JURA

Journal of Undergraduate Research in Alberta

UNIVERSITY OF CALGARY

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Photo taken by Thomas Lijnse

Table of Contents

Volume 10: August 2022

Editors’ Remarks

We are pleased to present the August 2022 full edition of the Journal of Undergraduate Research in Alberta. COVID-19 continues to be a significant challenge to the research ecosystem in Alberta, and resulted in several barriers. Nonetheless, the resilience of the undergraduate students has proven vital to the advancement of our journal and we thank them for their contributions.

This edition features a wide array of research themes and methodologies that highlight the ingenuity of the next generation of researchers. We at JURA are currently encouraging further submissions of research from undergraduate students within the science realm. We understand that current circumstances are in constant flux and continue to make in person research difficult. However, students are encouraged to submit written portions from sources beyond extracurricular laboratory research such as their thesis dissertations and class research projects. Furthermore, students are encouraged to continue submitting review papers, as these are equally vital contributions. Moving forward, JURA will aim to return to a regular publishing schedule with two issues per year in the summer and winter.

JURA has been proud to be involved within the Albertan scientific community and create initiatives to stimulate more research from students. We hope that readers enjoy the August 2022 JURA edition and we encourage you to share this journal with your peers.

Sincerely,

Jura Editorial Team

Thomas Lijnse

Sara Hassanpour Tamrin

Neuroscience Behind the Success of Cannabinoids in Treating Paediatric Epilepsy

Amarpreet Sangha[1]

1 Health Science, Department of Biology, Mount Royal University, Calgary, AB

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Abstract— Paediatric epilepsies are especially concerning as they can be resistant to standard antiepileptic drugs and have high mortality rates. Moreover, young children are naturally at an increased risk of developing epilepsy. In early life, the depolarizing effect of γ-aminobutyric acid (GABA) renders immature neurons incapable of adequate inhibition which predisposes them to becoming hyperexcitable. Until recently, the search has been ongoing for a suitable therapy that would work more effectively in severe childhood epilepsies. Fortunately, cannabidiol (CBD), the non-psychoactive part of cannabis, has recently surfaced as a successful anticonvulsant in rare and severe paediatric epilepsy disorders. CBD circumvents normal endocannabinoid signalling in which endogenous cannabinoids are released by a postsynaptic neuron to silence the activity of a presynaptic neuron at a synapse. Instead, CBD exerts its anticonvulsant effects by acting on a number of non-cannabinoid targets, namely the transient receptor potential vanilloid 1 channel, G protein-coupled receptor 55, and equilibrative nucleoside transporter 1. In clinical trials, CBD lowered seizure frequency in paediatric patients who had Dravet syndrome (DS), Lennox-Gastaut syndrome (LGS), tuberous sclerosis complex (TSC), and other epilepsy syndromes. For the past few decades, the illegalization of cannabis has halted research into cannabinoids, and we are only now starting to study cannabis further despite knowing for centuries that it relieves a variety of conditions. The Food and Drug Administration and European Medicines Agency have recently approved CBD as an additive treatment option for DS, LGS, and TSC patients. Our society is becoming more open to the prospect of cannabis' therapeutic potential. This is particularly promising for children suffering from severe forms of epilepsy in which most of our current antiepileptic drugs are often ineffective.

I. INTRODUCTION

A. Charlotte's Web

Have you ever heard of a child being a regular cannabis user? What if taking cannabis was the only way to live? This was the case of Charlotte Figi who had her first seizure at three-months-old and was diagnosed with Dravet Syndrome (DS), a severe form of epilepsy [1]. By age five, Charlotte experienced over 300 seizures a week, approximately one every 30 minutes [1-3]. Her doctors gave up hope after seven antiepileptic drugs failed to alleviate her condition, prompting the Figi's to sign a do-not-resuscitate order [1, 2]. Charlotte's mother, Paige Figi, eventually contacted the Stanley brothers who were attempting to create a unique strain of cannabis high in non-psychoactive cannabidiol (CBD) [1]. After receiving permission from the state of Colorado, Paige began administering CBD to Charlotte in 2012 [1]. With low doses of CBD on a daily basis, Charlotte's quality of life vastly improved as she went from having 300 plus seizures a week to only 2-3 episodes per month [1]. The Stanley brothers named their CBD-abundant-plant "Charlotte's Web" to help other paediatric epileptic patients [1]. In 2012, Charlotte's story essentially paved the way for CBD as a therapeutic alternative in severe forms of paediatric epilepsy [1, 3]. Her case exemplifies the need to pursue greater research on cannabinoids in epilepsy.

B. Epidemiology of Epilepsy

Epilepsy is a chronic condition characterized by recurrent and unprovoked seizures [4]. It impacts more than 50 million individuals globally [4]. The paediatric population has a greater susceptibility of developing seizures, with a rate of about 1 out of 150 children [5, 6]. In addition, children are less responsive to standard antiepileptic medication [5]. Two severe epilepsy syndromes that arise in early life include DS, as seen in Charlotte Figi's case, and Lennox-Gastaut syndrome (LGS) [7]. DS and LGS are rare as they occur in 10% and 1-4% of childhood epilepsy cases, respectively [7, 8]. The incidence of mortality is 15-20% for DS and 3-7% for LGS [9, 10]. Additionally, a rare genetic disorder called tuberous sclerosis complex (TSC) occurs in 1 in 6000 births and involves severe epilepsy in 85% of the cases [11, 12].

C. History of Cannabinoid Research

Approximately 3000 years ago, the use of cannabis to lessen pain and anxiety was documented in India and Egypt [13]. William O′Shaughnessy brought cannabis from India to Europe in the early 19th century and thus introduced it to the Western world [1]. He subsequently showed that the plant helped alleviate various conditions, one of which was epilepsy [14, 15]. This was based on his account of a 40-day-old girl whose epileptic seizures were relieved after she was orally given cannabis [15]. In the late 19th century, Sir

William Gowers demonstrated that cannabis could successfully treat epilepsy when it was administered with the anticonvulsant bromide [14]. However, for most of the 20th century, reports of cannabis' psychoactive side-effects stigmatized its therapeutic use and led to legal restrictions on cannabis production [15-17].

In the 1980s-1990s, researchers discovered cannabinoid receptors in the central nervous system (CNS) that could be modulated by both endogenous and exogenous cannabinoids [18]. This triggered a renewed interest in the biological effects of exogenous cannabinoids, mainly CBD and Δ9-tetrahydrocannabinol (THC) [18]. While THC's applications are limited due to its psychoactive properties, CBD has greater clinical potential in anxiety, inflammation, depression, neurodegenerative diseases, and epilepsy [18, 19].

D. Current State of Cannabinoid Research in Paediatric Epilepsy

After Charlotte's remarkable improvement of symptoms in 2012, the following years saw several clinical trials being conducted on CBD supplementation in DS and LGS, which subsequently demonstrated CBD as an effective anticonvulsant [20, 21]. In June 2018, the Food and Drug Administration (FDA) approved a CBD-enriched formulation, called Epidiolex, for the treatment of DS and LGS [16, 22]. In September 2019, the European Medicines Agency (EMA) approved Epidiolex in conjunction with a common antiepileptic medication for DS and LGS [18]. Later, the FDA and EMA also approved Epidiolex for paediatric patients who suffer from TSC based on promising clinical trial evidence [23]. The World Health Organization [21] released a report indicating that CBD is generally tolerable with no major side-effects.

Since paediatric epilepsies can be resistant to standard antiepileptic drugs and treatments, utilizing an exogenous cannabinoid, namely CBD, represents a safe and promising technique to dampen seizure activity [5]. This review will outline endocannabinoid signalling and exogenous cannabinoids, the physiological factors that put the paediatric population at a higher risk of epilepsy, the mechanistic effects of CBD in epilepsy, CBD's effectiveness in recent clinical trials conducted on paediatric epileptic patients, and the safety considerations of utilizing CBD to treat paediatric epilepsy.

II. INTRODUCTION TO CANNABINOIDS

A. Introduction to the Endocannabinoid System

The endocannabinoid system (ECS) is an endogenous system that is a critical regulator of homeostasis [24, 25]. The abundance of cannabinoid receptors throughout various cell types and body regions enables the ECS to influence all physiological systems [26, 27]. As such, the ECS is involved in the regulation of numerous physiological processes, some of which include synaptic plasticity, neuronal development, sleep, memory, addiction, stress, pain, appetite, mood, reproduction, metabolism, digestion, and cardiovascular function [28]. A defining characteristic of the ECS is its retrograde signalling mechanism that helps regulate synaptic transmission [29] (Fig. 1). Periods of high neural activity trigger postsynaptic neurons to produce endogenous cannabinoids, also known as endocannabinoids (eCBs), that inhibit the activity of presynaptic neurons, thereby dampening incoming signals acting on postsynaptic neurons [26] (Fig. 1).

