AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF BOVINE MILK EXOSOMES ON THE SODIUM POTASSIUM ATPASE OF HUMAN CACO-2 CELLS

by
ZENA SLEIMAN WEHBE

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ZENA SLEIMAN WEHBE

Approved by:

Dr. Elias Baydoun, Professor Biology	Advisor
Dr. Sawsan Kreydiyyeh, Professor Biology	Member of Committee
Dr. Mike Osta, Associate Professor Biology	Member of Committee

Date of thesis defense: August 19, 2020

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The year 2020. Thinking of it makes me speechless. It has been most recently defined by our latest "Beirutshima" explosion, the largest non-nuclear explosion in the history of the world- its force 3rd only to Hiroshima. Before that, the (still ongoing) Covid-19 pandemic which bound us to our homes and defined a new way of life, and previous to that our revolution triggered by massive corruption, lack of access to our money and depravation. Interlaced through all of this was my Master's. In a way it has been a constant rock for me, a source of stability. The doors to the labs remained opened if one could manage to reach and the professors always ready to offer guidance, support and most importantly patience. To attest to that, here I am, two days after the catastrophic explosion, finalizing my thesis. The American University of Beirut, the professors, and the current president Dr. Fadlo Khoury are all a pillar of strength- the STRONGEST academic force to exist, keeping the university somehow functional in a completely dysfunctional environment. I am lucky to have been under all of your wings.

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AN ABSTRACT OF THE THESIS OF

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Title: The Effect of Bovine Milk Exosomes on the Sodium Potassium ATPase of Human Caco-2 Cells

Given the species-specific formulation of milk, it is no surprise to find the increase in bovine dairy consumption by humans implicated in the development of several modern diseases of civilization. However, the milk constituents involved in chronic diseases have not been entirely identified. Within the last decade, increasing evidence is shedding light on a potential causative agent: exosomes. Exosomes are the smallest category of extracellular vesicles secreted by cells into body fluids like milk. They transport intact bioactive components to distant cells within an organism as well as to external recipients, including the mother's own offspring and human consumers. The recent identification of enclosed and displayed inflammatory cytokines implicates exosomes in inflammatory chronic diseases, like inflammatory bowel disease (IBD). One of the major targeted proteins in this condition is the sodium potassium ATPase, which is responsible for actively pumping 3 sodium ions to the outside and 2 potassium ions to the inside of the intestinal cells producing thus a sodium gradient used to drive secondary active transport processes and water movements by osmosis. The ATPase maintains consequently water homeostasis, and perturbations in its activity may result in diarrhea and/or constipation which are typical of IBD. Many cytokines are elevated in patients with IBD, such as Tumor Necrosis Factor alpha (TNFα), Interleukinβ and Transforming growth factor β (TGFβ), some of which have been identified on exosomes. These cytokines have also been shown to endogenously affect the pump. This work was conducted to investigate if bovine milk due to exosomal inflammatory cargo, may alter the activity of the pump in in the colon, using Caco-2 cells as a model. Exosomes were extracted from fat free milk by a series of ultra-centrifugation steps and filtration. Cells were treated with different concentrations of bovine exosome proteins and for different time intervals, and the activity of the pump was assayed by measuring the amount of inorganic phosphate liberated in the presence and absence of ouabain. A time response study revealed, at a dose of 550 μg exosome proteins, stimulation of the ATPase up to 6 hours with a maximal effect at one hour, followed by inhibition at later time points. Both stimulation and inhibition were found to be dose dependent. This work was restricted to the study of the stimulatory effect induced at one hour, at a chosen exosome concentration of 225 μg. Because PGE2 was previously demonstrated to exert a

similar dual and opposite effect on the pump, it was considered as a potential effector. The suspected involvement of the prostaglandin in the action of exosomes was confirmed when their effect was abrogated by indomethacin and imitated by exogenous PGE2. The exosomes' induced stimulation still appeared in presence of a blocker of EP1 and EP2, was enhanced further when EP4 was blocked, and changed to an inhibition in presence of an EP3 antagonist, suggesting an involvement of both EP3 and EP4. The activation of the Na+/K+ ATPase was significantly enhanced or reduced when cells were treated respectively with an inhibitor or an activator of PKA. Since EP4 and EP3 act via Gs and Gi respectively, they exert opposite effects on PKA and consequently on the ATPase. The stimulatory effect of EP3 on the ATPase predominates over that of EP4 resulting in a net activation of the pump. To our knowledge this study highlights for the first time a molecular mechanism potentially linking dairy consumption to the Na+/K+ ATPase activity and may be chronic diseases like IBD.

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ABBREVIATIONS

AC: adenylyl cyclase (AC)

AKI: acute kidney injury

Alix: Programmed cell death 6-interacting protein

ANOVA: analysis of variance

ATCC: American Type Culture Collection

ATF3: cAMP-dependent transcription factor 3

ATP: adenosine triphosphate

ATPase: adenosine triphosphatase

BGC 20-1531: EP4 antagonist

BSA: Bovine serum albumin standards

Caco-2: heterogeneous human epithelial colorectal adenocarcinoma cells

cAMP: cyclic adenosine monophosphate

CCL-12: chemokine ligand 12

CD: cluster of differentiation

CD: Crohn's Disease

Cos-7: fibroblast-like cell line derived from monkey kidney tissue

COX: cyclooxygenase

DA: dopamine receptor

DAG: diacylglycerol

DC: dendritic cell

DCx: dendritic cell derived exosomes

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimethyl sulfoxide

DNMT-1: DNA methyltransferase-1

EDTA: ethylenediamine tetra-acetic acid

EP: prostaglandin E2 receptor

ERK: Extracellular Signal-regulated Kinases

ESCRT: endosomal sorting complexes required for transport

FBS: fetal bovine serum

GPCRq: G protein coupled receptor ‘q’

HMEC-1: human microvascular endothelial cells

HRP: horseradish peroxidase

IBD: inflammatory bowel disease

IL15R: IL-15 receptor

ILVs: intraluminal vesicles

Ilβ1: interleukin beta-1

ILγ: interleukin gamma

INFγ: interferon-gamma

IP3: inositol 1,4,5-trisphosphate

IP3R: inositol-1,4,5-triphosphate receptor

K-RAS: ras protein isolated from Kirsten murine sarcoma viruses

LDL: low density lipoprotein

MAPK: mitogen activated protein kinase

MEK: mitogen activated protein kinase

MHC: major histocompatibility complex

miR: microRNA

MVB: multivesicular body

NK: natural killer immune cells

PBMCs: peripheral mononuclear blood cells

PBS: phosphate buffer saline

PGE: prostaglandin

PGE2: prostaglandin E2

PIP2: phosphatidylinositol 4,5-bisphosphate

PK: protein kinase

RUNX2: Runt-related transcription factor 2

TGFβ: transforming growth factor beta

THP-1: human monocytic cell line derived from an acute monocytic leukemia

TSG101: Tumor susceptibility gene 101

UC: Ulcerative Colitis

VECs: vascular endothelial cells

VSMCs: vascular smooth muscle cells

CHAPTER 1

INTRODUCTION

The complex and heterogeneous mixture of milk produced by a lactating mammalian mother, is directed by the species' unique genome for the purpose of nourishing and directing the healthy growth of its own offspring (Melnik & Schmitz, 2017). In the context of evolution, dairy produced from cow milk is a fairly recent addition to the human diet (Cordain *et al.*, 2005). Dairy, processed grains and refined sugars combined provide 72% of the total energy intake of individuals in the West (Cordain *et al.*, 2000). Conversely milk would have been almost absent from our Hominin ancestor's diet. Not only are Neolithic humans the only mammals to ingest the milk of another species, mainly cow, but they singularly continue to do so beyond the weaning period (Melnik & Schmitz, 2017).

Given the species-specific formulation of milk, it is no surprise that the increase in dairy consumption by humans has been corollary to several inflammatory diseases including diabetes, metabolic syndrome and auto-immune diseases like arthritis (Melnik & Schmitz, 2019). Besides the known food intolerance to lactose and allergy to milk protein, the specific components in milk attributed to chronic diseases are not completely elucidated. However, within the last 6 years, evidence is increasingly emerging which sheds light on a possible source: the smallest extracellular vesicles found in milk, exosomes (Melnik & Schmitz, 2017). Due to the integrity provided by the bilayer lipid membrane of exosomes, they can endogenously transport intact and bioactive cargo, like proteins, lipids, and nucleic acids, to distant cells within an organism. In the context of milk, exosomes

additionally enable the transfer of their cargo to external recipients, including the mother's own offspring and the human consumer.

After a thorough research on the available literature on bovine milk exosomes, we found evidence that was highly suggestive, yet unexplored, of their role in inflammatory diseases, particularly inflammatory bowel disease (IBD). Primarily, bovine milk exosomes were shown to be efficiently absorbed into human colon cells and their cargo, such as microRNA, have been detected in human plasma after consumption (Wolf *et al.,* 2015. Although scarce and limited to microRNA, evidence exists that some of the cargo is bioactive in humans and affects the translation of known target genes (Baier *et al.,* 2014; Wolf *et al.,* 2015*)*. More recently, a pivotal finding was that exosomes enclose and display a library of cytokines with shared homology across species (Fitzgerald *et al.,* 2018). Incidentally, many of these cytokines like TNFα, Ilβ1 and TGFβ are elevated in IBD patients (Turner *et al.,* 2014). A major disrupted protein in IBD and other intestinal diseases includes the Na+/K+ ATPase (which will be referred to as pump, Na^+/K^+ ATPase or ATPase hereafter), as it is the main regulator of ion and water absorption in the colon. The activity of this ATPase in the large intestine was shown to be altered by the aforementioned inflammatory cytokines (Kreydiyyeh *et al*., 2007; Turner et al., 2014). Although there are many factors that support the involvement of these exosome-derived cytokines in disruption of the pump's activity, there have been no studies that examined any potential underlying molecular mechanisms. Because many regulatory signaling modules of this ATPase commonly include prostaglandin E2 (PGE2) and the protein kinases A (PKA) (Al-Sadi & Kreydiyyeh, 2003; Markossian & Kreydiyyeh, 2005; Kreydiyyeh et al., 2007; El Moussawi *et al.,* 2018;), we suspected that bovine milk exosomes may alter the activity of

the pump via these mediators. The following literature review thoroughly explores these concepts that led us to form our hypothesis.

CHAPTER 2

LITERATURE REVIEW

A. Exosomes are Bioactive Components Detected in Human and Bovine Milk

1. Milk is a species-specific formulation

Since the early 1900s, there have been numerous components of milk identified (Hernell, 2011). Comparison of milk constituents between species, like humans and cows, undeniably demonstrates the vast differences between the two. For example, while human milk contains 70 grams of lactose per liter of breast milk, bovine milk contains only 48 grams (Hernell, 2011). Additionally, cow's milk contains approximately 4 times more total protein than human milk. There are also major discrepancies in the type of proteins found in the various milk fractions. For instance, β-lactoglobulin is detected in cow's milk, whereas it is absent in human milk, which alternatively carries α-lactalbumin (Hernell, 2011). MicroRNA (also known as miR) composition is also remarkably different, with over 1400 miRs detected in human milk and only 250 found in bovine milk, of which 171 miRs common between the two (Melnik *et al.*, 2013).

Prolonged consumption of cow's milk are correlated to several diseases like diabetes, obesity, neurodegenerative diseases, auto-immune diseases and cancer , although the reason for this association is not clear (Melnik & Schmitz, 2017). Thus, it has the potential to impart damaging effects on adults who continue to consume it- particularly if it is from another species. However, besides correlation studies, there is no research that directly highlights the underlying molecular mechanisms through which milk imparts its

effects. Within the last decade, however, increasing evidence is emerging, shedding light on a potential causative agent: exosomes.

2. Exosome biogenesis and endogenous function

a. What are exosomes?

Exosomes are the smallest category of extracellular vesicles that have emerged as important mediators of intercellular communication (Simons & Raposo, 2009). They are enclosed by a phospholipid bilayer yet lack organelles. Moreover, they display a disc-like morphology and are approximately between 60-100 nm in size (**Figure 1**).

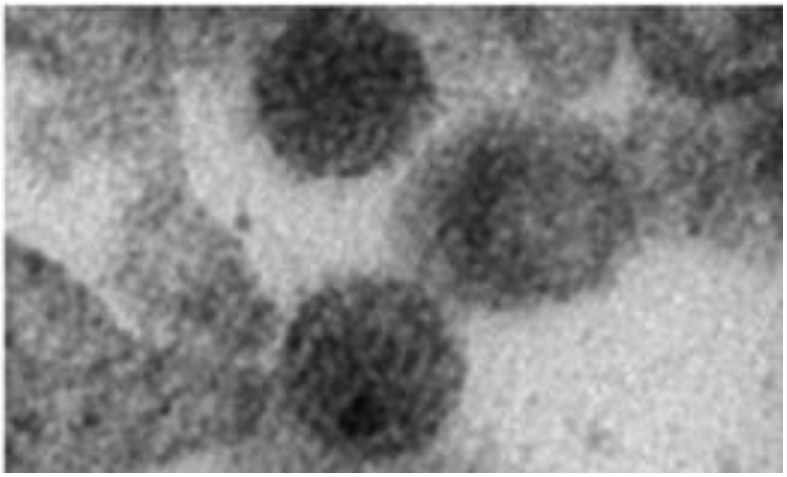

Figure 1. Scanning Electron Micrograph of Exosomes. Their diameter is up to 100 nm and often display a disc-like or sphere-like morphology. Image is adapted from Reinhardt *et al.*, 2012.

Exosomes are produced by virtually every living organism and are present in all physiological fluids like milk, blood, saliva and urine (Manca *et al.*, 2018). Following their detection in human milk, they were also identified in bovine milk (Théry, Z, 2002;Valadi et al., 2007; Simons & Raposo, 2009;; Hata et al., 2010). Due to the integrity provided by the bilipid membrane of exosomes, they confer protection for their cargo against RNAses, heat and acid, ultimately enabling the transport of intact bioactive cargo to near and distant cells within an organism (Manca *et al.*, 2018). Exosomes transport a variety of cargo, predominantly transport lipids, proteins and nucleic acids to recipient cells where they can potentially alter cell activity, including transcription and translation **(Figure 2)** (Anand *et al*., 2019; Lonnerdal, 2019; Melnik & Schmitz, 2019).

