment on the vesicle composition, production, and cargo contents and how the extracellular vesicle membrane changes with these changes in process of cargo transportation (Rosso & Cauda, 2023).Natural extracellular vesicles have been refined through evolutionary pressures to optimize functionality within homeostatic microenvironments with unparalleled capacity for navigating the transition from localized to systemic environments while maintaining rigorous functional integrity. They exhibit a capacity for autonomous regulation and complex intercellular cooperation that persists across a diverse physiological spectrum, ranging from baseline healthy function to states of hostile invasion, chronic inflammation, malignancy, and systemic pathology. Reductionist pharmacology has yet to develop an agent with a similar level of plasticity, relying heavily on blockades of mechanisms. The mechanics of extracellular vesicles show signs of interlinked, codependent pathways characterized by compensatory signaling. There is a need for deeper research of codependence between the mechanisms and a transition from target-centric inhibition to a systems-oriented approach when studying the internal and external environment of the vesicles. It is improbable that a definitive consensus on baseline states for vesicle cargo composition will be fully realized, given the inherent complexities involved in the validation and confirmation of such diverse datasets. But as we gain more mechanism insights and instruments develop, the Multi-Machinery Model. System-oriented approach can help to describe vesicles within the environment, which might aid in distinguishing between drastically opposite states, such as healthy and diseased, which is important in diagnostics. We need to decode the integrated molecular language of cellular communication within its broader functional context to truly gain insight into cellular function.

The limitations of the reductionist model are particularly discussed in living clinical situations of interlinked and codependent behavior. In oncology, during malignant transformation, the tumor is increasingly conceptualized as a dynamic ecosystem rather than a monoclonal population. Redundancy in receptor tyrosine kinase signaling allows malignant cells to bypass specific inhibitors by "rewiring" intracellular circuitry. The tumor microenvironment further facilitates this evasion by utilizing extracellular vesicles to transmit survival signals and pro-angiogenic factors between heterogeneous cell populations. Another example is the emergence of antibacterial resistance through horizontal gene transfer and

anthelmintic resistance driven by the upregulation of P-glycoprotein efflux pumps and metabolic reprogramming, which underscores the urgent need for a systems biology perspective as we are facing healthcare and agricultural crises (Zhang et al., 2025).

In academic literature, cellular signaling is traditionally categorized into four spatial groups: autocrine, paracrine, endocrine, and juxtracrine; in complex eukaryotic organisms, the extracellular vesicles act as integrated information carriers across these distinct signaling layers. Cross communication disrupts the academic classification and clinical strategies of single cause impact, causing laboratory challenges with significant background noise (Salomon et al., 2022). To meet these challenges, this thesis proposes to develop a validation strategy that includes consideration of cellular state and vesicle adaptability to stressors like hypoxia, thermal fluctuations, and pH.

Hypoxia, characterized by a reduction in oxygen tension, as a stressor is clinically present in the microenvironments of breast cancer (Petrova et al., 2018; Semenza, 2016), pancreatic carcinoma (Sun et al., 2021), glioblastoma (Heddleston et al., 2009), ischemic stroke (Kamel & Iadecola, 2012), obstructive sleep apnea (Dong & Yue, 2025), and high-altitude pulmonary edema (Rodway et al., 2003).Metabolic stress arises when a cell's energy demand exceeds supply, leading to the offloading of damaged proteins via extracellular vesicles (EVs) to maintain proteostasis. This phenomenon and its associated signaling cascades are observed in type 2 diabetes and obesity-associated insulin resistance (Xu et al., 2024), skeletal muscle atrophy (Gao et al., 2025), non-alcoholic fatty liver disease (Grossini et al., 2025), and cachexia in advanced malignancies (Khan et al., 2026). Thermal stress significantly impacts membrane fluidity, representing a critical clinical reality when parasites or bacteria infiltrate a host (Evans et al., 2015). This form of stress is central to the pathophysiology of systemic fever, clinical heat stroke (Alawad et al., 2025), and malignant hyperthermia (Rosenberg et al., 2007). Furthermore, temperature-based interventions, encompassing both cold and warm thermal modalities, are extensively utilized in healthcare settings—primarily within physiotherapy—as tools for injury prevention, recovery, and the regulation of the autonomic nervous system (Cheshire, 2016; Vrindten et al., 2025). Oxidative stress, primarily characterized by a systemic imbalance of reactive oxygen species ROS, serves asl driver in the pathophysiology of diverse clinical conditions. In dermatology, this imbalance significantly accelerates skin aging and compromises cutaneous integrity (Chen et al., 2021). In the central nervous system, oxidative damage is considered the core pathogenic mechanism underlying the progression of neurodegenerative disorders, specifically Alzheimer's disease (Bai et al., 2022) and Parkinson's disease (Dionísio et al., 2021). ROS-mediated injury contributes to the