B. Components of the ECS

The ECS consists of cannabinoid receptors, eCBs, and enzymes involved in the synthesis and degradation of eCBs [28].

The two receptor types found in the ECS are cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2) receptors [30]. Although both receptor types are G protein-coupled receptors (GPCRs) and distributed throughout the body, they are selectively concentrated in certain regions [30]. The CB_1 receptor is the most abundantly expressed GPCR in the mammalian brain [31]. CB_1 and CB_2 receptors are differentially expressed in various body tissues [28, 31] (Table 1).

Table 1. Distribution of the CB_1 and CB_2 receptors of the ECS throughout different body regions. The three columns represent the presence of either CB_1, CB_2, or both CB_1 and CB_2 receptors in specific tissue types.

CB_1 Receptors	**CB_2 Receptors**	**CB_1 and CB_2 Receptors**
-Brian -Lungs -Gastrointestinal tract -Reproductive system -Muscle -Cardiovascular system	-Bones -Spleen -Skin	-Immune system -Liver -Pancreas -Bone marrow

The distribution of cannabinoid receptors also varies with cell type [19]. Whereas the CB_1 receptor is highly expressed on presynaptic terminals in the CNS and peripheral nervous system (PNS), CB_2 receptor expression is more limited to leukocytes and microglia [19, 32]. Additionally, CB_1 and CB_2 receptors are not the sole targets through which eCBs exert their actions, further emphasizing the complexity of the ECS [19]. Common non-cannabinoid receptor targets of eCBs include transient receptor potential channels, orphan receptors, peroxisome proliferator-activated receptors, serotonin receptors, as well as other metabotropic and ionotropic receptors [28, 33].

CB_1 and CB_2 receptors are activated by two endogenous agonists, namely anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG), which constitute the body's primary eCBs [19]. AEA and 2-AG are lipid metabolites, allowing them to cross cell membranes via simple diffusion [19, 34, 35]. Despite 2-AG being the most prevalent eCB in the ECS, it still has a lower binding affinity for CB_1 receptors than AEA [36]. eCBs function as retrograde messengers that bind to CB_1 receptors on presynaptic terminals to modulate synaptic activity, as well as regulate immune function by activating the CB_2 receptors on leukocytes [28, 37].

C. eCB Retrograde Signalling

Unlike typical neurotransmitters, eCBs are not pre-synthesized, but only produced upon demand [38] (Fig. 1). Intracellular calcium levels rise in the postsynaptic cell either due to calcium influx via the glutamate receptor or the opening of voltage-gated calcium channels

(VGCCs) when the postsynaptic cell depolarizes [19] (Fig. 1, step 1). Elevated postsynaptic intracellular calcium stimulates the biosynthetic enzymes NAPE-specific phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL) to synthesize AEA and 2-AG from their phospholipid precursors N-acylphosphatidylethanolamine (NAPE) and 1,2-diacylglycerol (DAG), respectively [28, 39] (Fig. 1, step 2).

eCBs cross the postsynaptic membrane and diffuse across the synaptic cleft to bind to CB_1 receptors on the presynaptic terminal [39] (Fig. 1, step 3). CB_1 receptors are coupled to an inhibitory G (Gi) protein [36] (Fig. 1). CB_1 receptor activation results in inhibition of VGCCs and opening of potassium channels, which lowers calcium influx and increases potassium efflux, respectively [39] (Fig. 1, steps 4a and 4b). The presynaptic membrane hyperpolarizes and calcium-dependent neurotransmitter exocytosis stops [19, 39] (Fig. 1). By diminishing presynaptic neurotransmitter release into the synaptic cleft, eCB stimulation of presynaptic neurons can dampen both excitatory and inhibitory inputs acting on the postsynaptic neuron [39].

Once steady-state neural activity has been restored, eCBs are taken up from the synaptic cleft to be degraded by enzymes within the cell to terminate the eCB signal [30, 31] (Fig. 1, step 5). AEA and 2-AG are hydrolyzed by fatty acid amide hydrolase (FAAH) in the postsynaptic neuron and monoacylglycerol lipase (MAGL) in the presynaptic neuron, respectively [40] (Fig. 1, steps 5a and b).

D. Phytocannabinoids

In addition to eCBs, there are also various exogenous cannabinoids, called phytocannabinoids (pCBs), that influence the ECS [18]. There are currently 113 pCBs identified from the *Cannabis sativa* plant [28]. When ingested or inhaled, pCBs exert their biological effects by interacting with cannabinoid receptors and/or eCB biosynthetic/degradative enzymes [28]. THC and CBD are the most abundant and heavily researched pCBs [18].

THC is the principal psychoactive pCB that binds to CB_1 and CB_2 receptors, with a particularly high affinity for CB_1 receptors [41]. At neuronal synapses, THC mimics the function of eCBs by activating Gi-linked CB_1 receptors on the presynaptic terminal to inhibit neurotransmitter release [26, 42] (Fig. 1). This in turn disrupts normal eCB retrograde signalling [26]. As a result, THC induces euphoria, slower reaction times, memory impairment, heightened sensory perception, and tachycardia [43, 44].

CBD is a non-psychoactive and non-intoxicating pCB as it does not induce euphoria [27, 40]. Compared to THC, CBD elicits anti-inflammatory and neuroprotective benefits despite its lower affinity for both CB_1 and CB_2 receptors [45]. CBD modulates the activity of non-cannabinoid receptors, possibly constituting the underlying mechanisms by which CBD alleviates pathological processes [18].

E. Pathology of the ECS

Disrupted ECS signalling has been implicated in various neurodegenerative disorders, including multiple sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, and epilepsy [19]. pCBs are currently being utilized in therapies for these conditions, particularly in the treatment of paediatric epilepsy [18, 20].

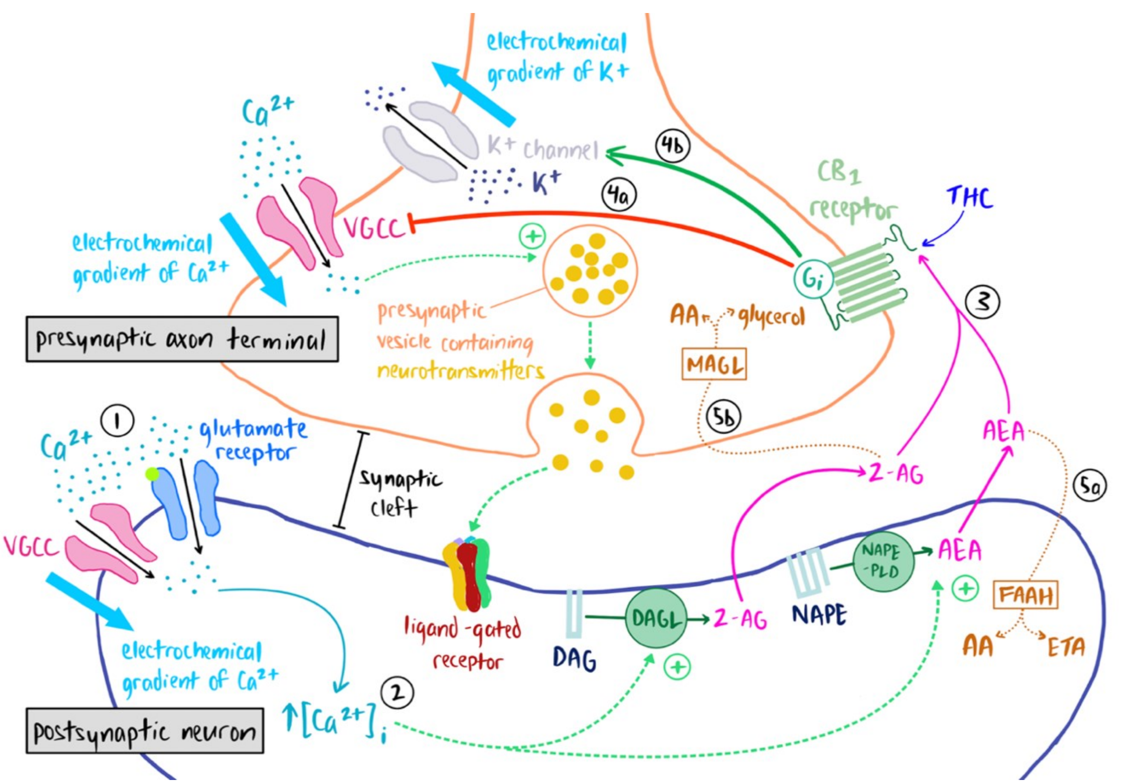

Fig. 1. ECS retrograde signalling mechanism. eCBs produced postsynaptically or exogenous THC can bind to the CB_1 receptor on the presynaptic terminal to inhibit neurotransmitter release. 2-AG, 2-arachidonoyl-glycerol; AA, arachidonic acid; AEA, anandamide; CB_1, cannabinoid 1; DAG, 1,2-diacylglycerol; DAGL, diacylglycerol lipase; ETA, ethanolamine; FAAH, fatty acid amide hydrolase; Gi, inhibitory G protein; MAGL, monoacylglycerol lipase; NAPE, N-acylphosphatidylethanolamine; NAPE-PLD, NAPE-specific phospholipase D; THC, Δ9-tetrahydrocannabinol; VGCC, voltage-gated calcium channel.