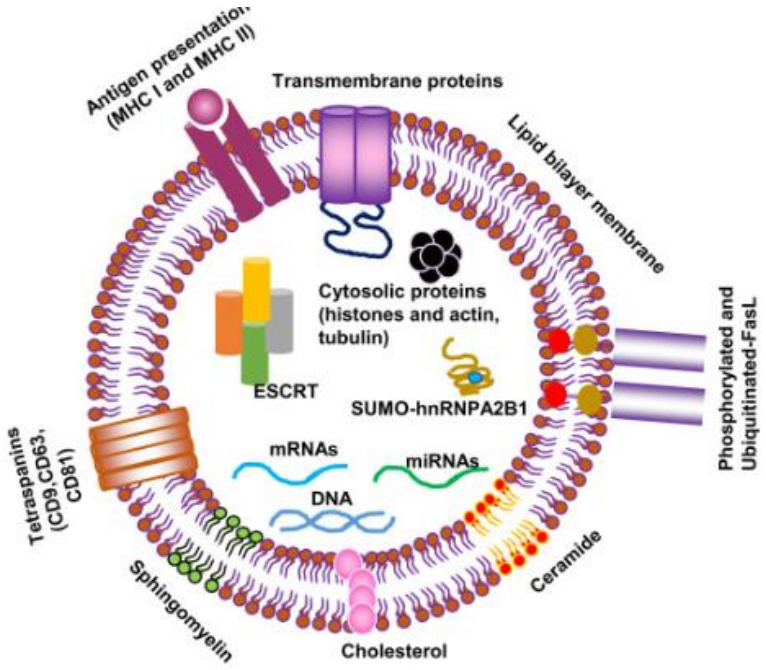

Figure 2. Image depicting the various cargo of exosomes. Most abundantly they carry proteins like cytoskeletal components, those involved with exosome formation, biomarkers, and cytokines. They also notably enclose nucleic acids, especially microRNA. Several proteins are used as biomarkers such as tetraspannins. Image is adapted from Anand *et al.*, 2019.

b. Exosome formation

Compared to other extracellular vesicles which pinch off from the lipid bilayer membrane into the plasma, exosomes are endosomal in origin. Initially, inward invaginations of the plasma membrane pinch off forming intracellular endosomes. These vesicles undergo further inward budding, eventually forming several intraluminal vesicles (ILVs) enclosed within the endosome, now termed a 'multivesicular body' (MVB) (Abels & Breakefield, 2016). The late endosomal ILVs within the MVBs may eventually either become degraded via lysosomes or undergo further maturation to become exosomes. In

order to commit specifically to becoming an exosome, its membrane is re-organized to become more saturated with tetraspannins, especially CD9, CD63 and CD81 (Abels & Breakefield, 2016). Next, proteins from the endosomal sorting complexes required for transport are drawn to the ILVs to initiate the process of export. Finally, once the MVBs fuse with the cell's bilayer, they subsequently release the enclosed ILVs now termed "exosomes" (Chen *et al.*, 2012).

The release of exosomes is considered an active-transport process affected by the presence of ATP in a cell (Wang *et al.*, 2010). A unique feature of exosomes compared to other extracellular vesicles is that they are constantly released, both in the presence and absence of triggers (He *et al.*, 2018).

c. Uptake of exosomes by target cells

What factors direct the released exosomes towards a particular tissue rather than another? Scientists are in favor of the theory that exosomes may display surface and ligand binding proteins mirroring those of their parent cells and specific to those of their target cells (Wiklander *et al.*, 2015). It has been suggested that motifs on the exosomes' membrane recognize the target cell's surface receptor proteins, yet it is still unclear which specific surface glycoproteins of the exosome facilitate targeting and uptake into their recipient cells (Kusuma *et al.*, 2016).

Once reaching a recipient cell, exosomes may fuse with the plasma membrane, conduct ligand-cell communication or become internalized via endocytosis (Théry *et al.*, 2002). Endocytosis of exosomes has been demonstrated by human vascular endothelial

cells, whereby elimination of cytochalasin D on the cells, a protein integral to endocytosis, significantly reduced exosome uptake (Kusuma *et al.*, 2016). Additionally, surface proteins on the exosomes are also involved in the uptake procedure as removal of their surface proteins by proteinase K significantly reduced exosome internalization by cells, particularly within intestinal and vascular endothelial cells (Kusuma *et al.*, 2016; Manca *et al.*, 2018). Interestingly, exosomes are not necessarily confined to the initial target cell, as they have been shown to exit endothelial cells and travel to various tissues (Kusuma *et al.*, 2016).

The specific endocytic pathway used to internalize exosomes appears to be cell dependent. For example, epithelial cells uptake exosomes via caveolin-mediated endocytosis while neurons favor phagocytosis (Abels & Breakefield, 2016). Interestingly cancer cells maintain a low pH, which seemingly favors fusion of the exosome and subsequent release of its contents, rather than endocytosis of intact exosomes (Abels & Breakefield, 2016). Importantly, extracellular vesicles have also been shown to trigger signaling cascades by ligand and receptor interaction on the cell's membrane (Al-Nedawi *et al.*, 2008).

Within an organism, uptake of exosomes is possible in cells with similar functions as the donor cell but also in those which differ from than the original donor cell. For example, the monocyte human THP-1 cells have been shown to export exosomes that are taken up by human microvascular endothelial cells (HMEC-1) and exert associated effects (Zhang et al., 2010). On the other hand, exosomes appear to gravitate to cells which are somehow related to the function of their cell source (Wiklander *et al.*, 2015). For example, in mice, bone marrow-derived and immune cell-derived exosomes are remarkably elevated in the spleen. This highlights that exosomes may display surface and ligand binding

proteins mirroring those of their parent cell and specific to those of their target cell (Wiklander *et al.*, 2015).

d. Exosome Cargo

Throughout the sequential steps of inward invaginations to form exosomes, cell membrane and cytosolic contents become entrapped, yet the exosome is devoid of organelles like the Golgi apparatus, endoplasmic reticulum, mitochondrial and nuclear proteins (Théry *et al.*, 2002). Over thousands of exosome contents have been detected which predominantly include proteins, nucleic acids and lipids **(Figure 2)** (Anand *et al.*, 2019). Exocarta, an online database, categorizes exosome cargo according to various factors such as species, type of content (like protein or nucleic acid), original cell source and physiological fluid from which they were derived (Keerthikumar *et al.,* 2017). Upon interaction with or uptake of the exosomes, their cargo can carry out intracellular regulation of numerous endogenous physiological activities, just as they did within their original cell (Kosaka *et al.*, 2010). Once the contents or the exosome itself is internalized, the cargo may either exert an effect in the cytoplasm or nucleus, endure lysosomal degradation or be repackaged for external transport once again (Abels & Breakefield, 2016). The specific pathways and mechanisms directing the fate of the contents have yet to be elucidated.

Due to their endosomal origin and the fact that they are exported and received by cells, exosomes, regardless of their cell source, share common proteins involved in the endosomal, export and uptake procedures (Théry et al., 2002; Simons & Raposo, 2009). These proteins include tetraspannins CD63, CD9, CD81, annexin, flotillin, ankyrin, ESCRT

components and heat shock proteins (hsp70 and hsp90) (Simons & Raposo, 2009; Lawson *et al.* , 2017;). Other proteins include Alix and TSG101 which are unique to the formation of multivesicular bodies (MVB) and integrins which facilitate their uptake by recipient cells (Anand *et al.*, 2019). Of paramount importance, exosomes were shown to display and carry a library of pro-inflammatory cytokines (Fitzgerald *et al.*, 2018) - the implications of which will be discussed in following sections. Many of these proteins are unique and abundant in exosomes, relative to other extracellular vesicles, and are therefore used as exosome identification markers in vivo and in vitro, namely CD63, CD9, CD81, Alix and TSG101 (Simons & Raposo, 2009). Exosomes also feature an abundance of lipids common to lipid rafts, like sphingolipids, cholesterol and ceramides (Théry *et al.*, 2002). Nucleic acids detected in exosomes include DNA, mRNA and especially microRNA (miR) (Anand *et al.*, 2019). MiRs are among the most copious category of nucleic acids detected in exosomes and these short RNA fragments usually downregulate translation by binding to the target seed sequence in mRNA transcripts (Anand *et al.*, 2019).

Although exosomes share these common aforementioned constituents and markers, their contents also vary depending on their original cell source and the body fluid from which they are derived (Schey *et al.* , 2015). For example, urinary exosomes are derived from epithelial cells of the urogenital tract and their contents are associated with these tissues. Proteins indicating renal origin are uniquely found in these exosomes including numerous aquaporins and solute carrier family 12 member proteins (Schey *et al.*, 2015). On the other hand, exosomes found in blood plasma are mainly produced by hematopoietic cells like platelets, peripheral mononuclear blood cells (PBMCs), B lymphocytes, dendritic cells (DCs) and mast cells. As such, they are especially abundant in

class II major histocompatibility complex (MHC) proteins (antigen presenting surface proteins that initiate the immune system) (Caby *et al.* , 2005). Meanwhile, exosomes detected in mammalian breast milk are predominantly produced by mammalian epithelial cells, and their cargo effectively reflects this. Proteins like lactadherin, lipopolysaccharide binding protein, MHC class I, calcium binding protein and osteopontin are majorly detected in both human and bovine milk derived exosomes. However, it is worth noting that over 500 proteins are differentially expressed between the two species, further corroborating the species-specific design of mammalian breast milk (Yang *et al.*, 2017).

3. General physiological roles of endogenous exosomes as viable intercellular communicators

a. Biomarkers of disease

As previously discussed, several exosome contents like proteins and microRNA are unique depending on their cell source and changes in their profile have been detected during disease states (Lin *et al.*, 2015). This quality enables exosomes to function as reliable biomarkers of various pathologies. For example, ATF3 (cAMP-dependent transcription factor 3) is especially elevated in urinary exosomes of patients with acute kidney injury (AKI) and urinary exosomes derived from prostate cancer cells enclose a relatively higher abundance of proteins like TMPRSS2 (transmembrane protease serine 2) (Wang *et al.*, 2012). Exosomes released from several diseased cells ultimately enter the bloodstream and their characterization may reflect an associated pathology in a particular

tissue or organ. For example, detection of epidermal growth factor VIII could be indicative of glioblastoma while survivin may suggest prostate cancer (Lin *et al.*, 2015).

MicroRNAs extracted from exosomes have become promising tools of cancer diagnosis. For example, 8 miRs typically elevated in ovarian biopsies are also correspondingly elevated in exosomes derived from ovarian cancer cells, while absent in healthy cells and exosomes (Taylor & Gercel-Taylor, 2008). Moreover, lung adenocarcinoma patients express an altered miR profile, relative to healthy individuals, in the tumor cells themselves and their released circulating exosomes (Rabinowits *et al.*, 2009).

b. Immunoregulatory function

Immunologically, exosomes are involved in antigen presentation, immune suppression and activation (Greening *et al*., 2015). Much of the immune activation is due to exosomes released by dendritic cells (DCx). Their abundant display of cytokines and antigen presenting molecules like MHC render them effective triggers of the immune response. For example, DCx display the cytokine tumor necrosis factor (TNF) (Reiners, Dassler, Coch, & Pogge von Strandmann, 2014). This interacts with TNF receptors on natural killer (NK) immune cells, inducing them to produce and secret interferon-γ (INFγ). The presence of MHC and tetraspannins like CD40 and CD86 on DCx also render them potent activators of T cells (Reiners *et al.*, 2014).

c. Disease progression

Exosomes also offer intercellular communication that enables disease progression, like atherosclerosis (Tang *et al.* , 2016; Wang et al., 2019) . Many of the cells involved in atherosclerosis like platelets, vascular endothelial cells (VECs), vascular smooth muscle cells (VSMCs), macrophages, among others, release and receive exosomes which participate in development of the disease (Tang *et al.*, 2016). The exosomes can either slow down progression of atherosclerotic lesions or further drive plaque formation. For instance, monocyte-derived exosomal miRs like miR-126 activate chemokine ligand 12 (CCL-12) on VECs, thereby attenuating endothelial cell apoptosis and stabilizing the lesion (Carr *et al.* , 1994). On the other hand, exosomes released by foam cells (LDL cholesterol-laden macrophages which cause the atherosclerotic fatty streak in the subendothelial layer) notably display integrins β1 and α5 and directly supply them to the VSMCs, without increasing endogenous mRNA production (Niu *et al.*, 2016). It is known that atherosclerotic VSMCs uniquely revert and display these particular integrins as they enable their migration towards the clot- a key step in atherosclerotic lesion progression. As such, exosomes in this context represent intercellular communication between foam cells and VSMCs in order to specifically enhance migration of the VSCMs (Niu *et al.*, 2016).

The detection of prions in exosomes, aggregated toxic proteins that should have been destined for degradation, also heavily implicates them in neurodegenerative diseases (He *et al.*, 2018).

d. <u>Tumor progression and metastasis</u>

Cancer progression is another context in which exosomes function. For example, in lung cancer, tumor derived exosomes (TEX) are the main mediators of intercellular communication between tumor cells and their surrounding healthy host cells (Alipoor *et al.*, 2018). Specifically, TEX transport transcription factors, tumor associated antigens, oncoproteins like K-RAS and oncogenic miRs to promote tumor formation in its microenvironment. Additionally, associated integrins direct TEX to distant cells which seems to play a role in metastasis of the cancer (Alipoor *et al.*, 2018).

e. Drug delivery

Endogenous exosomes are stable in physiological conditions and are biocompatible within the native host organism (Bunggulawa *et al.*, 2018). Thus, they are increasingly being considered as efficient low risk vehicles of drug delivery (Manca *et al.*, 2018). Several methods for drug loading into exosomes exist such as electroporation (applying an electric field to create pores to allow drug entry), simple co-incubation and ultrasonication (Bunggulawa *et al.*, 2018). Moreover, they remain viable and can effectively deliver the loaded drug, eliciting a physiological response. For example, curcumin-loaded exosomes were able to exert anti-inflammatory effects in brain inflammation, tumors and auto-immune diseases (Bunggulawa *et al.*, 2018). The ability to present antigens on their bilayer membrane also renders exosomes as promising tools for vaccine development (He *et al.*, 2018).The ability to present antigens on their bilayer membrane also renders exosomes as promising tools for vaccine development.