development of atherosclerosis (Batty et al., 2022) and is a leading cause of diabetic retinopathy-induced blindness (Zou et al., 2026). This oxidative burden also extends to pulmonary and maternal health, playing a central role in the inflammatory mechanisms of chronic obstructive pulmonary disease (Xu et al., 2024) and the vascular complications associated with preeclampsia (Afrose et al., 2025). Acidic stress shift to acidosis is connected to inflamed and neoplastic tissues, it leads to modulation of cellular homeostasis by impacting intracellular trafficking of multivesicular bodies toward plasma membrane fusion, under physiological pH, the transit it toward lysosomes. Clinical examples include the microenvironments of melanoma, enhancement of exosome secretion, which facilitates immune evasion and promotes a pro-metastatic niche (Gieniusz et al., 2024). In rheumatoid arthritis extracellular vesicles accumulate in the synovial fluid under conditions of joint acidity, which uphold chronic inflammation and contributes to tissue degradation (Kupczyk et al., 2025; Parolini et al., 2009). In renal tubular acidosis metabolic acidification, Fanconi syndrome, impairs endolysosomal trafficking in proximal tubule cells. This dysfunction results in a characteristic failure of protein reabsorption and intracellular trafficking defects (Hennings et al., 2026). Radiation stress is catalyst for radiation-induced bystander effects), a phenomenon where non-irradiated cells exhibit damage responses due to molecular signals, including DNA fragments, proteins, and microRNAs, shuttled from area of radiation to surrounding cells by extracellular vesicles (Martin et al., 2009). In oncology, radiation therapy has been shown to induce metabolic shifts, such as the promotion of unsaturated fatty acids, which facilitate the survival of glioblastoma cells in the post-irradiation microenvironment (De Martino et al., 2023). Beyond intentional therapy, radiation stress is a significant factor in accidental nuclear exposure, necessitating complex radiological decontamination strategies (Bodin & Menetrier, 2021), and is present during routine diagnostic procedures such as CT scans (Martin et al., 2009). Chronic exposure to low-level radiation, such as ultraviolet light, induces oxidative stress and autophagy, which are foundational mechanisms in the clinical progression of skin aging (Gu et al., 2020). Electromagnetic stress modulates cellular homeostasis primarily by disrupting equilibrium of calcium signaling and redox balance (Alawad et al., 2025; Hosseini & Kianifard, 2023). These biophysical perturbations are increasingly recognized as drivers of chronic inflammation and neurodegeneration by medication of extracellular vesicles (Zhang et al., 2024). Cell death is a network of programmed pathways, most notably apoptosis and necroptosis, where extracellular vesicles serve as dynamic indictors of death progression and modulators of the immune response (Yuan & Ofengeim, 2024). Dysregulation of programmed cell death pathways is central to the pathogenesis of systemic lupus erythematosus (Yang et al., 2019). Apoptotic bodies and necroptotic extracellular vesicles play central roles in communication within the systemic landscape of multiple pathologies. In hematological

malignancies like acute leukemia, inflammatory cell death mechanisms influence treatment efficacy and the tumor microenvironment (Ishaq et al., 2025). Induction of these death pathways is central in therapeutic development, focusing on monitoring of tumor burden and treatment response through development of EV profiles (Chung et al., 2020).

Industrial monocultures and factory farming create genetic bottlenecks that, when coupled with climate-induced thermal stress and hypoxia, destabilize microbial ecosystems. This shift places immense chemical pressure on the environment, necessitating a strategic focus on innovative antibiotic and anthemic therapies to safeguard public health. At a cellular level, for the prokaryotes and eukaryotes, we are observing changes in the biogenesis of vesicles loaded with virulence factors and resistance genes, facilitating rapid niche adaptation and the bypass of traditional antimicrobial defenses. Highlighting a need for effective vaccines and holistic frameworks, as we are studying escalating and drastic recalibrations in the microscopic environment around us and within our own microscopic ecosystem.

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Appendix 1

Nanotechnology and extracellular vesicle research face not only technical challenges but also structural and infrastructural bottlenecks that demand interdisciplinary collaboration and methodological pragmatism.

In emerging research fields, prioritization of research objectives is critical. While increasing classification resolution and refinement of isolation protocols remains scientifically valuable, these goals must be weighed against the scalability, cost-efficiency, and translational applicability of existing methodologies.