III. PHYSIOLOGY OF PAEDIATRIC EPILEPSY

A. Excitation-Inhibition Imbalance Hypothesis

One idea about the proximate cause of epilepsy is the excitation-inhibition imbalance hypothesis [46]. Disrupting the normal balance between excitation and inhibition in the CNS contributes to hyperexcitability in epilepsy [47] (Fig. 2). Neurons can become hyperexcitable with either an increase in excitatory inputs and/or a decrease in inhibitory inputs [46] (Fig. 2).

In the adult nervous system, the major neurotransmitters that elicit excitatory and inhibitory postsynaptic potentials are glutamate and γ-aminobutyric acid (GABA), respectively [48]. The cerebral cortex is composed of approximately 70-80% glutamatergic neurons and 16-30% GABAergic neurons [47, 49]. Excitatory glutamatergic neurons can synapse on inhibitory GABAergic neurons or other glutamatergic neurons [50]. GABAergic neurons can innervate glutamatergic neurons to inhibit their excitability [50]. A constant balance between excitation and inhibition ensures that a steady action potential frequency is maintained within a safe range, which is vital for normal neuronal signalling [47] (Fig. 2a).

When glutamatergic excitatory signalling increases, the net excitation outweighs the net inhibition, leading to a higher action potential frequency and a hyperexcitable state [47] (Fig. 2b). The flipside of this is a loss of inhibition that can occur with impaired GABA signalling that would normally dampen the excitability of glutamatergic neurons, again resulting in hyperexcitability [51] (Fig. 2b). Even if a relatively small number of neurons initially become hyperexcitable, this can spread to neurons located both adjacently and further away, severely affecting an entire neuronal network [50].

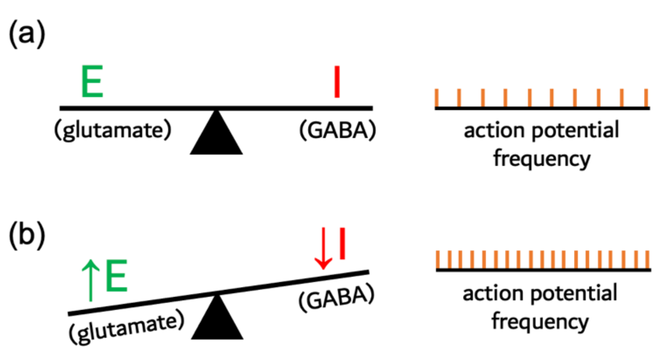

Fig. 2. An alteration of the excitation-inhibition balance can result in hyperexcitability. (a) Normal excitation-inhibition balance coordinated by glutamatergic and GABAergic neurons maintains a constant action potential frequency. (b) Enhanced activity of glutamatergic neurons and/or a loss of inhibition of GABAergic neurons results in net excitation outweighing net inhibition, which leads to hyperexcitability.

Hyperexcitability can result from abnormal functioning at multiple levels [46]. At the molecular level, genetic mutations can alter receptor structure, composition, or abundance [52]. For instance, gain of function mutations in glutamate receptors can lead to overexcitation, whereas mutations involving a loss of subunit(s) in $GABA_A$ receptors can impair hyperpolarization [5]. At the neuronal network level, injury resulting from seizures can induce axonal sprouting in which axons grow and form new connections between intact neurons [53]. With greater connections formed between glutamatergic neurons after seizures, neuronal excitability is increased [53].

B. Why are Children at a Greater Risk of Seizures?

Children are more susceptible to seizures than adults as GABAergic inhibition is naturally impacted in children, which increases their chances of developing a hyperexcitable state [5]. This is due to developmental changes in the ratio of sodium-potassium-chloride cotransporter 1 (NKCC1) to potassium-chloride cotransporter 2 (KCC2) [54].

NKCC1 and KCC2 are secondary active cotransporters that continually transport chloride into and out of the cell, respectively, in order to maintain an optimal intracellular chloride concentration [5, 54]. This contributes to a stable resting membrane potential along with the action of other pumps and transporters [5] (Fig. 3). The relative proportions of these cotransporters change throughout development, leading to GABA signalling being excitatory in early life and inhibitory later in life [5, 54, 55].

NKCC1 transports chloride into the neuron, resulting in an increased intracellular chloride concentration and an outward electrochemical chloride gradient [5] (Fig. 3a). Therefore, when GABA binds to the $GABA_A$ receptor, chloride effluxes, resulting in depolarization and increased excitability of the neuron [5] (Fig. 3a). KCC2, on the other hand, maintains a low intracellular chloride concentration and inward electrochemical chloride gradient by transporting chloride into the extracellular fluid [5] (Fig. 3b). With more KCC2, $GABA_A$ receptor activation causes a chloride influx, consequently hyperpolarizing the neuron and inhibiting its activity [5] (Fig. 3b).

Immature neurons derived from postnatal human and rat brain tissue express more NKCC1 transporters than KCC2 [51] (Fig. 3a). Thus, early in development, GABA signalling is excitatory [54, 55]. However, later in development, the relative abundance of NKCC1 to KCC2 reverses, and neurons express more KCC2, rendering GABA signalling inhibitory [54, 55] (Fig. 3b). The capacity of immature neurons to inadequately silence electrical activity due to excitatory GABA signalling tips the balance towards a hyperexcitable state, ultimately predisposing young children to developing epileptic conditions [54, 55]. Standard treatment protocols, such as benzodiazepines and barbiturates, that target $GABA_A$ receptors have reduced effectiveness in children compared to adults [5]. Fortunately, researchers are investigating pCBs as possible anticonvulsants for childhood epilepsies [18, 20].

IV. PHYTOCANNABINOIDS IN PAEDIATRIC EPILEPSY

A. Why Would pCBs be Useful in Paediatric Epilepsy?

pCBs could be effective therapeutic options for paediatric epilepsy as they can modulate the ECS and non-cannabinoid targets to lessen hyperexcitability [16, 18]. In the case of Charlotte Figi who experienced 300 seizures a week, although antiepileptic drugs failed, a pCB helped her become essentially seizure-free [3]. Of the two main types of pCBs, both of which have anticonvulsant effects, THC seems to be effective at lower doses, but not at higher doses [20, 56]. CBD has emerged as a successful anticonvulsant based on its ability to interact with non-cannabinoid receptors without causing psychoactive side-effects [20, 57].

B. CBD's Anticonvulsant Mechanistic Effects in Epilepsy

CBD exhibits greater binding affinity for several non-cannabinoid targets, including the transient receptor potential vanilloid 1 (TRPV1) channel, G protein-coupled receptor 55 (GPR55), equilibrative nucleoside transporter 1 (ENT1), serotonin 1A receptor, voltage-gated sodium channels, and T- and L-type VGCCs [20, 57]. There is greater research supporting CBD's actions on TRPV1 channels, GPR55, and ENT1 in the treatment of epilepsy [57, 58] (Fig. 4).

In a recent study utilizing a seizure-induced mouse model, Gray et al. [59] treated wild-type mice and TRPV1-knockout mice with CBD. The TRPV1-knockout mice displayed impaired responses to CBD, thereby indicating that TRPV1 is a critical target of CBD's anticonvulsant action [59]. TRPV1 channels are ligand-gated cation channels expressed in PNS and CNS neurons [60]. When activated by vanilloids, such as capsaicin, these channels permit the influx of calcium and sodium, enhancing neuronal depolarization [60]. TRPV1 expression is elevated in epilepsy [58, 59]. CBD initially activates TRPV1, but then quickly desensitizes it, thereby limiting calcium influx [58, 61] (Fig. 4). This reduces presynaptic glutamate exocytosis and neuronal hyperexcitability [20, 58] (Fig. 4).

GPR55 is a GPCR located on excitatory presynaptic terminals [20] (Fig. 4). GPR55 regulates excitability by coupling to a Gq pathway to increase calcium release from the ER when stimulated by its primary endogenous ligand, l-α-lysophosphatidylinositol (LPI) [20, 58] (Fig. 4). By functioning as an antagonist of GPR55, CBD attaches to LPI's ligand site to prevent LPI from binding to GPR55 [20, 58] (Fig. 4). CBD inhibits the activation of GPR55, which leads to calcium sequestration in the ER and minimized presynaptic glutamate release [20, 58] (Fig. 4).