Hijacking exosomes as drug delivery vehicles is not straightforward, as their innate targeting ability to certain cells is not exact. However, exosome membranes have effectively been engineered to display specific ligands, compatible with the desired target tumor cells (He *et al.,* 2018).

f. Stem cell niche management

Exosomes are also involved in the management of a stem cell niche (Collino *et al.,* 2010). For example, mesenchymal and liver stem cells release exosomes that are taken up by neighboring recipient cells, highlighting a possible mechanism by which stem cells utilize exosomes to communicate and direct their environment (Collino *et al.,* 2010).

Exosomes are involved in several more processes, like reproduction, fetal development, neural cell communication, cell maturation and proliferation (He *et al.,* 2018). Because of their bioactive cargo, physiological and pathological roles, exosomes are now established as important mediators of intercellular communication with an undeniable ability to alter the activity of the target cells.

4. Bovine Milk Exosomes

a. Intact bovine milk exosomes are absorbed by humans

The detection of exosomes in human and bovine milk warrants exploration of an additional and important question for these small extracellular vesicles: what is their unique

cargo and can they epigenetically regulate physiological processes in their recipients, whether they are neonates extracting nourishment from their mother or humans consuming cow's milk as part of an established diet.

Exosomes are found in raw, pasteurized and powdered cow's milk (Baier *et al.*, 2014). Pasteurization (78°C) has no significant effect on the integrity of cow milk derived exosomes, however boiling (100°C) and ultra-heat treatment (130°C) appear to reduce the content of exosomes. Similarly, fermentation of milk reduces the size of exosomes and diminishes the protein and miR content (Melnik & Schmitz, 2019). Importantly, cow milk exosomes can be absorbed within foreign species (Alsaweed *et al.* , 2015). In fact, human colon cancer cells (Caco-2) efficiently absorb cow milk exosomes primarily via endocytosis (Baier *et al.*, 2014). In a related in vivo study, bovine milk exosome contents were elevated in the blood of humans who orally consumed cow's milk, indicating successful translocation across the intestinal epithelium (Wolf *et al.*, 2015). Contents were shown to remain in the blood for up to 9 hours and organs like the liver and spleen for 24-48 hrs., while endogenous extracellular vesicles have been shown to have a half-life of 5.5 hrs. in circulation (Melnik et al., 2013; Yáñez-Mó et al., 2015; Manca et al., 2018;). Once absorbed across the intestinal mucosa, foreign exosome contents are detected in human target cells, similar to endogenous exosomes. For example, miR-200c and miR-29b were detected in human peripheral blood mononuclear cells (PBMCs) after milk consumption (Melnik *et al.*, 2013).

Bovine milk exosome contents are elevated 100% more in the blood of humans after milk ingestion (Wang *et al.*, 2018). Moreover, bovine and human milk exosomes share sequence homology among their cargo (Melnik & Schmitz, 2017).

b. Potential bioactive components of bovine milk derived exosomes in humans

As previously discussed, exosomes enclose collectively over thousands of cargo spanning nucleic acids, proteins and lipids (Théry *et al.*, 2002). Of these, miRs in bovine milk exosomes have received the most attention for potential adverse effects on human consumers (Pauley, Cha, & Chan, 2009; Zempleni, 2017; Zempleni *et al.*, 2019). In addition, the recently identified cytokines are also worth examining due to the inflammatory responses they may illicit.

i. MicroRNAs

Over 60% of coding genes are regulated by microRNAs (miRs), rendering them the most copious category of genetic modulators (Manca *et al.*, 2018). Up to two percent of the entire mammalian genome encodes for evolutionarily conserved miRs, yet they can target over 20,000 genes, implicating the promiscuity of a single miR in regulating several different mRNAs (Alsaweed *et al.*, 2015; Vidigal & Ventura, 2015). MiRs function locally within a cell to regulate endogenous genetic expression and can be shuttled to different regions of an organism, also directly influencing the phenotype of a seemingly intangible cell (Chen *et al.*, 2012).

The mature miR is up to 22 nucleotides and can bind to the mRNA seed region (nucleotides 2- 7) with partial or complete sequence complementarity (X. Wang, 2014). Depending on the extent of sequence complementarity the mRNA transcript is either cleaved or, more commonly, blocked from translation (Chen *et al.*, 2012). Usually, the

miRs bind with partial complementarity thus resulting in the downregulation of translation, without actual obliteration of mRNA (Vidigal & Ventura, 2015).

Knockout studies of certain miRs have highlighted their relevance in homeostasis and normal physiological function, and their disruption is associated with various pathologies. For example, lack of miR-90, miR-17, miR-26 impairs embryonic and perinatal cardiac development. miRs-1 and 2 can lead to fatality at weaning if they are absent and in miR- 275 yield to disruptions in glucose metabolism (Vidigal & Ventura, 2015). By 2014, approximately 2500 human miRs had been identified which perform important regulatory roles in a number of physiological processes like bone health, immunity and reproduction (Wang, 2014; Alsaweed et al., 2015; Kusuma et al., 2016).

In contrast to the variety of miRs detected in human milk, cow's milk encompasses a library of up to 251 miRs, most of which have identical sequences to their human orthologues (Baier *et al.*, 2014). Correspondingly, preliminary analysis suggests that over 11,000 human genes may be affected by bovine miRs, yet this has not entirely been verified by thorough studies (Baier *et al.*, 2014). Many of the most abundant miRs found in human milk share homology to those found in cow's milk, the most prevalent one being miR-148 (Benmoussa *et al.*, 2016). Importantly, this miR targets DNA methyltransferase-1, an enzyme which prevents the expression of many genes. Upon miR binding to DNMT-1 transcript, then its usual inhibition of genes is no longer evident, and transcription may ensue. This can be problematic especially if the gene in question is a tumors promotor or oncogene. MiR-21 is another prevalent miR in milk and is considered tumorigenic, particularly in the initiation of melanoma (Benmoussa *et al.*, 2016). Although almost all potential harmful effects of bovine milk exosome cargo on humans have been linked to

miRs, there are no definitive studies that directly portray this. One of the scarce experiments indicated that bovine milk exosome-derived miR-200c and miR-29b absorbed into human cells were able to exert a physiological effect. Specifically, bioactivity was confirmed by assaying targets of these miRs. Consistent with the role of miR-29b, RUNX2 was significantly upregulated within the cells (Melnik *et al.*, 2013). Much of the controversy surrounding the absorption of milk exosome miRs is propagated by the sequence homology shared between the two species in a large proportion of miRs and their detection in the cells of human consumers, as previously discussed (Baier *et al.*, 2014).

ii. Cytokines

Only within the last two years was is it discovered that exosomes also display and enclose a variety of cytokines, key immunomodulatory proteins (Fitzgerald *et al.*, 2018). Previously, it had been widely established that cytokines exert their effect by interacting with highly specific cell surface receptors that trigger internal signaling cascades, resulting in a response from the cell. They function in an endocrine, autocrine, or paracrine manner. The primary role of cytokines is as immune modulators, activating or suppressing the immune system, as well as shaping the development and maturation of cells (Turner *et al.*, 2014). It has been already established that free soluble cytokines exist in mammalian milk, the most prominent being Transforming Growth Factor β (TGF β) which is critically involved in anti-inflammatory response as well as metabolic growth and development in the new-born (Ballard & Morrow, 2013). However, their recent detection also within exosomes warrants exploration of their effects in milk consumers.

In human milk, eleven cytokines are predominantly found in the enclosed rather than soluble version, including a number of interleukins (mainly secreted by immune T helper cells in order to enhance an immune response) , transforming growth factor (TGF β), interferon gamma (INF-γ) and tumor necrosis factor alpha (TNF α), all of which are involved in the immune and inflammatory response. Moreover, the enclosed cytokines show biological activity once internalized by cells (Fitzgerald *et al.*, 2018). Many cytokines like TGF β are evolutionarily conserved across species, which is a cause for concern for young humans that are incessantly exposed to these inflammatory modulators in cow's milk, even well into adulthood (Saito *et al.*, 2018). What is especially concerning is that 75% of milk exosomes escape absorption and remain within the colon (Manca *et al.*, 2018), and -as previously discussed- can be taken up by colon cells via endocytosis (Wolf *et al.*, 2015). However, until now examination of any adverse effects on the activity of these cells remains obsolete.

B. Inflammatory Bowel Disease and the Link to Bovine Milk Exosomes

Inflammatory Bowel Disease (IBD) is a collection of intestinal tract chronic diseases in which environmental factors seem to trigger an auto-immune inflammatory response in genetically susceptible individuals (Lucendo & De Rezende, 2009). Onset can be as early as childhood or can peak between the age of 20-30 (Cashman & Shanahan, 2003). It includes Ulcerative Colitis, a continuous section of inflammation within the large intestine and Crohn's Disease, which can arise anywhere across the intestinal tract. Regardless of the type, IBD commonly results in an altered and disproportionate long-lasting inflammatory response of the intestinal mucosa (Lucendo & De Rezende, 2009). In

between episodes of inflammation are quiescent periods during which the symptoms subside until the next triggering factor (H. Zhang et al., 2019). Of the two main types of IBD, Ulcerative Colitis is between 2-5 times more common than Crohn's Disease *(*Zhang *et al.,* 2019).

The intestinal inflammation that occurs in IBD mainly reduces sodium and chloride net absorption, and increases K+ secretion, as indicated by elevated levels of Na+ and reduced levels of K+ within the mucosal secretions of the intestinal lumen. Lack of proper Na+ absorption subsequently disrupts normal water uptake and secretion within the colon (Barkas *et al.,* 2013). Hence common symptoms associated with IBD include diarrhea, tenesmus and even proximal constipation (James *et al.,* 2018). Water absorption is mainly predetermined by sodium absorption and ultimately flows in the same direction as the ion. One specific mediator of basolateral sodium extrusion is the sodium-potassium-ATPase (Na+/K+ ATPase) (Barkas *et al.,* 2013). It is located mainly at the surface of the epithelium as well as the upper crypts of the distal colon. Several studies have indicated that impairment of electrolyte uptake, including the function of this ATPase, is life-threatening in patients with UC (Ejderhamn *et al.,* 1989; Bernstein & Shanahan, 2008; Barkas *et al.,* 2013).

Triggering factors are still under scrutiny but correlation studies have revealed an increase in the incidence of Inflammatory Bowel Disease (IBD) in relation to certain change in lifestyle and environmental factors in the West (Bernstein & Shanahan, 2008). Microflora and food intake are the major environmental factors that are implicated in IBD, in addition to genetic predisposition and unique immune response of the individual. Also, the incidence of IBD was significantly reduced in children who were exclusively or at least

partially breast fed (Acheson & True Love, 1961; Cashman & Shanahan, 2003). It has been suggested that the protective qualities of breast milk against infections and enhancing the microflora may attribute to the reduced IBD incidence. Possible explanations for reduced IBD incidence for breast-fed individuals include the anti-microbial properties of breast-milk, early enhancement of the infant's intestinal mucosa and simply by delaying the infant's exposure to cow's milk (Cashman & Shanahan, 2003).

Several chronic diseases have been linked to the consumption of cow's milk, a major staple in the diet of humans (Melnik & Schmitz, 2017). Mainly inflammatory diseases like asthma, rheumatoid arthritis and inflammatory bowel disease (IBD) are associated with dairy (Melnik & Schmitz, 2019), yet the exact mechanism is unknown. Importantly, several inflammatory molecules like prostaglandin E2 (PGE2), TNFα and TGFβ are elevated in patients with IBD and incidentally have been shown to interfere with Na+ regulation in the colon, which will be discussed further in the following section (Al-Sadi & Kreydiyyeh, 2003; Dey, Lejeune, & Chadee, 2006; Markossian & Kreydiyyeh, 2005; Turner *et al.*, 2014).

Given the major interaction of milk exosomes with colon cells and the trove of pro-inflammatory cytokines which they entail, they could offer useful insight into the pathogenesis of IBD and its association with milk consumption.

C. The Na+/K+ ATPase

The Na+/K+ ATPase is especially abundant on the basolateral membrane of colonocytes where it pumps Na+ into the blood and pumps K+ from the plasma into the

colonocyte. In fact, most of the ion and water transport extracted from food and water intake occurs within the colon (Fondacaro, 1986). The transport of Na+ from the colon lumen, through the colonocyte and into the plasma simultaneously establishes the movement of water in the same direction. Therefore, any disturbances in the activity of the pump is directly correlated with alterations in water flow, which ultimately manifests as blood pressure dysregulation as well as diarrhea, constipation, or tenesmus- all of which are major symptoms of IBD (Windsor & Kaplan, 2019). Understanding the factors that regulate the pump may serve as the interface at which milk, IBD and this ATPase all converge.

1. Structure and Function of Na+/K+ ATPase

a. Ion transporter

The sodium potassium ATPase (which will be referred to as 'pump' from now on) is a ubiquitous oligomeric transmembrane protein. It is mainly composed of the α catalytic subunit and the β subunit and a much smaller FXYD family single membrane spanning protein. While the β subunit is important in localization of the pump to the membrane (Clausen *et al.*, 2017), the α subunit has binding sites for ATP, Na+ and K+ ions and thus enables active transport of 3 Na+ ions extracellularly for every 2 K+ pumped intracellularly, per molecule of ATP **(Figure 3)** (Aperia, 2007).