In parallel, it is essential to acknowledge that structural inequities persist within the global research ecosystem. Empirical studies demonstrate that disparities in access to funding, leadership opportunities, patent participation, and venture capital investment correlate with gender, racial identity, and sexual orientation, even after controlling for scholarly productivity (Huang et al., 2020). Further stratification has been reported within LGBTQ+ populations, where transgender and non-binary individuals experience higher rates of discrimination than cisgender LGBQ individuals, and compounded disadvantage is observed among queer individuals of color (Bilimoria & Stewart, 2009).

Institutional reports continue to document elevated levels of exclusion and discrimination affecting trans and intersex individuals across education and employment sectors (European Union Fundamental Rights Agency, 2021).

Within scientific research environments specifically, non-inclusive laboratory and conference cultures have been shown to diminish professional visibility and participation among marginalized researchers, thereby indirectly reducing the dissemination and perceived impact of their scientific contributions (Ruzycki & Ahmed, 2022).

At a structural level, global research output is further shaped by geopolitical and institutional asymmetries, reinforced through publication norms and editorial practices which remain strongly centralized within high-income regions (Larivière & Sugimoto, 2023).

Linguistic barriers also contribute substantially to uneven participation, as the dominance of English in scientific publishing restricts effective access and contribution for a majority of the global population (Bahji et al., 2022).

Methodological access is particularly relevant in EV research. Over the past two decades, ultracentrifugation has been widely adopted as a reference standard for EV purification (Théry et al., 2006). However, this approach requires substantial infrastructural investment, with ultracentrifuge instruments commonly exceeding USD 150,000–250,000, per-sample consumable costs estimated at USD 5–10, and processing times ranging from 4 to 12 hours per run (Li et al., 2017; Market Report Analytics, 2025). Limitations related to reproducibility, throughput, and scalability have positioned ultracentrifugation as a topic of sustained methodological evaluation in current EV research.

Alternative scalable approaches have been proposed. In 2017, Niu and colleagues reported a precipitation-based EV isolation protocol utilizing a water-excluding polymer system. In this study, EVs were harvested from conditioned medium of Ishikawa endometrial adenocarcinoma cells using a polyethylene glycol–based reagent following sequential low-speed centrifugation. EV preparations were characterized using electron microscopy and Western blot detection of CD63, TSG101, and HSP70, with quantitative assessment based on acetylcholinesterase activity and particle enumeration. Purity was evaluated using EV-to-protein ratios determined by BCA assays.

This polymer-based precipitation strategy exploits the hydrophobic properties of lipid assemblies in aqueous environments, enabling selective isolation of EVs under centrifugal forces orders of magnitude lower than those required for ultracentrifugation. The method therefore reduces dependence on specialized high-cost instrumentation and improves scalability for laboratory settings with limited infrastructural resources. Challenges related to polymer formulation and post-isolation polymer removal remain areas for continued methodological optimization.

Collectively, these observations illustrate that methodological choices in EV research are inseparable from broader considerations of resource accessibility, scalability, and global participation. The continued diversification of EV isolation strategies may therefore play a critical role not only in advancing technical reproducibility but also in expanding equitable research participation across institutional and national boundaries.

Popular science summary

This thesis presents a systems-level perspective on extracellular vesicle (EV) cargo, where the cargo is understood as a dynamic reflection of integrated cellular communication networks at a given point in time. As a theoretical framework, the Multi-Machinery Model of Cellular Communication is introduced, describing EV cargo formation as an emergent property of coordinated molecular processes. The model links theoretical analyses of the biological functions of EVs with practical bioinformatic applications, with the aim of contributing to the development of novel anthelmintic vaccines.

Extracellular vesicles are nanoscale, membrane-enclosed particles that transport biomolecules between cells and thereby serve as a central mechanism for intercellular signaling. Despite extensive research, the mechanisms governing cargo sorting, targeting, and delivery remain only partially understood. However, it is evident that EVs display a remarkable degree of specificity in recognizing recipient cells and transferring their contents. This property makes them particularly attractive for biotechnological and pharmacological applications, as they enable the development of therapeutics that combine high efficacy with low dosage requirements and minimal side effects.

EVs primarily originate from multivesicular bodies, which are responsible for sorting and packaging prior to release. During this process, vesicles may actively alter their membrane protein profile, for instance via tetraspanins, which play a key role in vesicle formation. Multiple mechanisms contribute to this regulation, among which the ESCRT complex is a well-studied example, driving membrane sculpting and vesicle budding. Notably, ESCRT-like mechanisms are conserved across all domains of life, including bacteria, archaea, and parasites.

Against this background, the present study developed a bioinformatic analysis pipeline based on Python and NCBI databases. This pipeline enables the systematic selection of antigen candidates for rational vaccine design, thereby representing a concrete step toward the development of new anthelmintic vaccines.

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