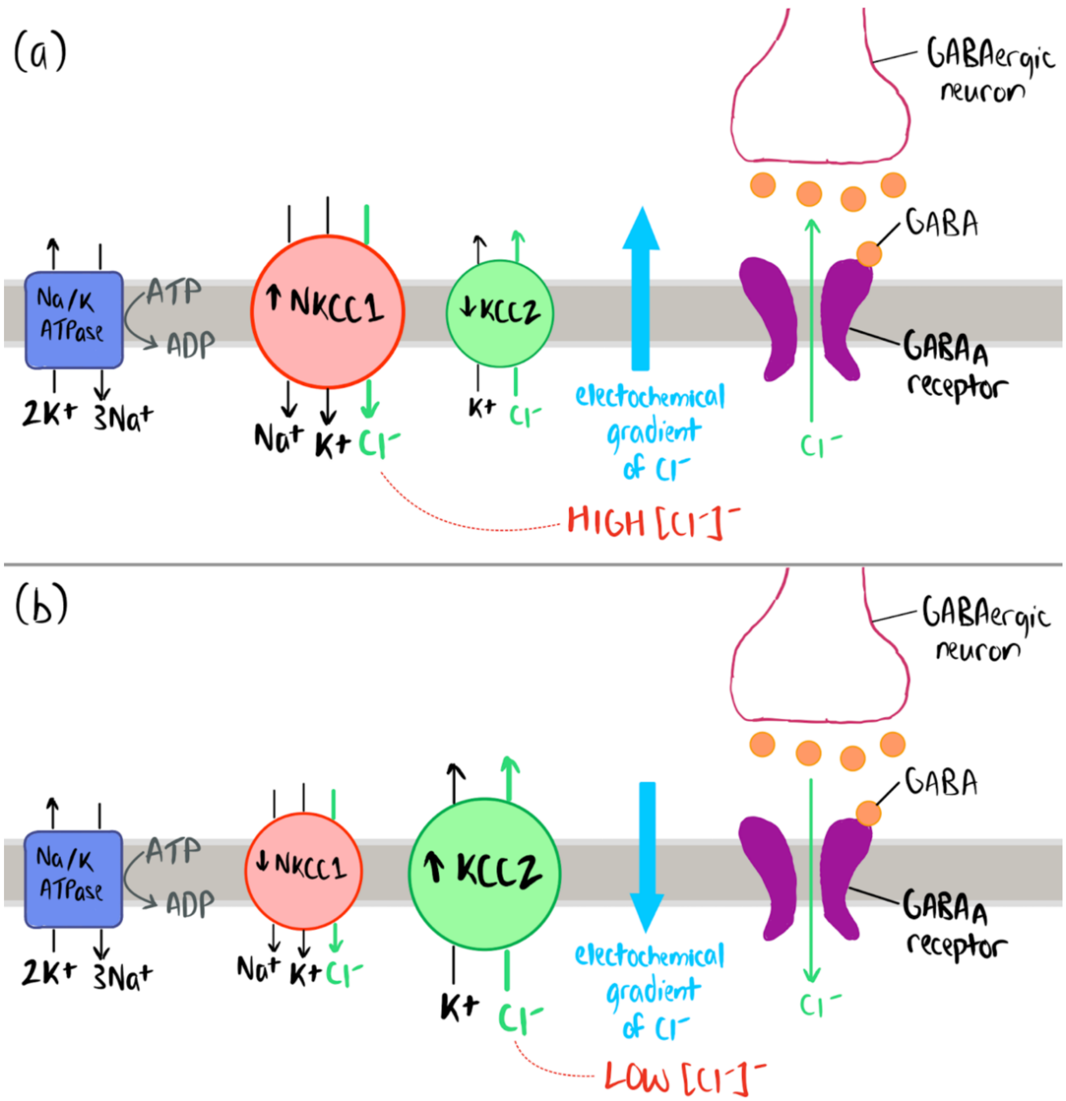

Fig. 3. Chloride homeostasis in early life versus later life. (a) In early life, greater NKCC1 increases intracellular chloride, resulting in depolarization when GABA binds to the $GABA_A$ receptor. (b) In later life, more KCC2 keeps the intracellular chloride low, leading to hyperpolarization upon $GABA_A$ receptor activation. ADP, adenosine diphosphate; ATP, adenosine triphosphate; KCC2, potassium-chloride cotransporter 2; NKCC1, sodium-potassium-chloride cotransporter 1.

During seizures, adenosine is released into the extracellular environment to help dampen hyperexcitability [62, 63]. Extracellular adenosine can activate the A_1 receptor located on presynaptic terminals to initiate an intracellular signalling cascade, which lowers glutamate exocytosis and neuronal excitability [20] (Fig. 4). CBD enhances adenosine's anticonvulsant effect by binding to neuronal and microglial ENT1 to prevent adenosine reuptake, resulting in an increased extracellular adenosine concentration [20, 58] (Fig. 4).

C. Clinical Trial Evidence of CBD Supplementation in Paediatric Epilepsy

In 2017-2018, randomized, double-blinded, and placebo-controlled clinical trials conducted on DS and LGS patients demonstrated purified CBD to be an effective anticonvulsant [64-68]. CBD supplementation in 61 DS patients, who were 2-18 years old, reduced seizure frequency by more than 50% [64]. 5% of the DS patients became seizure-free [64]. In two trials involving LGS paediatric and adult patients, CBD decreased the occurrence of seizures by approximately 40% [65, 66]. In the three aforementioned trials, researchers administered CBD to patients in conjunction with the patient's prescribed antiepileptic medication [64-66]. The positive outcomes of these studies influenced the FDA to approve purified CBD for treating DS and LGS in 2018 [16, 20]. A more recent 2020 study of adjunctive CBD supplementation in 133 DS paediatric patients reported a 45-48% reduction in seizure frequency, which supports previous findings [69].

In the case of TSC, a 2016 non-randomized study showed that CBD lowered seizure frequency in 9 of 18 patients who were 2-31 years old [12]. More recently, a randomized clinical trial revealed a 54-68% reduction of seizures in TSC paediatric and adult patients who were taking CBD [70].

CBD's effectiveness in other paediatric epilepsy syndromes has been reported by a 2018 study [71]. Researchers analyzed previous data of 55 patients who had varying epilepsy syndromes, including CDKL5 deficiency disorder, Aicardi syndrome, Doose syndrome, and Dup15q syndrome [71]. CBD administration alleviated seizure frequency in each condition [71].