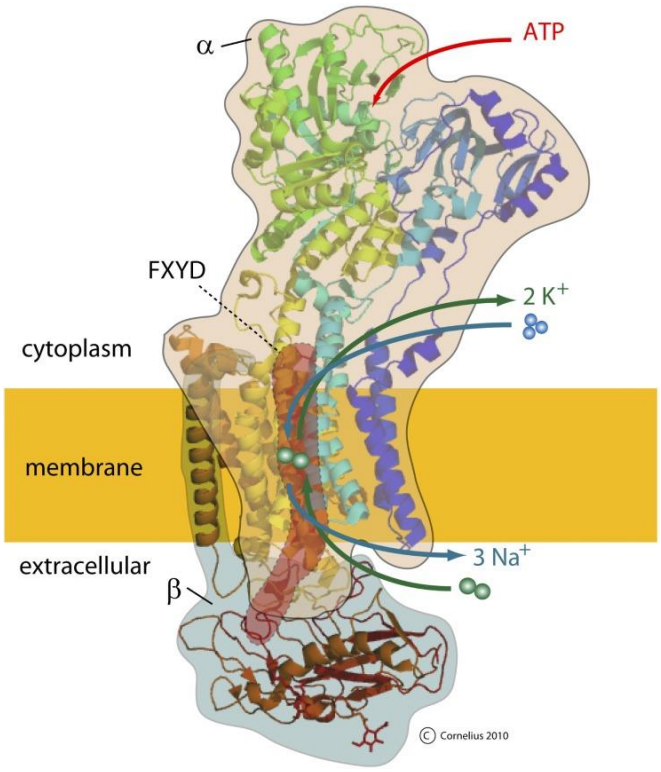

Figure 3. Molecular structure of the Na+/K+ ATPase. It is comprised mainly of the alpha subunit facing the cytoplasmic side and the beta subunit which protrudes extracellularly. FXYD further enables proper localization of the pump. Image is adapted from Clausen *et al.*, 2013.

FXYD has mainly been studied for its ability to alter the affinity of the pump to the ions (Poulsen *et al.*, 2010). By maintaining the sodium and potassium concentrations against their gradient, the pump is essential for vital functions like establishing membrane potential (important for neural signal transmission, cardiac and muscle function) physiological water and ion homeostasis (predominantly by colonocytes and the kidney), osmotic pressure in all cells and enabling secondary active transport of glucose and amino acids (Therien & Blostein, 2000; Poulsen *et al.*, 2010). Given the essential roles of the

pump, dysregulation in its activity can result in a spectrum of pathologies (Poulsen *et al.*, 2010).

b. Signal Transducer

Not only is the pump an ion transporter, but it is also increasingly emerging as an important tool in signal transduction (Aperia, 2007; Poulsen et al., 2010; Clausen et al., 2017). Specifically, the cardiac glycoside, ouabain, has a specific binding site on the extracellular domain of the α subunit. In specific doses, ouabain can inhibit the ion pumping activity. However, mammalian endogenous levels of ouabain are minimal and below the threshold required to affect the transportive function. Nevertheless, this minimal blood concentration is sufficient for triggering signaling activity of the pump that is associated with cell adhesion, motility, growth and apoptosis (Aperia, 2007). In fact, this sub-inhibitory concentration of ouabain has been established to activate two main signaling pathways, independent of any alteration in ion transport: calcium and the non-receptor tyrosine kinase, Src-dependent signaling.

Ouabain uniquely causes intracellular calcium oscillations mediated by direct interaction of the pump with the inositol-1,4,5-triphosphate receptor (IP3R), located on the endoplasmic (or sarcoplasmic) reticulum (Aperia, 2007). This represents an alternative mechanism for activating the IP3R, which has typically been associated with G protein coupled receptor ‘q’ (GPCRq)/phospholipase C (PLC) signaling cascade that ultimately results in production of IP3, a soluble molecule that then binds to and activates IP3R. The resulting calcium oscillations are sufficient enough to activate calcium dependent

transcription factors like NFƘB, translocating it the nucleus and inducing transcription of anti-apoptotic genes and genes involved in cell growth (Aperia, 2007).

Another signaling pathway triggered by ouabain involves the non-receptor tyrosine kinase Src (Xie & Askari, 2002). Normally, the pump binds and inhibits Src. Upon treatment with ouabain, the inhibition is lifted, enabling it to initiate downstream phosphorylation (Tian *et al.*, 2006). Specifically, it has been shown to phosphorylate the intracellular domain of the epidermal growth factor receptor (EGFR), thereafter recruiting Shc/Grb2/SOS and ultimately inducing two pathways- the phospholipase C γ (PLCγ)/Protein kinase C (PKC) and the mitogen activated protein kinase (MAPK) Ras/MEK pathways. These finally culminate in the production of the transcription factors that upregulate protein synthesis. This pathway is most visibly demonstrated in cardiomyocytes undergoing hypertrophy (Xie & Askari, 2002). Other signaling pathways mediated by the pump are summarized in Xie *et. al* (Xie & Askari, 2002).

2. Molecular regulation of the Na+/K+ ATPase

a. Physiological regulation

There are numerous factors which regulate the physiological activity of the pump, including hormones, catecholamines and steroids, and they are thoroughly reviewed by Therien *et al.*, 2000. Regulation is often characterized as short-term and long-term effects, the former involving an alteration in kinetic activity or trafficking of the pump and the latter usually affecting its expression or degradation. Especially in short-term regulation, the ultimate phosphorylation of the pump is usually the end result of a triggered signaling

event. Phosphorylation on various sites on the enzyme can trigger conformational changes, whereas phosphorylation of other components may trigger increased membrane localization or endocytosis of the pump. Most commonly, phosphorylation is achieved by the two serine/threonine protein kinase A and C (PKA and PKC). PKA and PKC are commonly regulated by various families of G protein coupled receptors (GPCR), as will be described below. PKA has been shown to phosphorylate the serine residue Ser943 on the intracellular domain of the α subunit, but it is important to stress that there are likely other undiscovered phosphorylation sites. The exact phosphorylation by PKC is less understood. Because phosphorylation plays an important role, understanding the various upstream modulators of the PKs are indispensable for characterizing the regulation of the pump (Poulsen *et al.*, 2010).

Numerous hormones have been shown to exert short-term and long-term effects (Therien & Blostein, 2000). Steroids (like aldosterone and dexamethasone), peptide hormones (like insulin) and catecholamines (norepinephrine and dopamine) have all been examined for their roles in Na+ and K+ regulation and their effects vary according to tissue, ligand concentration, duration of ligand exposure and type of receptor. For example, the steroids aldosterone and dexamethasone mainly exert long-term regulation of pump in epithelial cells, including those of the colon. The steroid hormone/receptor complexes are believed to interact with cAMP-inducible factors, directly affecting gene expression of the ATPase subunits. This highlights the importance of a phosphorylation cascade involving adenylyl cyclase (AC)- which catalyzes the production of cAMP and thereafter activate PKA- in long-term pump regulation (Therien & Blostein, 2000).

Catecholamines like dopamine and norepinephrine are also prominent physiological regulators of the pump and their effect is illustrated in various tissues. Unlike steroid hormones which are complexed and internalized with their receptors, dopamine exerts its effect through its GPCRs, DA1 and DA2 and downstream PKA or PKC dependent pathways, culminating in a phosphorylation event that usually inhibits activity of the pump. Again, the type of receptor and kinase involved is tissue specific. PGE2, induced by the activation of phospholipase A2 (PLA2) is involved in mediating this effect. For example, dopaminergic GPCRs have been shown to differentially affect PKA and downstream pump activity depending on the specific type of GPCR, concentration of ligand and duration of ligand exposure. For instance, in kidney cells (Cos-7) short-term treatment of cells with the dopaminergic ligand, morphine, inhibits PKA and leads to enhanced pump activity, whereas long-term exposure activates PKA, leading to diminished pump activity (Poulsen *et al.*, 2010).

Adrenergic catecholamines like norepinephrine have been shown to affect the pump via GPCR adrenergic receptors that also trigger downstream PKC or PKA in a tissue specific manner. In Caco-2 cells, epinephrine resulted in reduced pump activity via the α2 adrenergic receptor in a dose and time dependent manner (maximum effect was shown at 0.5 mM at 20 minutes) (El Moussawi *et al.*, 2018). Specifically, it induced activation of a Src/p38 MAPK/ERK/COX-2 pathway which ultimately increased production of PGE2. The adrenergic receptor α2 is established to be a GPCR coupled to Gi, the G protein which AC and inhibition of PKA resulted in diminished pump activity. Thus in this context, it is implicated that PKA is needed for basal stimulation of the pump and its inhibition due to

epinephrine plays a partial role in the diminished activity of the pump (El Moussawi *et al.*, 2018).

Of the peptide hormones that regulate the pump, insulin is the most thoroughly characterized. Like the other hormones, its effects are tissue specific. Short-term effects of insulin include increased trafficking of the pump to the membrane or increasing affinity of Na+ to the pump. Long-term effects of insulin involve phosphatidylinositol-3 kinase (PI3K), PKC and p38 MAPK and although they all may involve separate pathways, they all seem to converge at the activation of PLA2, which ultimately results in production of arachidonic acid and subsequently PGE2 (Therien & Blostein, 2000).

In fact, a common convergence point for several of the aforementioned regulatory molecules includes the formation of PGE2 and further insight into its role is important for understanding how both endogenous and exogenous sources may affect production.

b. <u>The role of PGE2</u>

Prostaglandins (PGs) are eicosanoid lipid molecules enzymatically derived from the fatty acid arachidonic acid (Ricciotti & FitzGerald, 2011). They are produced and secreted by most cells in body, yet they behave in an autocrine or paracrine manner, exerting a spectrum of effects, especially those related to inflammation like fever, vasodilation, regulation of smooth muscle contraction, as well as inducing synthesis of eicosanoid derived hormones. PGE2 is the most common PG and it is specifically synthesized by cyclooxygenase (COX) enzyme and its substrate arachidonic acid (derived from phospholipid breakdown to diacylglycerol (DAG) by phospholipase A2 (PLA2). Many

external factors can trigger activation of PLA2, namely tyrosine kinase receptors – common cytokine receptors- and GPCRs (Dey *et al.*, 2006). While COX-1 maintains normal physiological levels, COX-2 is susceptible to stimulation by exogenous factors like stress, cytokines, inflammation and growth (Markossian & Kreydiyyeh, 2005; Ricciotti & Fitzgerald, 2011). After its production and release from cells, PGE2 can interact with any of the 4 PGE2 GPCRs, EP1-4, depending on its concentration and duration of interaction with the receptors. Importantly the different types of coupled G proteins include Gq, Gi and Gs. Whereas Gi inhibits AC and thus reduces production of cAMP, Gs induces the activity of AC, thereby increasing cAMP production and thus enhancing downstream activity of PKA. Gq, on the other hand, is mainly associated with phospholipase C- β (PLC-β) activation, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol triphosphate (IP3). DAG primarily activates PKC which interacts with the pump, while IP3 induces intracellular calcium release from the endoplasmic reticulum. Usually EP2 and EP4 activate PKA, while EP3 inhibits, but sometimes activates PKA, and EP1 activates the DAG/PKC or IP3/Ca2+ pathways (Dey *et al.*, 2006).

PGE2 is widely present in the intestinal mucosa and is the most important PG responsible for intestinal basal physiological regulation for functions like motility, protection, gastric acid and mucus regulation (Dey *et al.*, 2006; Ricciotti & FitzGerald, 2011). PGE2 is also implicated in intestinal diseases like IBD and colorectal cancer (Dey *et al.*, 2006). Its ability to exert a vast array of roles stems from its interaction with all four of its EP receptors which effect numerous important signaling pathways via their direct effects on PKA, PKC and PI3K The tissue and location of the cells in the tissue also produces

differential effects of PGE2. Of all the EP receptors, PGE2 has the least affinity to EP1, whereas EP3 has a relatively high affinity for its substrate, followed by EP4. Interestingly, EP3 is not always consistent in its effect on downstream cAMP as it can both increase and diminish its levels. Factors like the length of the EP receptor, its state of coupling or uncoupling to the G protein, variations in receptor desensitization and internalization all contribute to the different effects of these GPCRs. In inflammatory conditions, there is differential expression of EP receptors on epithelial cells, particularly elevated levels EP2 and EP3, in addition to significantly elevated levels of PGE2 (Dey *et al.*, 2006).

Importantly, PGE2 has been shown to regulate the sodium potassium ATPase via its various EP receptors **(Figure 5)** (Kreydiyyeh *et al.*, 2007; Rida & Kreydiyyeh, 2018).

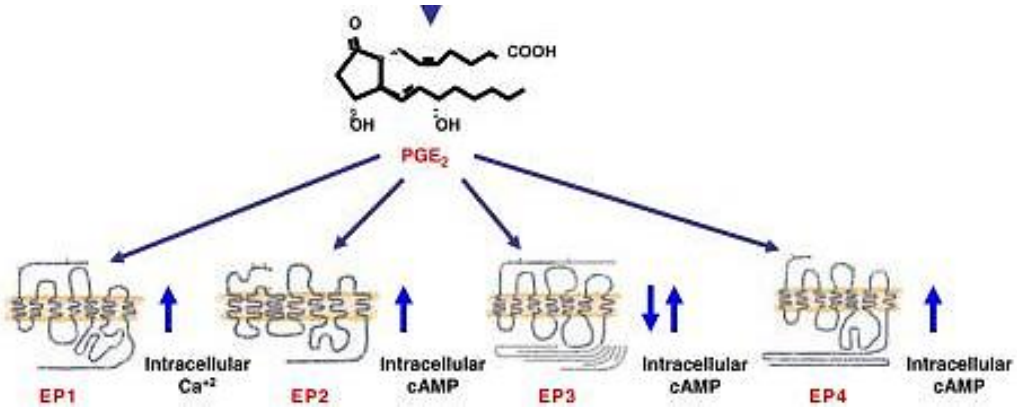

Figure 4. Depiction of PGE2 signaling pathway. EP receptors commonly regulate downstream production of cAMP and thus PKA activity. EP1 is uniquely involved in Calcium signaling. Image is reproduced from Dey *et. al* (Dey *et al.*, 2006).