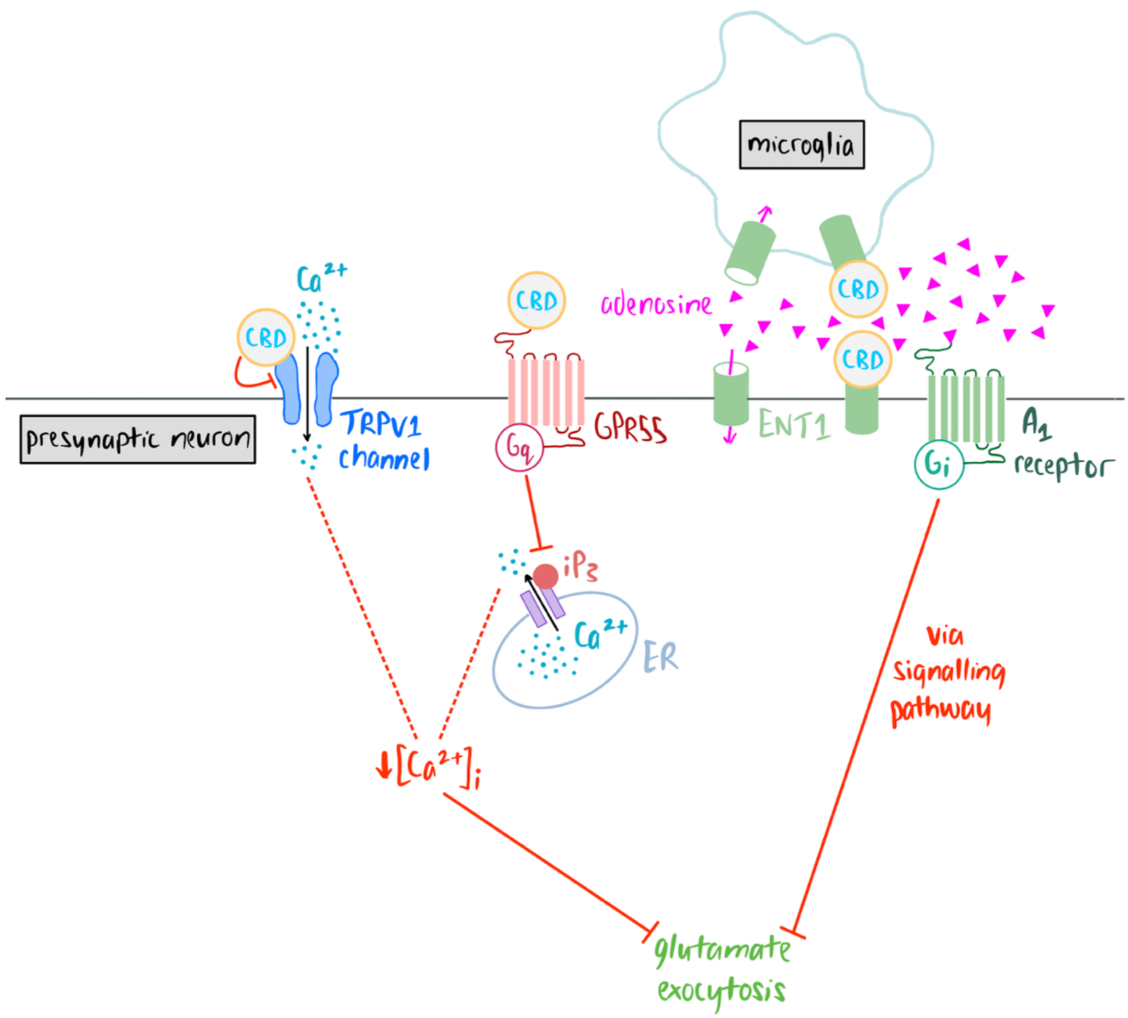

Fig. 4. CBD's anticonvulsant actions mediated by desensitization of TRPV1, antagonism of GPR55, and blocking the reuptake of adenosine by neuronal and microglial ENT1. CBD, cannabidiol; GRP55, G protein-coupled receptor 55; iP3, inositol 1,4,5-trisphosphate; ENT1, equilibrative nucleoside transporter 1; ER, endoplasmic reticulum; TRPV1, transient receptor potential vanilloid 1.

D. Limitations and Safety Considerations of pCB Use in Paediatric Epilepsy

Where most antiepileptic drugs fail, CBD can be effective at dampening hyperexcitability and seizure frequency in difficult-to-treat epilepsy syndromes [64-68]. However, it is vital to ensure that CBD is a safe therapeutic alternative, especially in the case of paediatric patients.

Firstly, CBD does not work for all types of seizures [72-74]. It is more effective against certain forms of seizures, but not other forms [73, 74]. Currently, CBD has not been approved as a treatment option for most epileptic disorders [72, 73]. It has only been approved for treating paediatric patients with DS, LGS, and TSC, which are rare conditions [73, 74]. In other epileptic conditions, treating a patient with CBD could further aggravate the patient's health [75].

The chemical composition of the CBD formulation is also critical to consider in the treatment of paediatric epilepsy. CBD is the only active ingredient in the pharmaceutical formulation called Epidiolex which has been approved for DS, LGS, and TSC paediatric patients [76, 77]. Commercial CBD products are not regulated by governmental agencies, such as the FDA [78, 79]. As such, many commercial CBD products are mislabeled and contain significant amounts of THC that are enough to cause severe effects in children [78, 80]. Treating paediatric epileptic patients with unregulated CBD products may not be safe [78, 80]. It was recently reported that pharmaceutical CBD and commercial CBD resulted in a 39% lower and 70% higher seizure frequency in paediatric epileptic patients, respectively [81]. This is likely because of the THC within commercial CBD products which can elicit seizures at higher doses [56, 79, 81, 82].

Despite its psychoactive and seizure-inducing effects, another reason why THC is not recommended for paediatric patients is because THC can adversely impact brain development [83, 84]. In early childhood, significant brain development occurs in which one million synapses are formed each second [85]. During the first few years of life, THC exposure can lead to abnormal brain structure and function, as well as unfavourable neurodevelopmental outcomes [83, 84]. THC activates CB1 receptors in a nonspecific manner and interferes with the ECS, which can prevent synaptic connections from forming, especially in the prefrontal cortex and hippocampus [86]. This can impair cognitive functioning and emotional reactivity [86].

In comparison to THC, the evidence of CBD's effects on brain development is still fairly limited [87]. A study conducted over a period of three months showed that cognitive performance in the areas of learning and memory did not decline in paediatric epileptic patients who were given pharmaceutical CBD [88]. Another study demonstrated that over a longer period of one year, continuous pharmaceutical CBD use in paediatric epileptic patients did not adversely impact their cognitive performance, specifically in the areas of memory and attention [89]. As well, a group of researchers analyzed changes in brain structure in response to CBD administration [90]. They found that found that grey matter volume and cortical thickness in paediatric epileptic patients did not significantly change over a 12-week period [90].

While CBD may not negatively affect brain development and cognition as THC does, there is evidence that CBD can impact other body systems, resulting in unhealthy side-effects [64-66, 91, 92]. In clinical trials involving child epileptic patients, the most common side-effects were drowsiness, fatigue, diarrhea, vomiting, and diminished appetite [64-66, 91, 92]. These side-effects were short-term and less severe [64-66, 91, 92]. In contrast, a low proportion of patients experienced more serious side-effects, including seizures and weight loss [64-66, 91, 92].

Studies have also shown that CBD has significant interactions with a number of antiepileptic medications, leading to altered serum levels of these drugs and their metabolites [93]. This may impact the effectiveness of antiepileptic medications taken concurrently with CBD, which increases the risk of side-effects [93, 94]. In the liver, cytochrome P450 enzymes are responsible for metabolizing drugs, including CBD and antiepileptic medications [93]. CBD can inhibit cytochrome P450 enzymes, which is believed to be one way that CBD can influence the metabolism of antiepileptic medications [94]. CBD can also impact the absorption, distribution, and clearance of antiepileptic medications [94].

Most of the studies testing the efficacy and safety of CBD in child epileptic patients are relatively short in duration, typically only lasting a few months [64-66, 91, 92]. As such, the potential effects that may occur after the prolonged use of CBD in these patients are still not well known [91, 92, 95]. Compared to most studies, a few studies that occurred over a slightly longer period have reported that CBD is generally well tolerable for paediatric epileptic patients [89, 95-97]. In one study, CBD supplementation over one year did not adversely impact cognitive performance in the paediatric epileptic patients [89]. In another study, paediatric epileptic patients who were taking CBD for two years mainly experienced mild side-effects, with the most common being diarrhea and drowsiness [97].

A few strategies could be employed to acquire more evidence on the potential harmful effects of CBD in paediatric epileptic patients. Researchers could utilize animal models to evaluate the safety of CBD during the first few years of life [98]. Since animal models are easier to manipulate, they would be useful to study CBD's interactions with other drugs and the specific mechanisms by which CBD may impact body systems [93, 94, 99]. Of course, the main drawback with animal models is that the results obtained may not be directly applicable to humans [100]. As with most other studies, in order to investigate the safety of CBD in young children, the best and safest approach would be to check if paediatric epileptic patients who are admitted into clinical trials experience adverse effects [64-66, 91, 92]. Additionally, follow-up with patients should be done for a longer duration after the study period is over to allow researchers to collect more data on the long-term effects of CBD [89, 92, 96, 97, 101].

CBD use in the paediatric population does carry risks [95]. CBD is not an effective anticonvulsant in most epileptic disorders [72, 73]. Commercial CBD products frequently contain THC that can increase seizure frequency in epileptic patients [56, 79, 81, 82]. CBD is also associated with side-effects in paediatric epileptic patients and can interact with antiepileptic medications, potentially altering their effectiveness [64-66, 91-94]. Therefore, CBD should only be used as a treatment option for children with certain paediatric epilepsies, such as DS, LGS, and TSC, who are not responding to standard antiepileptic drugs [95]. If a child suffers from seizures, it is critical that his/her family consults a doctor to receive appropriate treatment [95].

V. CONCLUSION

Young children are at an increased risk of developing epilepsy due to GABA's excitatory role in the immature brain [5]. Paediatric epilepsy syndromes can be seriously debilitating, and common antiepileptic medications that doctors prescribe can fail to reduce seizure frequency in paediatric epilepsy patients [5]. In recent years, CBD has emerged as an effective and tolerable anticonvulsant based on recent clinical trials and anecdotal cases [1, 64-71]. CBD has been approved in the United States and Europe for DS, LGS, and TSC patients [20, 23]. With greater research, therapeutic approval can expand to other nations as well, which can help paediatric epilepsy patients worldwide [20]. This tremendous change was pioneered by young Charlotte Figi, whose story taught our society to become more accepting of cannabis' therapeutic potential, especially as a life-altering treatment for children suffering from epilepsy [1, 20].

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Isolation of Exosomes Derived from Mesenchymal Stem Cells Using Ultracentrifugation

Heather Booth[1,2], Sara Hassanpour Tamrin[1,2], Jolene Phelps[1,2], Arindom Sen[1,2*]

1 Pharmaceutical Production Research Facility, Department of Chemical and Petroleum Engineering, Schulich School of Engineering, University of Calgary, Calgary, AB, Canada

2 Department of Biomedical Engineering, University of Calgary, Calgary, AB, Canada

**Email: asen@ucalgary.ca*

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Abstract— Exosomes, which are membrane-enclosed extracellular vesicles (30 - 200 nm) secreted by cells, may be responsible for the therapeutic benefits associated with the implantation of mesenchymal stem cells. Currently, ultracentrifugation is commonly used to isolate extracellular vesicles and characterize the exosome fraction. In some studies, a second ultracentrifugation step for "washing" is included in the isolation process to increase purity by removing contaminating proteins. This study attempted to investigate the validity of using an additional step to increase the purity of samples as well as examine the effect on recovery by comparing a single ultracentrifugation step to when a second ultracentrifugation step is added to the isolation procedure. The results obtained indicated that the additional step increased the purity of the isolated exosomes by removing contaminating proteins, however, the total yield was lower. The reduced yield is likely the result of misplacing exosomes during the extra step because it is difficult to ensure all exosomes are pelleted and re-suspended after ultracentrifugation. Therefore, the inclusion of an additional ultracentrifugation step in research is dependent on the application. For example, higher purity is a priority in therapeutic applications, while a higher yield is often preferred in diagnostic applications.

I. INTRODUCTION

Mesenchymal stem cells (MSCs) are unspecialized, multipotent stromal cells that can be isolated from adult tissues such as bone marrow and fat [1, 2] making them a promising option for immunomodulation and regeneration [2]. MSCs were first discovered in bone marrow by Friedenstein in the 1970s [3] and have become the most extensively used cell in the field of regenerative medicine [4], primarily because they show potential as a therapeutic tool to treat and increase the regenerative capacity of damaged tissue that can result from degenerative diseases [4, 5]. MSCs have been widely tested as nanotherapeutics, including for the treatment of diabetes [6], stroke [7] and wound healing [4]. The potential associated with MSCs is the result of their ability to migrate to injured sites (homing ability) and differentiate into the required local components [5]. However, clinical applications of MSCs have been limited by additional challenges, including poor-quality control and inconsistent cell characteristics, such as immunocompatibility, stability, heterogeneity, differentiation, and migratory capacity [3]. These challenges have led to increased interest in the use of extracellular vesicles for cell-free therapy as they offer the potential for higher sterility, lower immune response and improved transport and storage [1]. Recent studies suggest components secreted by MSCs may be responsible for their perceived therapeutic effects, specifically exosomes (EXs) [1].

Cells secrete biological nanoparticles of various sizes and classifications which are collectively referred to as extracellular vesicles (EVs) [8]. EV populations include apoptotic bodies, microvesicles and EXs [9]. EXs have become a prominent focus and can be distinguished from other vesicles based on their biogenesis, content, function, surface markers and size [10]. EXs are of endocytic origin, known to be produced by all mammalian cells and present in nearly every bodily fluid [10]. These membrane-bound vesicles are spherical and have a diameter that ranges between 30 and 200 nm [1]. EXs were first discovered in the 1980s by Pan and Johnstone and were previously thought to transport waste from the cell [10]. However, recent studies have demonstrated that EXs underpin a critical form of communication between cells and carry contents such as proteins, messenger RNA and microRNA, that elicit various biological effects [10, 11, 12].

EXs secreted by MSCs (MSC-EXs) have gained traction as they may be responsible for the therapeutic benefits associated with the implantation of MSCs [13, 14]. As of July 2022, there are 19 clinical trials reported on the U.S. National Library of Medicine database related to MSC-EXs [15]. These studies are using MSC-EXs to treat conditions such as COVID-19, acute respiratory distress syndrome, multiple organ failure, Alzheimer's, and diabetes mellitus Type I, among others. The authors encourage the reader to refer to a concise review conducted by Phinney and Pittenger [16] which summarizes translational studies employing MSC-derived microvesicles and EXs. In this summary, MSC-EXs are used in models such as stroke, fibrosis, traumatic brain injury, amyotrophic lateral sclerosis, and breast cancer [16].

The administration of MSC-EXs could also eliminate the hurdles currently hindering the formulation of MSC-based cell therapies [3, 17]. As a result, cell-free therapies are being further explored and includes the utilization of EVs, such as EXs. Benefits of cell-free therapies involving MSC-EXs includes the small size of the EXs which allows them to circulate easily and elicit therapeutic effects at more distant locations compared to MSCs [16, 18]. Additionally, MSC-EXs are promising carriers for drugs [19] and could offer a higher or even scalable dosage as a result of their ability to circulate, whereas the dose of infused MSCs quickly diminishes after transplantation and their larger size limits circulation [16, 18]. However, the potency of EXs is an evolving area of research [16]. Finally, using MSC-EXs is considered safer as this method avoids the transfer of mutated or damaged cells into patients [16] and MSC-EXs are considered non-immunogenic [19] and cannot self-replicate, therefore they do not have endogenous tumor-formation potential [18].

A significant challenge associated with EXs is the development of standardized isolation methods and validated controls. To assist in the proper standardization of methodologies, the International Society for Extracellular Vesicles (ISEV) released two resources: the Minimal Information for Studies of EVs Checklist [20], and the EV-TRACK knowledgebase (https://www.evtrack.org/index.php). Both resources aim to enhance the transparency and interpretation of experimental results obtained from EV-related studies.

The checklist summarizes considerations made by the ISEV for nomenclature, collection and pre-processing, EV separation and concentration, EV characterization, functional studies, and reporting [20]. The checklist includes items which are classified as either "mandatory", "mandatory if applicable" or "encouraged" to assist in the preparation of standardized and reproducible documentation for EV related studies [20].

The EV-TRACK knowledgebase is a tool that provides an overview of the 9 experimental parameters contained in the EV-METRIC, which was developed to improve the interpretation and reproducibility of EV studies [21]. An EV-TRACK summary add-on was introduced in 2019 to extend the practices of transparent reporting as well as the utilization of the EV-METRIC to other databases in the field of EVs such as EVpedia, EVmiRNA and ExoRbase [21].

Currently ultracentrifugation (UC) is the most common method to isolate EXs from cell culture or bodily fluids [10, 12] and has been reported as the method used in 81% of EX studies worldwide [22]. However, UC is time-consuming, tedious, and often yields low purity of EXs [8]. UC at 100,000 *g* or higher for 2 h can be used on the supernatant from cell cultures as well as bodily fluids to isolate EVs after differential centrifugation has been employed to remove living cells, dead cells, cellular debris, and other components with densities higher than EVs [8]. The components isolated by UC are referred to as EVs because various vesicles and impurities (contaminating proteins) are isolated during the process due to similarities in size and density. However, subsequent characterization of the EVs can determine the size distribution, purity, and yield which can be correlated to EXs based on our knowledge of EX characteristics. Differences in protocols including centrifugal force, duration and rotor type have been observed for differential centrifugation and UC techniques, which can be linked to inconsistent results for recovery rate and purity after the isolation of EVs [23]. Characterization techniques also greatly influence the recovery rate and purity of EVs; however, single particle interferometric reflectance imaging allows for analysis on a single vesicle basis which promotes the identification of EX specific characteristics such as size and surface markers.

To examine the various differential centrifugation and UC techniques currently being employed to isolate MSC-EXs, a literature review was conducted on PubMed and a summary of the protocols reviewed is provided in Table 1. Some isolation protocols utilize filtration or ultrafiltration (characterized by a 10 to 100 kDA molecular weight cutoff [10]) steps alongside differential centrifugation and UC techniques to help increase the purity of the EVs isolated [8]. However, this literature review focused on UC protocols for MSC-EXs that did not incorporate filtration or ultrafiltration steps. Isolation through differential centrifugation and UC typically occurs in 3 stages, based on the level of centrifugal force applied, which can each incorporate multiple steps: a low stage (A) that removes living and dead cells as well as cellular debris, a mid-stage (B) that removes large structures with densities higher than EVs, such as apoptotic bodies, and a high stage (C) which is the UC stage that pellets EVs. In Table 1, the stages are outlined for the relevant papers found.

From the literature review, it was noted that there were differences in the centrifugal force, duration and rotor as suggested in literature [23]. Additionally, the mid-stage was not included in paper [28] of Table 1. The mid-stage is designed to remove apoptotic bodies and remaining contaminants that have higher densities than EVs (after cells and cellular debris have been separated). By not including this step these contaminants could appear in the EV pellet isolated and interfere with EX specific results. In [28], this step may have been skipped because isolation was focused on EVs rather than on EXs specifically.