For example, in neuronal cells, PGE2 triggered a cyclic adenosine monophosphate (cAMP)/PKA-mediated Ser943 phosphorylation on the ATPase and resulted in its diminished activity. This has been attributed to altering the conformation of the pump in a manner which reduces its affinity to sodium ions. In colonic Caco-2 cells, Tumor Necrosis Factor α (TNFα) was shown to inhibit the activity of the pump via PGE2 (Markossian & Kreydiyyeh, 2005). Notably, TNFα exerted its effect in a time and dose dependent manner. Interleukin-1 β is another inflammatory cytokine shown to decrease the activity of the ATPase in a time and dose dependent manner (Al-Sadi & Kreydiyyeh, 2003). Its effect was mediated by the mitogen activated protein kinase (MAPK) p38/COX-2/PGE2 pathway. These pivotal findings demonstrate that inflammatory cytokines and mediators, like those associated with inflammatory bowel diseases (IBD), can alter an essential regulator of water and ion movement. As such, it offers underlying molecular pathways by which inflammation affects the activity of the colon.

D. Gap, Hypothesis and Aims

In summary, the important points that emerge from the literature include (i) bovine milk is species specific, (ii) it contains exosomes that are intact and absorbed via colon cells into the blood, (iii) some exosome contents are bioactive but the majority of the cargo remains unexplored, (iv) milk consumption is correlated to IBD, among other auto-immune diseases, (v) inflammatory cytokines have been detected in milk exosomes including those which can affect the activity of the pump and (vi) various regulators of the pump usually involve common components including PGE2, EP receptors, PKA and PKC. Although,

milk consumption is correlated to autoimmune diseases, including inflammatory bowel disease, the underlying molecular mechanism establishing the connection has never been identified. We hypothesize that bovine milk exosomes can alter the activity of the Na+/K+ ATPase in a signaling pathway that involves PGE2 and its GPCRs.

The specific aims of this work are to:

1. Investigate the effect of exosomes on the Na^+/K^+ ATPase in Caco-2 cells
2. Conduct a time and dose response study to determine the optimal time and dose at which the exosomes exert their effect on the ATPase.
3. Investigate the involvement of PGE2 in mediating the effect of exosomes on Caco-2 cells.
4. Determine the signaling pathway by which exosomes may alter the effect of the Na^+/K^+ ATPase of Caco-2 cells.

CHAPTER 3

MATERIALS AND METHODS

A. Preparation of Exosomes

To obtain exosomes, the protocol developed and verified by Wolf *et al.* was used (Wolf *et al.*, 2015). Fat-free fresh pasteurized milk was obtained from a local supermarket. Using the super-centrifuge SORVALL RC 5C PLUS (fixed rotor SORVALL F-28/50), 200 mL of milk were centrifuged (4-8°C, 13,200 *g*, 30 minutes) to pellet somatic cells and debris. Thereafter, the pellet was discarded, and 120 mL of supernatant was mixed with an equal volume of 120 mL of ethylenediamine tetra-acetic acid (EDTA, 0.25 M, pH 7) and placed on ice for 15 minutes in order to precipitate casein. Next, we used the SORVALL Discovery 100 SE ultra-centrifuge (swinging rotor AH-629) to spin the supernatant/EDTA mixture (100,000 *g*, for 1 hour, 4-8°C) to pellet fat globules, larger extracellular vesicles, and protein. Upon completion, the supernatant was obtained ultra-centrifuged once more (90 minutes, 120,000 *g* , of 4-8°C) to pellet the exosomes.

The exosome pellet was washed twice with 500 µL of phosphate buffer saline (PBS 1x, pH 7.4) containing 0.01% Sodium Azide (PBS, 0.01% Sodium Azide). The pellets were collected by sequentially transferring the PBS, 0.01% Sodium Azide from the initial to the last centrifuge tube, so that all exosomes were present in one suspension of 500 µL PBS, 0.01% Sodium Azide. A sterile syringe was used to remove the final collection of exosome/PBS suspension which was then microfiltered through a 0.22 µM micromembrane filter into a 5 mL sterile falcon tube. The suspension was thereafter stored

at -20°C for future use (up to 1 month) or at 4°C for next day use. It did not undergo more than 2-3 defrost-refreeze cycles.

B. Quantification of exosome protein

The Bradford Assay, a colorimetric technique, was used to quantify total proteins in the exosome-rich fraction. The Biorad Bradford protein assay dye reagent concentrate (Bio-Rad, 5000006) was diluted in deionized water (1:3 v/v) and added to the samples. Protein concentration of the samples was measured at 595 nm (Multiscan^{TM} GO Microplate Spectrophotometer). A standard curve obtained using different concentrations of Bovine serum albumin, prepared from a stock (1.45 µg/µL) as indicated in Table 1 below.

Table 1. Preparation of BSA standards

Standard	Double distilled water Ml	BSA stock µL (1.45 µg/µL)	Concentration µg/µL
Blank	100	0	0
2	95	5	0.0725
3	90	10	0.145
4	85	15	0.217
5	80	20	0.29
6	75	25	0.3625
7	70	30	0.435

As such, the various concentrations of exosomes were adjusted according to the desired treatment.

C. Identification of exosomes and exosome uptake by cells

In order to verify the presence of exosomes after exosome isolation, we used the Bovine CD81 antigen (CD81) ELISA Kit (mybiosource, MBS7233742). The kit applies the competitive enzyme immunoassay technique using the solid phase Elisa to identify CD81, a known marker of bovine milk derived exosomes.

1. Exosome identification

The average concentration of exosome protein upon quantification was approximately 30 μg exosome protein/ μL of exosome suspension. 50 μL of this solution was added to an equal volume of PBS (pH 7.4) and then incubated together with CD81-HRP (horseradish peroxidase) conjugate in pre-coated wells of the CD81 ELISA kit for one hour. Thereafter, the wells were washed 5 times with the washing solution of the kit and incubated with a substrate (not identified by supplier) for the HRP enzyme forming a product that turns blue. A stop solution was added, turning the solution yellow. The intensity of the color was determined colorimetrically at 450 nm using a microplate reader. The color intensity is inversely proportional to the amount of CD81 concentration since CD81 from samples and CD81-HRP conjugate compete for the anti-CD81 antibody binding site. CD81 concentration is reported as ng/ mL of solution and is interpolated using a standard curve with the provided standards.

2. Detection of exosome uptake by Caco-2 cells

Caco-2 cells were seeded into four 10 cm cell dishes at a density of 60,000 cells/ 1 mL media and upon 80% confluence, 2 were treated with exosome vehicle (PBS, 0.01% Sodium Azide) and 2 were treated with a concentration of 112.5 μg exosome protein/1 mL media for 1 hr, After 1 hr, cells were detached using Trypsin - 0.53 mM EDTA solution (Sigma Aldrich, T4049-100ML) and centrifuged (following the same method described in section E for cell culture and seeding). The pelleted cells of each sample were resuspended in 600 μL of PBS and were ultrasonicated for 90 seconds. Thereafter, cell protein was quantified using the Bradford Assay and each sample was adjusted to a final volume of 100 μL(PBS) with a concentration of 0.26 μg cell protein/ μL of suspension. The same protocol for identifying exosomes using the Bovine CD81 antigen (CD81) ELISA Kit was used, as described above except lysis of exosomes was not necessary.

D. Cell Culture

Human colorectal adenocarcinoma epithelial Caco-2 cells (ATCC®, HTB-37™) between passages 25-32 were used for our experiments. The cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM) (high glucose) containing 10% fetal bovine serum (FBS), 4500 mg/L glucose, 1% Penicillin (100 μg/mL)/ Streptomycin (100 μg/mL) (Sigma Aldrich, D6546). Incubation was under humidified conditions at 37°C and in presence of 95% O_2 and 5% CO_2. Cells were seeded in 10 cm cell culture dishes at a density of 60,000 cells/1 ml media and the medium was replaced every 2 days. Once the cells reached 80% confluence, they were washed with 5 mL of sterile phosphate buffered saline (PBS 1x) and treated with 1.5 mL of Trypsin-EDTA (37°C) for 1 minute at room temperature. Half the trypsin was removed, and cells were placed in the humidified

incubator until all cells detached (approximately 4 minutes). 5 mL of DMEM complete medium was added to the culture plate and cells were aspirated and placed in a 15 mL falcon tube, centrifuged (5 minutes, 4°C ,1500 rpm). The pellet was resuspended in 1 mL of DMEM complete media. The cells were counted using trypan blue (1:2 v/v cell suspension: trypan blue) and once again were seeded into 10 cm culture plates to continue propagating the cell line.

E. Cell Seeding

For cell seeding, upon centrifugation of the pellet of cells as explained in the previous section, cells were instead resuspended in 1 mL of exosome-free DMEM complete medium obtained by ultrasonication of FBS for 1 hour at 4°C in order to rupture exosome membranes. Cells were seeded into 6 well plates at a density of 15,000 cells/1 mL to prepare for the various treatments. At 80% confluence, the media were removed and replaced with fresh exosome-free media, and cells were treated according to the desired experiment (for example time response, dose response and signaling studies).

F. Obtaining Supernatant Containing Caco-2 Cell Membranes

At the end of the treatment, the plates were placed on ice in order to collect the cells. Specifically, 60 µL of Lysis Buffer [9.9 ml of 150 mM histidine buffer pH=7.4, 400 µl protease inhibitor (0.5 tablet/1 ml H_2O), 100 µl Triton-X (1mg/ml H_2O)] were added to each well. After scraping, the cells were resuspended in 60 µL of Histidine buffer (150

mM, pH 7.4), homogenized and spun at 20,000 *xg* for 30 minutes at 4°C to remove large debris. The supernatant was collected and its protein concentration adjusted to 0.5 μg / μL in Histidine buffer (150 mM, pH 7.4) and the activity of the pump was assayed as described under section J.

G. Time Response Studies

For the time response study, the following time points were selected: 0.5 hr., 1 hr., 2 hrs., 3 hrs., 6 hrs., 12 hrs., and 24 hrs. For each time point, one 6 well plate was used, where 3 wells were designated as controls while the other 3 were designated as exosome treatment wells. After replacing the media with fresh exosome-free complete DMEM media, the cells were treated with 225 μg exosome protein/1 mL media (2 mL per each well in the 6 well plate). For the control treatments, an equivalent volume of exosome vehicle (PBS, 0.01% Sodium Azide) was added. Thereafter, the plates were placed in a humidified incubator for the allocated time period. Next, cell homogenates were obtained according to the previously described protocol and sodium potassium ATPase assay was conducted as described under Section J.

H. Dose Response Studies

Dose response studies followed the same protocol for time response studies. However, cells were treated for a 1 hr. and 24-hour time period only given that these time points resulted in the most optimal responses to the exosomes. Various concentrations of

exosome protein were used in separate experiments: 25, 37.5, 122.5, 225 and 375 μg exosome protein/ mL media per well for 1 hour; and 37.5, 62.5, 150, 225 and 330 μg exosome protein/1 mL media for 24 hours. After 1 hour or 24 hours, cell supernatants were obtained according to the previously described under Section F. The activity of the sodium potassium pump was then assayed as described under section J.

I. Signaling Studies

1. Involvement of PGE2

To determine the involvement of COX-2 enzyme, and thus the involvement of PGE2, cells were pre-treated with the inhibitor of COX-2, indomethacin. Treatments were conducted in triplicates. Indomethacin was added at a concentration of 200 μM, 30 min before addition of the exosomes (122.5 μg exosome protein/1 mL media). All treatments received an equal volume of the exosome vehicle,(PBS,0.01% Sodium Azide) and the indomethacin vehicle, (DMSO). After the addition of exosome protein, cells were incubated for a period of 1 hr. Similar to time and dose response studies, cells were scraped and quantified for protein, as described under Section F and the sodium potassium ATPase activity was then assayed as described under Section J.

To assess if the exosomes act via PGE2, cells were treated with 100 nM PGE2. An equal volume of the vehicle (DMSO) was added to the control. At the end of the one-hour incubation period, the cells were scraped, homogenized, centrifuged, and assayed for their Na^+/K^+ ATPase activity.

2. Involvement of PGE2 receptors

In order to identify which of the 4 EP receptors are involved, cells were treated with a specific blocker of each receptor type, 30 min before the addition of the exosomes. The blockers are namely: SC-19220, (EP1 antagonist, 100 μM in DMSO), PF-04418948 (1 μM, DMSO), EP2 antagonist, L-798106 (10 μM,DMSO), EP3 antagonist and BGC 20-1531 (10 mM, DMSO), EP4 blocker. Treatments were conducted in replicates of 3 or more and all had an equal volume of the blocker's and exosomes' vehicle. Upon addition of exosomes, cells were incubated for 1 hr., followed by acquiring supernatants as described under Section F and the pump Assay as described under section J.

3. Involvement of PKA

To assess the involvement of PKA in the exosome mediated activation of the pump, the inhibitor RPcAMP (30 μM, H_2O) was added to cells 30 min prior to the exosomes. After the addition of exosome protein, cells were incubated for 1 hr. The activity of the ATPase (described under Section J) was assessed after obtaining the supernatants (under Section F). To confirm the effect of PKA, cells were treated with the PKA activator dibutryl cAMP (dbcAMP) (10 μM, DMSO). After one hour of incubation in humidified incubator, the cells were scraped, homogenized, centrifuged, and prepared for inorganic phosphate assay.