These findings primarily reflect the need for further standardization of UC protocols for EV isolation. A more prominent difference was the incorporation of an additional UC step. This additional step, often called the "washing" step, includes re-suspending the EV pellet in phosphate buffer saline (PBS) and conducting another UC step [9, 31, 32, 33, 34]. Given the need to standardize isolation techniques, assessing the relevance of a second UC step is critical to determine the appropriate protocols for EV isolation. Therefore, comparing characteristics such as the purity, yield, and size distribution of the isolated EV pellet, including the EXs contained within it, is critical to understand whether a second UC step is necessary.

II. MATERIALS AND METHODS

A. Materials and reagents

Dulbecco's PBS (10X), TrypLE™ Express Enzyme (1X) and a Pierce™ BCA Protein Assay Kit were purchased from Thermo Fisher Scientific, Canada. Gelatin from porcine skin was purchased from Sigma-Aldrich®. Serum-free PPRF-msc6 medium, was developed in the Pharmaceutical Production Research Facility (PPRF), as outlined by Jung et al. [35] and was utilized for cell culture by preparing it on site.

B. Cell Culture

MSCs were isolated enzymatically from human abdominal subcutaneous adipose tissue as described by Jung et al. [36] with ethical approval from the University of Calgary Health Research

Table 1. UC stages for MSC-EX isolation from PubMed

Stages			Ref.
A	**B**	**C**	
Single UC protocol			
2000 *g* for 30 min	10,000 *g* for 30 min	100,000 *g* for 4 h	[24]
200 *g* 2000 *g*	10,000 *g*	110,000 *g* for 2 h	[25]
2000 *g* for 10 min	10,000 *g* for 30 min	110,000 *g* for 90 min	[26]
300 *g* for 10 min 2000 *g* for 10 min	10,000 *g* for 30 min	100,000 *g* for 70 min	[2]
2000 *g* for 20 min	-	100,000 *g* for 1 h	[28]
2500 *g* 15 min	16,500 *g* for 20 min	110,000 *g* for 70 min	[29]
300 *g* for 10 min	16,500 *g* for 20 min	120,000 *g* for 2.5 h	[30]
Double UC protocol			
300 *g* for 10 min 2000 *g* for 10 min	10,000 *g* for 30 min	100,000 *g* for 70 min Repeat in PBS	[9]
300 *g* for 10 min 2000 *g* for 10 min	10,000 *g* for 30 min	100,000 *g* for 70 min Repeat in PBS	[31]
300 *g* for 10 min	10,000 *g* for 10 min	100,000 *g* for 70 min Repeat in PBS	[32]
800 *g* for 10 min	10,000 *g* for 30 min	110,000 *g* for 2 h Repeat in PBS	[33]
3000 rpm for 5 min 3000 rpm for 15 min	7500 *g* for 40 min	100,000 *g* for 1.5 h Repeat in PBS	[34]

Note: PBS = Phosphate Buffer Saline

Ethics Board (ID: REB15-1005, 1 donor: female, age 24). The cells were cultured to isolate the EVs present in the culture medium (supernatant). Standard cell culture protocols, developed in PPRF, were utilized. The cells were inoculated in plastic T-75 tissue culture flasks (75 cm^2) at a cell density of 5000 cells/cm^2 using PPRF-msc6. The tissue flasks were coated with sterile 0.1% gelatin solution prior to inoculation. The inoculated flasks were incubated at 37°C, 5% CO_2 and 100% humidity. Passaging was completed after approximately 72 hours (3 days). Morphology was assessed and images were taken daily using a Axiovert 200M phase contrast inverted microscope. An example of the standard morphology for MSCs on day 3 is provided in Fig. 1. During passaging, TrypLE™ was utilized to detach the cells and a hemocytometer and trypan blue were used to perform cell counting according to PPRF cell counting protocols. The culture supernatant and the PBS used to wash the cells (4 mL; twice) was collected during passaging and stored at 4°C.

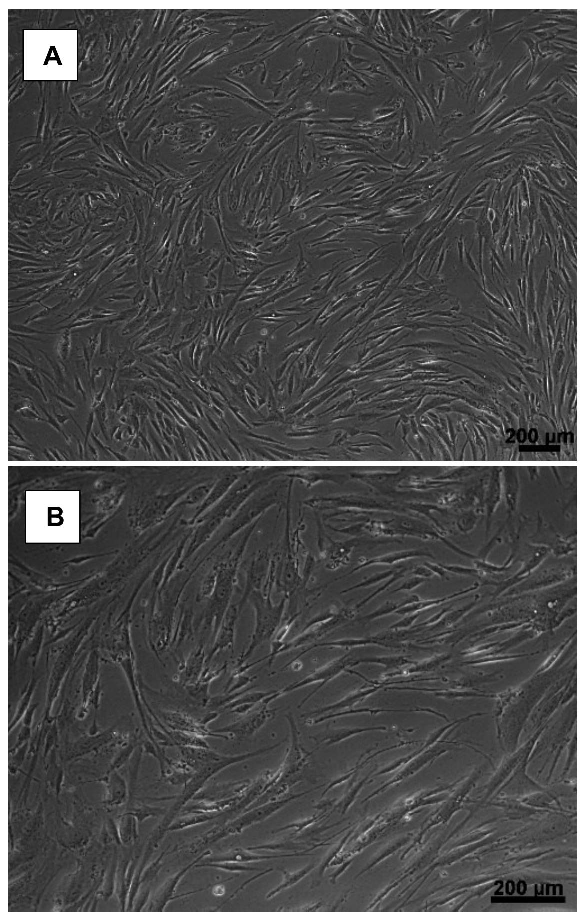

Fig. 1. Standard MSC morphology at (A) 5x and (B) 10x magnification on day 3 prior to passaging.

C. Isolation of EVs

Differential centrifugation followed by UC was employed to isolate the EVs from the collected supernatant. A visual depiction of the steps described in this section is provided in Fig. 2. First the supernatant was centrifuged at 4°C and 2000 *g* for 10 minutes (Sorvall ST 40R Centrifuge) to remove dead cells. The supernatant was removed and stored at -80°C while the pellet was discarded. When further processing could be conducted, the supernatant was thawed and processed at 10,000 *g* then 105,000 *g* on the same day to isolate the EV pellet. 10,000 *g* was conducted for 30 minutes at 4°C (Sorvall RC-5B Plus Refrigerated Centrifuge) using an HS-4 rotor to remove residual cellular components and large particles, such as apoptotic bodies. The supernatant was removed and divided into designated 25 mL polycarbonate UC tubes before PBS was added to dilute the supernatant to half the concentration (1:1 ratio of supernatant to PBS). The tubes were loaded and ultracentrifuged at 105,000 g for 2 h at 4°C (Beckman Coulter OptimaTM L-100K Ultracentrifuge) using a 70 Ti rotor after which the supernatant was removed and discarded while the EV pellet was kept. For the sample with the additional washing step, the EV pellet was re-suspended in PBS and ultracentrifuged again at 105,000 g for 2 h at 4°C. After UC (with or without the washing step) the pellet was re-suspended in 200 μL of PBS (50x concentration) and stored at -80°C until characterization could be conducted.

D. Characterization of EXs

For characterization, 3 samples were collected and analyzed during passage 6 (P6) including the supernatant collected after the 10,000 *g* centrifugation step (denoted CM′′) and the two EV pellet samples collected from 1 and 2 stages of UC (denoted 1 x UC and 2 x UC respectively). The supernatant was used as a reference for EX content to determine the approximate recovery of EXs during the isolation process.

Protein Content

Purity of the samples was observed using a bicinchoninic acid (BCA) protein assay which determines protein content. The Thermo Fisher Scientific Pierce™ BCA Protein Assay Kit was used according to the user guide for a microwell plate. For each sample, 3 wells were filled with 20 μL of the sample along with 200 μL of working reagent (50:1 of reagent A and B from the kit). The plate was then incubated at 37°C for 30 min before an absorbance reading was taken at 562 nm using a SpectraMax iD3 plate reader. The absorbance results were converted to mg/mL using a standard curve produced by an albumin standard serial dilution created using the albumin standard provided in the kit. Pure PBS (1X) was used as a control and its absorbance was applied to the sample absorbance readings to normalize them before analysis.