J. Na+/K+ ATPase Assay

The activity of the pump was assayed according to a protocol developed by Esmann (Esmann, 1988). A volume of 130 μL of the supernatant obtained as described under Section F was incubated for 15 minutes at room temperature with 1% saponin added at a ratio of 1:4 v/v . Subsequently phosphatase inhibitor cocktail (ratio of 1:6 v/v), prepared by mixing 10.7 mM glycerophosphate, 10.7mM pyrophosphate and deionized water (in a ratio of 3:3:4 v/v/v), was added to the supernatant. Then 12 μL of supernatant (0.5 μg cell protein/1 μL), were transferred to Eppendorf tubes together with 42 μL histidine buffer (150 mM, pH 7.4), 30 μL ion mix [(10 μL NaCl (121.5mM), KCl (10 μL 19.6 mM,), $MgCl_2$ (10 μL 3.92 mM), 1:1:1 v/v), and 10 μL of adenosine tri-phosphate (ATP, 2.94 mM)]. This was repeated 8 times per each sample (for example, cell samples treated with exosome protein ultimately yielded 8 Eppendorfs, each containing the above components). To 4 out of the 8 Eppendorfs, 12 μL of the specific inhibitor of the Na+/K+ ATPase, ouabain (1.47 mM) was added, and an equal volume of deionized water was added to the second half. The tubes were then incubated for 15 minutes at 37°C. The reaction was stopped by adding 10 μL of 50% trichloroacetic acid (ratio of 1:10 v/v) and the samples were centrifuged at 3000 *g* at room temperature for 5 minutes. The pellet was discarded and 90 μL of each supernatant containing the released inorganic phosphate (Pi) were mixed with 80 μL of ferrous sulfate molybdate reagent (0.5g ferrous sulfate + 1 ml ammonium molybdate (0.1g/L of 10N H2SO4). In parallel, a standard curve was prepared with various concentrations of KH_2PO_4. A blue molybdenum complex is formed and the intensity of color was determined colorimetrically in a microplate reader at 750 nm, against a standard

curve prepared by mixing 90 µL of various concentrations of KH_2PO_4 with 80 µL of ferrous sulfate –molybdate reagent.

The activity of the pump was calculated as follows and reported as a percentage of the control value

$$\frac{Pi(sample) - Pi(sample + ouabain)}{Pi(control) - Pi(control + ouabain)} X 100$$

K. Statistical analysis

Results are reported as means ± SEM. Data were tested for statistical significance by a one-way analysis of variance followed by a Tukey-Kramer multiple comparison test ,or by an unpaired student t test where applicable , using the statistical software GraphPad Prism 8.4.2

CHAPTER 4

RESULTS

A. Bovine milk-derived exosomes exert an opposite dual effect which is time dependent on the Na+/K+ ATPase in Caco-2 cells

To determine if bovine milk derived exosomes alter the activity of the pump in Caco-2 cells, a time response study was conducted in which cells were treated with 225 μg of exosome protein/1 mL of media for different time periods (0.5, 1, 2, 3, 6, 12 and 24 hrs.). The exosomes increased the activity of the ATPase as early as 0.5 hrs. **(Figure 5)**. By 1 hr., the activation reached its peak at approximately 230% relative to control. Importantly, at 12 and 24 hrs., the exosomes significantly inhibited the pump with a residual activity of around 30% only. The viability of the cells was not affected at this point. Because the most prominent effect was activation at 1 hour, we proceeded with dose response studies at this time point.

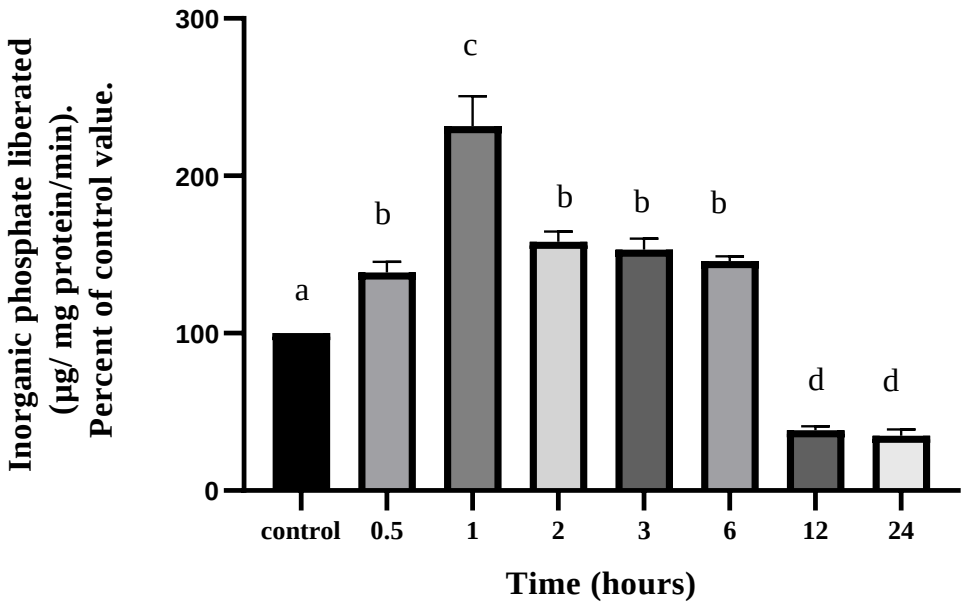

Figure 5. Time response study on the effect of bovine milk exosomes on Na+/K+ ATPase in Caco-2 cells. Cells were treated with 225 µg exosome protein/ 1 mL media. The results are reported as a percentage of the control value. Statistical significance was tested by a One-way ANOVA followed by Tukey Kramer test. ($P < 0.001$ for means with different letters. Values with same letters are not significantly different from each other. N = 6-9.

B. Bovine milk derived exosomes alter activity of the pump in a dose dependent manner

We treated Caco-2 cells with various concentrations of exosome protein for 1 hr and 24 hrs. To adequately assess the effect of concentrations, we began with the 225 µg exosome protein/1 mL used in the time response study. We also used concentrations above and below this value. If the result was still similar to the effect at 225 µg exosome protein/1 mL (suggesting we were working at plateau levels) we widened the gap of concentrations until it was apparent that we were no longer working at the plateau concentration.

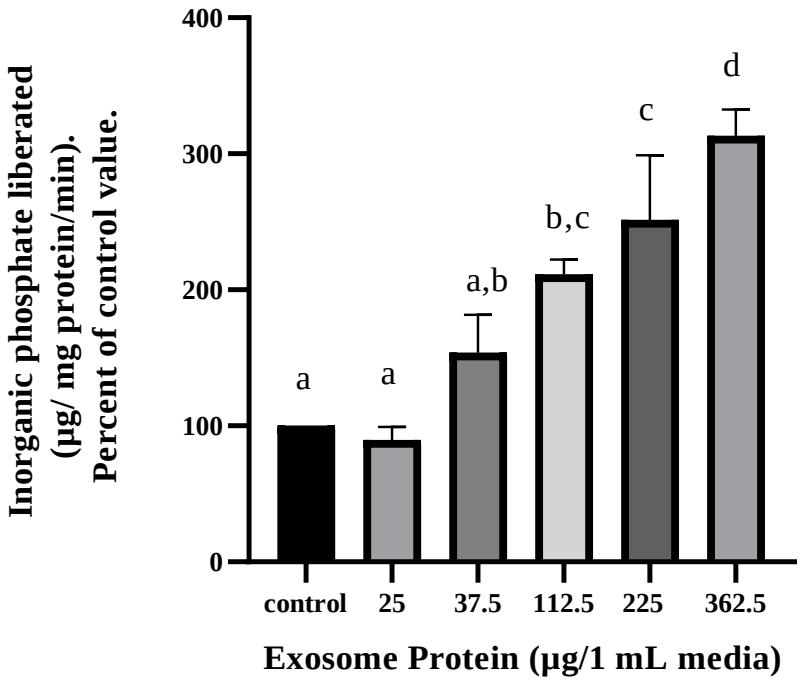

Figure 6. Dose Response study at 1 hr. Inorganic phosphate liberated at various concentrations of exosome protein. Statistical significance was tested using a One-way ANOVA followed by Tukey Kramer test. Values with different letters are significantly different from each other at $P< 0.05$. N = 6-9.

The minimum required concentration to induce significant activation at 1 hr. relative to control was 225 µg exosome protein/1 mL media **(Figure 6)**. Activation continued to increase directly according to concentration, indicating that exosomes increase the activity of the pump in a dose dependent manner. For the subsequent signaling studies we used the concentration of 112.5 µg exosome protein/1 mL media, rather than 225 µg exosome protein/1 mL to avoid functioning at plateau and to minimize waste of isolated exosomes, especially that the effect between these two concentrations was not statistically significant.

At 24 hrs, the results indicated that inhibition also intensified as exosome protein concentration increased **(Figure 7)**. Again, depending on the effect on the pump, we widened the gap between each concentration treatment to ascertain that we were no longer functioning at plateau. The minimum required concentration to produce a significant decrease in the pump's activity was 150 µg exosome protein/ 1 mL media. Concentrations greater than 225 µg exosome protein did not produce further significant inhibition, suggesting that at 225 µg and above, the inhibitory activity of the exosome remained at a constant plateau level.

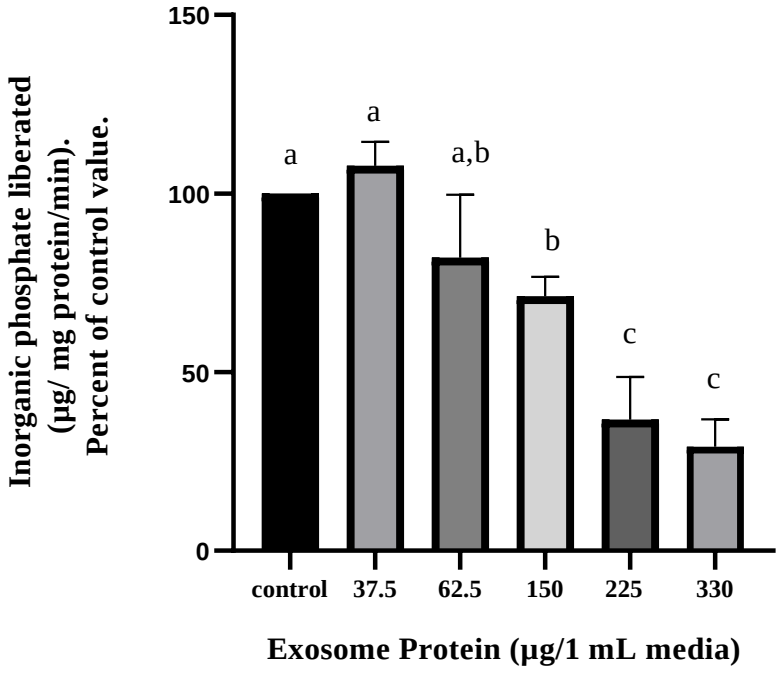

Figure 7. Dose Response study at 24 hrs. Inorganic phosphate liberated at various exosome protein concentrations. Statistical significance was tested using a One-way ANOVA

followed by Tukey Kramer test. Values with different letters are significantly different from each other at $P < 0.005$. $N = 3\text{-}6$.

C. The activation of the Na+/K+ ATPase is mediated by COX-2 and PGE2 at 1 hour

Because the most prominent effect was seen at 1-hour with over 200% activation of the pump by 112.5 μg exosome protein/1 mL media, this time point and dose were used to conduct signaling studies. Since PGE2 synthesis is catalyzed by the COX-2 enzyme, we used a specific COX inhibitor, Indomethacin (200 μM, DMSO), to block production of PGE2. Caco-2 cells were pre-treated with Indomethacin for 30 minutes and subsequently with 112.5 μg exosome protein/1 mL media, according to the previously described method. Our findings, which are summarized in Figure 8 **(Figure 8)**, indicate that inhibition of PGE2 production abolishes the effect of exosomes.

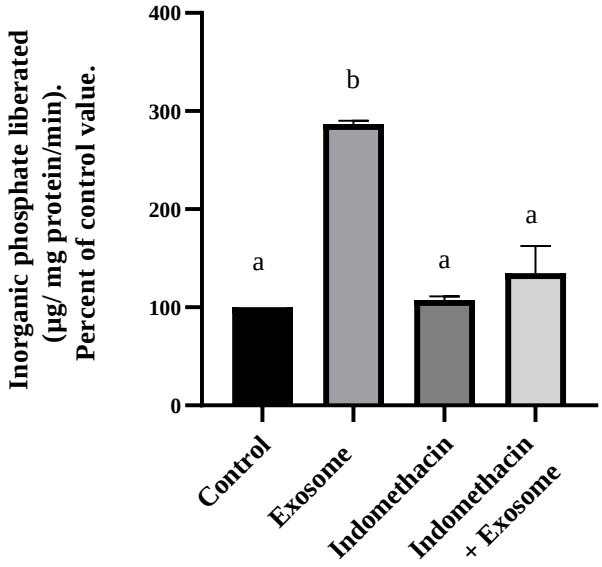

Figure 8. Effect of Indomethacin at 1 hr. Cells were pre-treated for 30 minutes with Indomethacin (Indomethacin: 200 µM dissolved in DMSO 1:10 v/v), prior to exosomes addition (112.5 µg/1 mL). Statistical significance was tested using a One-way ANOVA followed by Tukey Kramer test. $P < 0.0001$ for means with different letters. Values with same letters are not significantly different from each other. N =3.

These findings imply a role for PGE2 in the exosome-mediated activation of the pump at 1 hr. To further confirm the involvement of PGE2, Caco-2 cells were treated with 100 nM exogenous PGE2 (dissolved in DMSO 1:10 v/v) without exosome protein. The prostaglandin increased the activity of the pump by more than two folds, which further corroborates the findings with Indomethacin **(Figure 9).** Because PGE2 can act via 4 different GPCRs, EP1-4, we next determined which receptor is specifically involved in the activation at 1 hour.

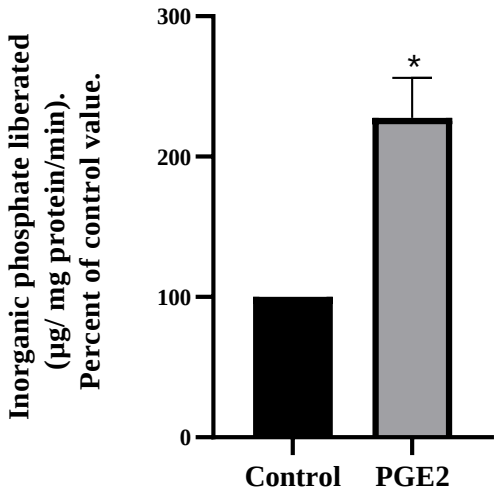

Figure 9. PGE2 stimulates the ATPase at 1 hr. Caco-2 cells were treated with exogenous PGE2 (100 nM). Statistical significance was tested using an unpaired t test. (P relative to control * P< 0.005; N =3).

D. Exosome-Induced PGE2 interacts with both EP3 and EP4 Receptors

The PGE2 produced by Caco-2 cells upon exosome exposure, modulates the activity of the pump via one or more of its G proteins- coupled receptors (GPCRs). The stimulatory effect of the exosomes in presence and absence of SC-199220, an inhibitor of EP1 receptors **(Figure 10)**, or in presence and absence of PF-04418948 , a blocker of EP2 receptors **(Figure 11)** , was not altered, suggesting that EP1 and EP2 receptors are not involved in the action of the exosomes.

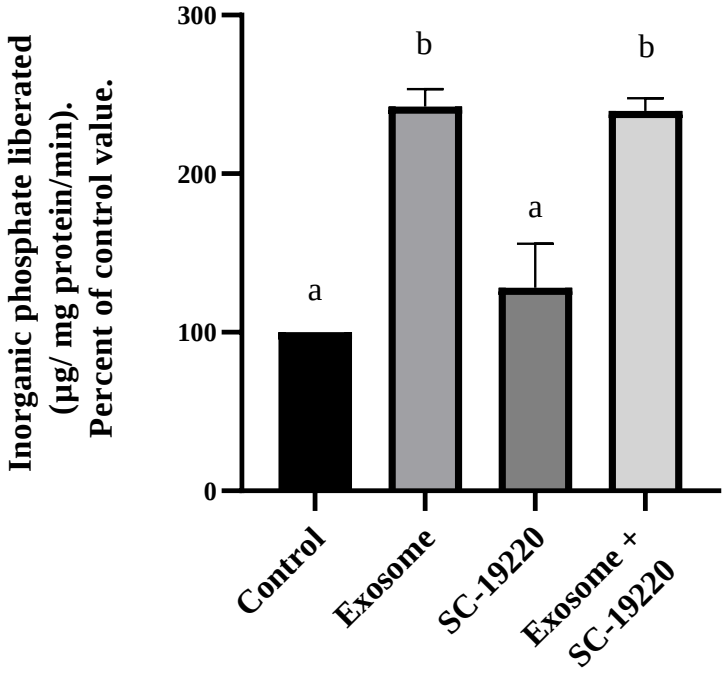

Figure 10. EP1 receptors are not involved. Caco-2 cells were treated with the exosomes (122.5 ug exosome protein/1 mL media; indicated as "Exosome"), in presence and absence of 100 µM of SC-199220, a blocker of EP1 receptors. Statistical significance was tested by a One-way ANOVA followed by Tukey Kramer test. ($P < 0.01$ for means with different letters. Values with same letters are not significantly different. N =3).

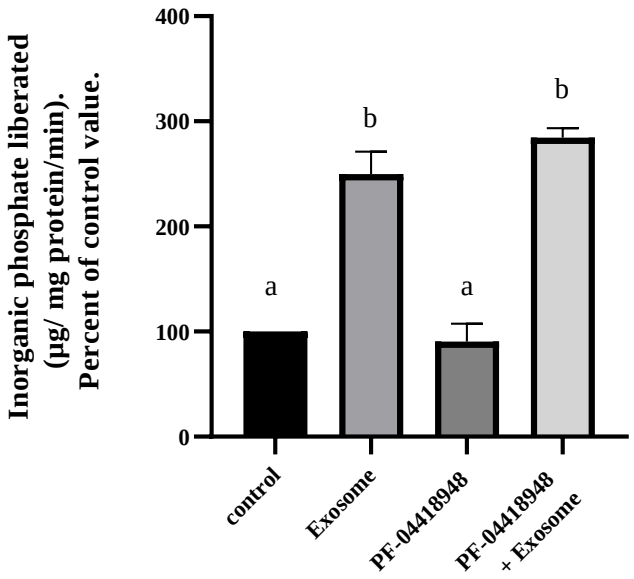

Figure 11. Effect of EP2 antagonist. Caco-2 cells were treated with exosomes (112.5 ug exosome protein/1 mL media; indicated as "Exosome") in presence and absence of 1 µM of PF-04418948, an inhibitor of EP2 receptors. Statistical analysis was conducted using One-way ANOVA, followed by Tukey Kramer test. ($P < 0.0001$ for means with different letters. Values with same letters are not significantly different; N =3)

Inhibition of EP4 in the presence of exosomes resulted in an activation which was higher than the one seen with the exosomes alone, suggesting a role of these receptors in the exosomes modulatory effect on the ATPase **(Figure 12).**

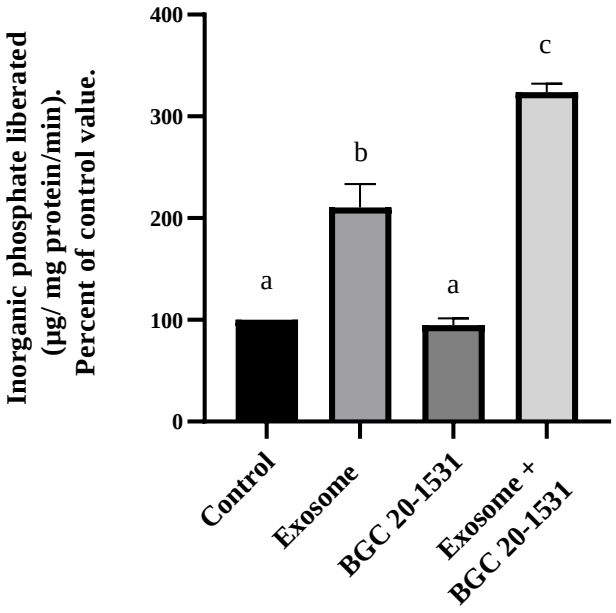

Figure 12. Effect of EP4 antagonist BGC20-1541. Caco-2 cells were treated with 10 µM of the inhibitor in presence or absence of the exosomes (112.5 ug exosome protein/1 mL media, indicated as "Exosome"). Statistical analysis was conducted using One-way ANOVA, followed by Tukey Kramer test. ($P < 0.001$ for means which have different letters. N =3).

When the EP3 receptors were blocked with L-798106, the stimulatory effect of the exosomes not only disappeared, but was changed into a significant inhibition (Figure 13).

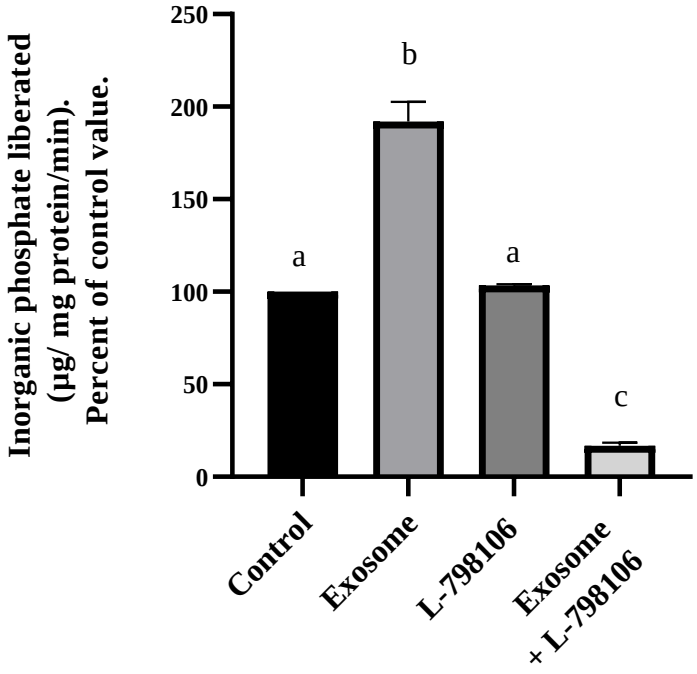

Figure 13. Effect of EP3 antagonist L-798106. Caco-2 cells were treated with 10 µM of the inhibitor to block EP3 receptors in presence or absence of exosomes (112.5 ug exosome protein/1 mL media, indicated as "Exosome"). Statistical analysis was conducted using One-way ANOVA, followed by Tukey Kramer test ($P < 0.0001$ for means with different letters. Values with same letters are not significantly different from each other; N =3).

E. PKA is a downstream mediator of PGE2

EP3 is known to inhibit PKA while EP4 activates it. To determine if PGE2 acts via PKA, we studied the effect of an inhibitor and an activator of the kinase. Inhibition of PKA with Rp-cAMP indeed resulted in a significant activation of the pump **(Figure 14)** and maintained the stimulatory effect of the exosomes**.** This

suggests that EP3 may also be acting, at basal levels, as an inhibitor of PKA. To further corroborate these findings, Caco-2 cells were treated with the PKA activator dbcAMP. The mere activation of PKA resulted in a significant decrease in the pump's activity **(Figure 15).**

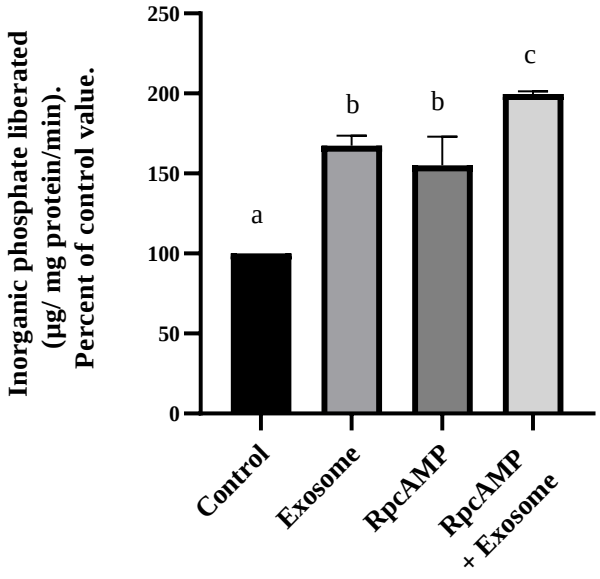

Figure 14. Effect of the PKA inhibitor RpcAMP. Caco-2 cells were treated, 30 min prior to the addition of exosomes, (112.5 µg exosome protein/1 mL media), with 30 µM of the inhibitor. Statistical analysis was conducted using One-way ANOVA, followed by Tukey Kramer test. ($P < 0.01$ for means with different letters. N =3).

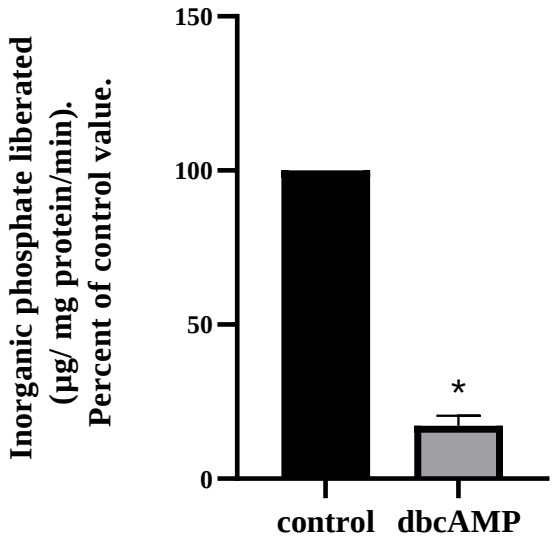

Figure 15. PKA activator dbcAMP (10 µM) inhibits the ATPase. Statistical analysis was conducted using unpaired t test (* P< 0.0001; N =3).

F. Exosome biomarkers were elevated in Caco-2 cells treated with exosomes

CD81 is a common marker used to confirm the presence of exosomes. However, since it is also present on mammalian cells, in order to determine if there was exosome uptake by the Caco-2 cells, it was necessary to compare CD81 levels in the cells treated with exosomes to that of the control. Initially, using the solid phase CD81 ELISA Kit (described in methods), we determined that there is an average of 6×10^{-4} ng CD81/ μg of exosome protein. After treating Caco-2 cells with 112.5 μg exosome protein/ mL media for 1 hour (against control), we performed an analysis of CD81 content in both control and treated cells. Our findings (summarized in **Figure 14**) indicate that Caco-2 cells treated with exosomes contained significantly higher levels of CD81 compared to control.

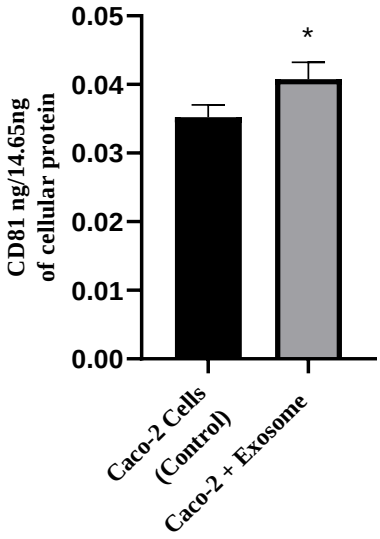

Figure 16. Quantitative assessment of bovine CD81. Values are reported as ng of CD81 per 14.65 µg of cellular protein. Statistical analysis was by applying the unpaired t test. ($P < 0.05$. $N = 3$).

CHAPTER 5

DISCUSSION

A. Bovine Milk Exosomes and the Time-Dependent Effect

It was previously demonstrated that Caco-2 cells can transport bovine milk exosomes via endocytosis at a maximum saturation concentration of 225 μg exosome protein/1 mL media (Wolf *et al.*, 2015). This was a major finding which propelled us to examine what are the full implications of consumption of cow's milk on the colon epithelial cells, with a special focus on the Na+/K+ ATPase given its indispensable role in ion and water homeostasis and the change in its activity in several intestinal diseases like IBD. Therefore, we began our experiment using the same concentration for our time response studies at 7 different time points. As early as half an hour after exposure to exosome proteins, a significant activation of the sodium potassium ATPase was detected, with the most prominent stimulation occurring at 1 hr. After this time point, there was still significant activation until 6 hrs, however this transitioned into inhibition of the pump by 12 hrs, and lasted well into 24 hrs.

A dose response study revealed that the exosome effect is also dose dependent. Using trypan blue to assess cell viability, we found no significant difference in cell viability between control and exosome treated cells, at the highest inhibitory concentration, supporting the conclusion that the reduced ATPase activity is not due to increased cell death.

The results indicate that the effect of bovine milk exosomes on the Na+/K+ ATPase is opposite and dual, with early activation and later inhibition **(Figure 5)**.

It would be interesting to examine, in future the effect on the pump after 36, 48 and 72 hrs. of exposure to bovine milk exosomes, especially that physiologically they may remain in contact with the colon epithelia for this length of time.

B. The dual role of PGE2

The activity of the pump at both 1 hr. (activation) and 24 hrs. (inhibition) directly intensified according to increasing concentration of exosome proteins **(Figures 3 and 4)**. Such an action trend with dual effects was previously noted with PGE2. In fact, concentrations of 0.1-1 nM PGE2 significantly reduce the activity of the pump to around 30% of the control value, while maximum significant activation of approximately 250% was obtained at 100 nM and was sustained at the same level for up to 300 nM PGE2, suggesting that at 100 nM a plateau is reached (Chakkour & Kreydiyyeh, 2019). Because we had a similar activation level at 1 hr., and since we observed a dual effect in our time response study, we decided to investigate the role of PGE2 as a mediator of the exosome effect. Indeed, inhibition of PGE2 production by antagonizing the COX-2 enzyme **(Figure 8)** with Indomethacin, completely abrogated the effect of the exosomes by reverting to control levels of the pump's activity. To further corroborate these findings, we treated Caco-2 cells only with 100 nM PGE2 for 1 hr. **(Figure 9)**

which resulted in a similar activation to the one achieved by Caco-2 cells exposed to bovine milk exosomes for 1 hr. This suggests that these extracellular vesicles are triggering the production of a high concentration (100 nM or above) of PGE2 in Caco-2 cells, at early times and lower quantities (around 1 nM) at later times. The inhibitory effect may be ascribed to a negative feedback mechanism in which high PGE2 levels reduce the expression of COX-2, the rate limiting enzyme needed for the production of PGE2 (Inoue, Tanabe, & Umesono, 2000).

C. The Various Effects of the EP Receptors

PGE2 can exert its effect in an autocrine or paracrine manner as a ligand for its GPCRs, EP1-4. After confirming the involvement of PGE2 in the activation of the pump, we sought to determine the type of EP receptors involved. It was apparent that both EP1 and EP2 **(Figure 10-11)** are not essential for activation since the stimulatory effect was still apparent when both receptors were blocked individually with a specific inhibitor. However, inhibition of EP3 changed the stimulatory effect of the exosomes into a prominent inhibitory one **(Figure 12)**, suggesting that when EP3 receptors are not active, the exosomes exert an inhibitory effect probably through EP4 receptors, since EP1 and EP2 were shown before not to be involved. The observed stimulatory effect of the exosomes when all receptors are active implies that EP3 receptors stimulate the pump to a very high level that surpasses significantly the inhibition induced by EP4 resulting in a net increase in the ATPase activity. According to this hypothesis, blocking EP4

receptors, should abolish their inhibitory effect that was counteracting the simulation induced by EP3, resulting in a higher activation of the Na+/K+ ATPase. In fact, this is what was observed upon blocking EP4 receptors with BGC20-1541. It can be concluded that at one hour and at a dose of 225 μg, exosomes act on the ATPase via EP3 and EP4 receptors. However, because the effect of EP3 is more pronounced, the role of EP4 was apparent only upon inhibition of EP3 in the presence of exosomes.

These findings are likely due to the nature of the GPCRs themselves where EP3 and EP4 are linked respectively to Gi and Gs. Gi is known to inhibit downstream AC and thus diminish production of cAMP, thereby inhibiting activity of PKA, while Gs activates AC, ultimately activating PKA (Neves *et al.*, 2002). In our case, PKA would be an inhibitor of the ATPase and EP3 and EP4 exert opposite effects on this kinase.

Our examination of the effect of PKA corroborates its involvement in mediating the effect of bovine milk exosomes **(Figures 14 and 15),** as inhibition of the enzyme in the presence of exosomes mimicked the stimulatory effect of EP3 and activation of PKA in the absence of exosomes mirrored the inhibition observed via EP4. Whether the EP4 effect may take greater precedence at 24 hrs. where the effect of the pump is reduced to almost 30% of the control value is a question that needs to be addressed in future work. It is worth noting that the order of affinities of the different receptors is as follows EP1<EP2<EP4<EP3 with EP1 having the lowest affinity and EP3 the highest (Dey *et al.*, 2006). So EP3 receptors are expected to be the most highly activated and impose their stimulatory effect.

Whether at later times PGE2 would have accumulated at high levels activating high and low affinity receptors and exerting thus opposite effect, is an issue to be investigated.

D. Homing in on the Effects of Bovine Milk Cytokines on PGE2 Production

Exosomes have been shown to carry several cytokines like TGFβ and TNFα, both of which can alter the activity of the pump (Markossian & Kreydiyyeh, 2005; Samuel *et al.*, 2017; Fitzgerald *et al*., 2018). Notably, cow milk exosomes carry TGFβ and incidentally it is capable of inducing PGE2 production via COX2. (Fang *et al*., 2014; Pieters *et al*., 2015). In addition TNFα is also capable of triggering PGE2 synthesis and modulates the activity of the pump in both colonocytes (inhibition after 2 hrs.) and hepatocytes (upregulation after 2 hrs.) (Markossian & Kreydiyyeh, 2005;Kreydiyyeh *et al.*, 2007). These studies illustrate that downstream effects of various ligands converge at the point of PGE2 synthesis.

Importantly, it has been demonstrated that the effect produced in isolated Caco-2 cells is translatable to *in vivo* conditions in animal studies. Specifically, similar results presented in both colon cells of rats that were treated with TNFα and in Caco-2 cells treated with the cytokine (Markossian & Kreydiyyeh, 2005). Perhaps it may be assumed that the displayed effect of exosome proteins on Caco-2 cells in our experiments are likely mimicked in the mammalian intestinal organ and translate to a tangible and detectable difference in ion and water flow.

Nevertheless, in future studies this would be a desirable aim to achieve and that would help in determining if increased pump activity does in fact manifest in increased ion and water movement across the colon and potentially contribute to constipation.

E. Physiological Relevance

The aforementioned cytokines represent major culprits in the symptoms of IBD (Turner *et al.*, 2014) and their presence in bovine milk exosomes further corroborates that cow milk consumption is associated with IBD (Bernstein & Shanahan, 2008). Humans as young as infants are introduced to cow milk derived infant formula, and many continue to drink it throughout adulthood. The USDA has demonstrated that children as young as two years of age consume almost 2 cups of milk daily, and many continue to do so well into their fifties although the quantity is substantially reduced with age. Furthermore the recommended daily intake states that a healthy diet contains 2-3 servings of dairy (Sebastian *et al.*, 2005; Gao *et al.*, 2006; Melnik & Schmitz, 2017).

In our study, the exosomes were obtained from 120 mL of fat-free fresh pasteurized milk and suspended in approximately 600 μL of PBS, 0.01% Sodium Azide. For each treatment containing 112.5 μg exosome protein/1 mL media, up to 18 μL of the suspension were used. The area of each well in a 6 well plate is 9.6 cm^2 (as per manufacturer) and that of the entire colon is approximately 10,000 cm^2. Our experiment exposed approximately cells in the well's surface area to 225

μg exosome protein/ 9.6 cm^2 of Caco-2 cells, translating to 23 μg of exosome/ cm^2. Given that our milk samples contained approximately 30 μg exosome protein/ μL of suspension, then 600 μL (obtained from 120 mL of milk) carry around 18,000 μg of exosomes. Consumption of 2 – 3 cups would deliver up to 108,000 μg exosome proteins to the colon. Importantly, our experiment showed that concentration as low as 37.5 μg exosome protein/1 mL media was sufficient to elicit significant activation, and this would be in proportion to around 78,000 μg exosome protein per the surface area of the colon. Another perspective to view this is that in just 1 mL of cow's milk, there are approximately 1.4×10^{11} particles of exosomes (Vaswani *et al.*, 2019). This would mean that in 1 serving (which equates to 250 mL of milk) an individual is ingesting 35 billion particles of exosomes, of which 30% are transported across the intestine and 70% remain in contact with the colonic epithelium- noting that exosome resist degradation due to acidification of the stomach (Vaswani *et al.*, 2017). Because milk consumption would deliver a quantity of exosomes more than what is required to alter the activity of the pump, it is important to consider the full implications of its prolonged- often decades long- consumption.

It is undeniable that there are critical developmental time frames where exposure to certain triggers may serve as epigenetic regulators. The most critical developmental time frames for humans are during embryonic development, the neonatal period and childhood. These periods of rapid growth and development are the most sensitive to environmental exposures. It is established that the mother's food intake can shape the body composition and health outcomes of the offspring

(Gluckman *et al.*, 2011). In fact, due to epigenetic regulation caused by food, the diet of pregnant mothers and infants serves as a risk factor for later adult non-communicable diseases such as type 2 diabetes and cardiovascular disease.

Exosomes transported in milk from a mother to her own infant, may carry cytokines involved in development and production of hematopoietic cells, an important component in infant immune programming (Saito *et al.*, 2018). On the other hand, young infants and pregnant women consuming dairy may be ingesting bovine cytokines enclosed in cow milk exosomes which are reaching the child or fetus, perhaps eliciting responses which would be undesired in a developmentally vulnerable human. Moreover, many cytokines are evolutionarily conserved across species, which is a cause for concern for the young human age groups that are incessantly exposed to these inflammatory modulators, even well into adulthood (Saito *et al.*, 2018). Additionally, because cytokines can be released in response to pathogens, it is likely that transported exosomes carrying cytokines are triggering an unnecessary immune response in the recipient, whether it is visible or not. Given the sustained exposure of human's to cow's milk, especially within the colon, but the lack of knowledge in its phenotypic effect, we found it almost obligatory to carry out our research.

F. Conclusion

Our findings reveal for the first time, the molecular mechanism by which milk disrupts one of the most important physiological proteins of humans- the sodium potassium ATPase.

Our findings home in on the major pathway, illustrated in Figure 14, that mediates the effect of bovine milk exosomes on the Na+/K+ ATPase in Caco-2 cells at 1 hr. In summary the exosome-linked cytokines activate COX-2 enzyme leading to PGE2 production. The prostaglandin activates EP3 and EP4 receptors which exert opposite effects on PKA. Because the inhibitory effect of EP3 is more prominent, there is a net inhibition of PKA which usually decreases the activity of the pump. The exosomes, by inhibiting the inhibitor (i.e. PKA) exert a stimulatory effect on the ATPase.

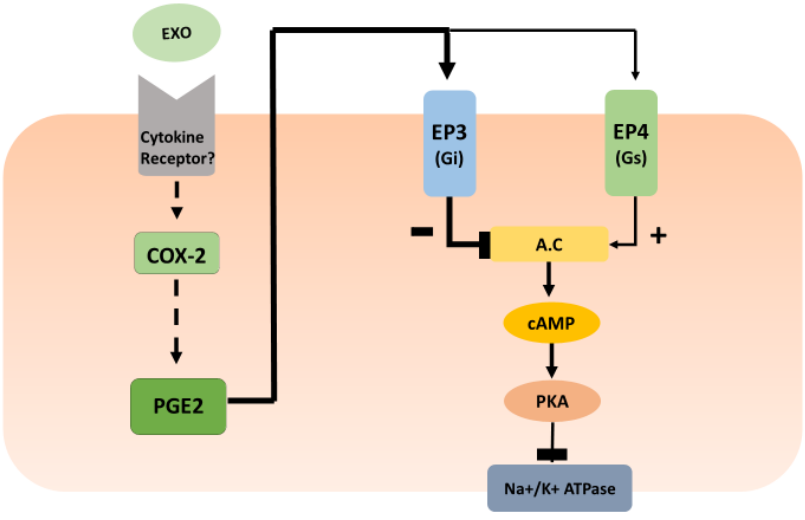

Figure 17. Pathway mediating the effect of exosomes on the Na+/K+ ATPase at one hour. Exosomes derived from cow's milk may possibly activate cytokine receptors. This eventually leads to activation of COX2 and production of PGE2.

The latter activates EP3and EP4 receptors which exert opposite effects on PKA. EP3 which is coupled to heterotrimeric Gi protein, inhibits adenylyl cyclase (AC), reduce cAMP production and inhibits PKA., while EP4 that couples to Gs activates the kinase. PKA exerts an inhibitory effect on the Na+/K+ ATPase either by direct phosphorylation or by modulating other components. The prominent inhibitory effect of EP3 on PKA masks the stimulatory effect of EP4 resulting in a net inhibition of PKA and activation of the Na+/K+ ATPase.

It is important to note that one study has elucidated that the pump's activity is inversely related to the degree of colonic inflammation in the Ulcerative Colitis form of IBD (Allgayer *et al.*, 1988). In fact, it was proposed that the activity of the pump may serve as an indirect measure of the degree of inflammation in the colon. The decrease in activity could be attributed to a decrease in the specific activity of the enzyme or to a decrease in its trafficking, as was the case in chronically inflamed small intestinal enterocytes (Saha *et al.*, 2015). As such it remains important to elucidate in the future whether the exosomes induce changes in the ATPase trafficking between the membrane and intracellular stores, at 1 and 24 hrs. Examining the molecular pathway of this state is important to fully characterize how exosomes may contribute to IBD. Moreover, the effect of exosomes may extend far beyond this pump and its disruption. In fact, this may only be a fragment of the full effect of milk consumption on humans. Their enclosure of cytokines, miRs, cytoskeletal proteins as well as prolonged exposure to these components may in fact alter the phenotype of various tissues in a silent manner of growing permanence that could appear later in adulthood. The ethical considerations of conducting such prospective experiments on humans means that

we need to rely more on examining the effect of exosomes on isolated tissue and cells and potentially on animal studies. As such, it would be interesting to further corroborate our findings by conducting animal studies in a technique summarized previously (Markossian & Kreydiyyeh, 2005).

Milk has been considered beneficial because it includes all 3 main macronutrients (proteins, fats, and carbohydrates) as well as minerals. However, the findings mentioned in this study illustrate that perhaps milk is a double edged-sword due to its potential to exert unwarranted epigenetic regulation.

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