Single Particle Interferometric Reflectance Imaging

Yield of EXs present in the samples was measured using the ExoView R100 (NanoView Biosciences, Boston, MA) which can determine size distribution, particle count and biomarker colocalization [37] of the samples. The expression of EX specific surface markers on a single vesicle basis could be performed using a multiplexed microarray chip and interferometric imaging. The number of particles expressing EX specific surface markers was determined [1]. These markers were tetraspanins CD63, CD81 and CD9 [1]. The results allowed for reasonable distinction and classification of the EXs from other EVs present within the samples examined. Manufacturer protocols were followed which involved diluting the samples with the incubation buffer provided by the manufacturer to standardize concentration (100 μL of supernatant per 1 mL of buffer and 2 μL of the 50x concentration EV pellet per 1 mL of buffer) before incubating 50 μL of the diluted samples on antibody coated chips overnight (16 h) at room temperature. The chips were then washed, labelled with fluorescent antibodies (CD63, CD81 and CD9) and scanned using the ExoView software, which developed relevant data and visuals based on digital counting of individual EXs immune-captured on the chips.

Dynamic Light Scattering (DLS)

DLS utilizes Brownian motion of the particles in solution and a monochromatic, coherent laser beam which passes through the solution [38]. When a particle interferes with the beam, the light is dispersed and the intensity of the scattered light as a function of time can be recorded. A Zetasizer Nano ZS (Malvern Instruments Ltd., Cambridge, UK) was used to determine the average diameter and polydispersity index of an EV sample isolated using 1 x UC.

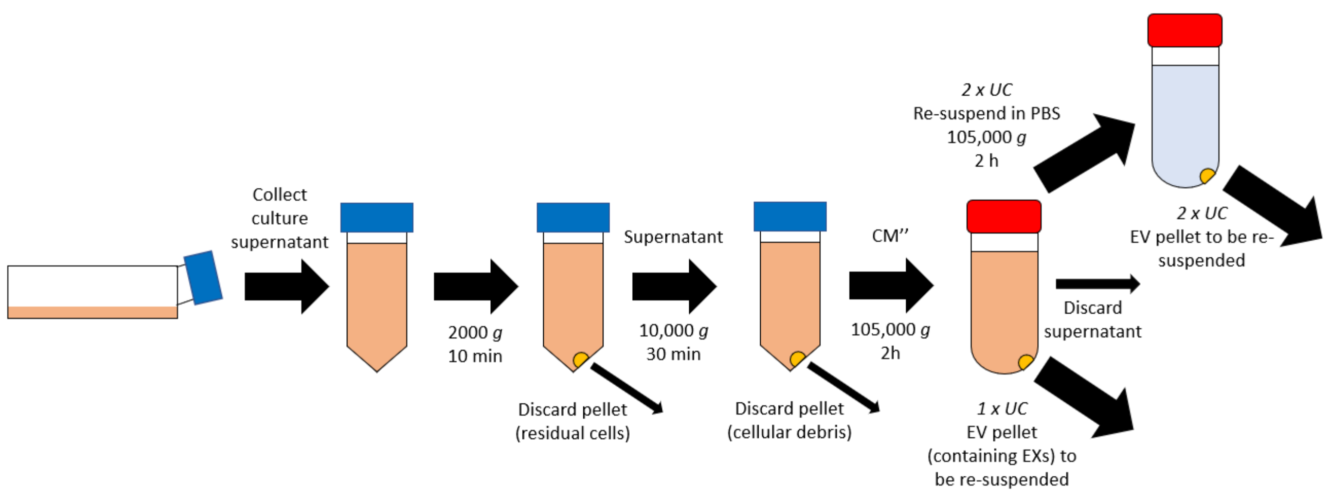

Fig. 2. Visual representation of the EV isolation process using differential and ultra- centrifugation. All steps occur at 4°C.

E. Statistical Analysis

Graphical data is presented as mean ± standard deviation (SD) where appropriate. Triplicates of each sample and two-tailed, unpaired t-tests were used to compare sample data between two experimental conditions. GraphPad Prism 9.4.0 was used to calculate statistics. A p-value less than 0.05 indicated that the difference was statistically significant.

III. RESULTS AND DISCUSSION

A. A second UC step increases the purity of the EV samples isolated

Using the BCA assay, the concentration of proteins co-isolated in the EV pellet samples (impurities) collected for 1 x UC and 2 x UC was determined, as depicted in Fig. 3. The percentage of proteins recovered from the CM'' is also provided in Fig. 3.

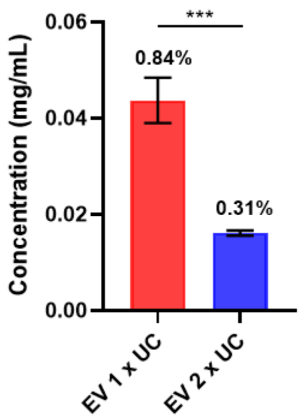

Fig. 3. Concentration (mg/mL) of proteins co-isolated with the EV samples using 1 x UC and 2 x UC. The percentage of proteins recovered from CM'' is provided for each sample above the corresponding concentration data (0.84% and 0.31%). The error bars are SD. *** p-value < 0.001.

From Fig. 3, 0.84% of the proteins present in the supernatant (CM'') were isolated during 1 x UC while only 0.31% were isolated for 2 x UC. Therefore, it can be suggested that a washing step decreases impurities by 63%. The higher purity is likely the result of residual contaminating proteins being further removed so a higher concentration of EVs remains, with respect to impurities.

B. A second UC step reduced the total yield of EVs isolated from a sample

Results for particle counts and colocalization of EX specific surface markers (tetraspanins CD63, CD81 and CD9) were obtained from the ExoView R100.

Fig. 4 demonstrates that the yield of EXs isolated during 1 x UC is higher than the yield obtained after 2 x UC as 55% of the total particles bound to CD63 and expressing EX specific markers were recovered for 1 x UC while only 26% were recovered for 2 x UC. To convert the total particle counts to particles/mL of sample (particles/mL) the raw data was multiplied by 10,000 as per the manufacturer's suggestion. Fig. 5 contains values for particles/mL and provides evidence that the yield of EXs isolated is lower for 2 x UC because the particles expressing each EX specific marker is lower than 1 x UC. Fig. 5 also indicates the average percent recovery of EXs from CM'' between CD63, CD81 and CD9 for 1 x UC and 2 x UC is 49% and 24% respectively.

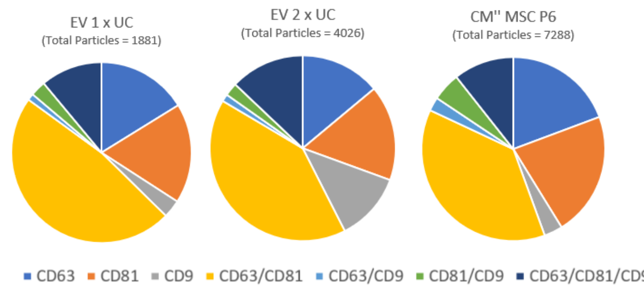

Fig. 4. Colocalization charts and total particle count for CM'' and the EV pellets after 1 x UC and 2 x UC bound to CD63. CD63 was used as it was more highly expressed than CD81 or CD9.

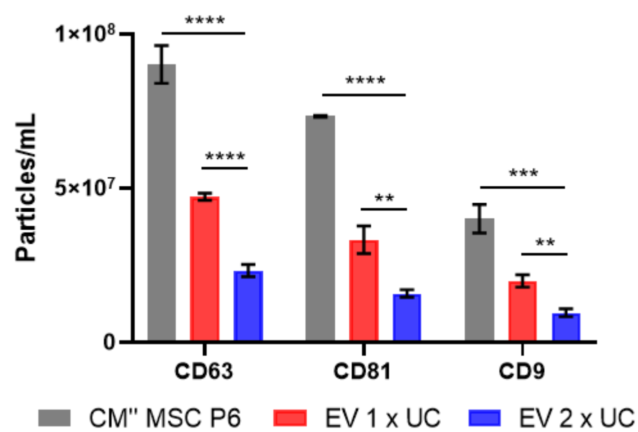

Fig. 5. Number of particles/mL of sample expressing EX specific markers for CM'', the EV pellet after 1 x UC and the EV pellet after 2 x UC. The error bars are SD provided by the ExoView software. **** p-value < 0.0001, *** p-value < 0.001 and ** p-value < 0.01.

From the results obtained from the ExoView, it was determined that despite the ability of a second round of UC to "wash away" contaminants, it also increases the chances of losing EXs. During the process, it is difficult to ensure all EXs are isolated and subsequently re-suspended in PBS, and an additional UC step increases the chances of misplacing EXs therefore reducing the yield. An additional concern is that UC is a stressful process for the EXs, and the washing step could subject the vesicles to increased damage [10].

C. The size distribution of isolated EVs is within a reasonable range for EXs

DLS was used to validate the size range of the particles present in an EV sample isolated using 1 x UC and the characterization plot can be found in Fig. 6. From the software, an average diameter of 170 nm with a polydispersity index of 0.3 was determined for the particles present using the plot generated. These values indicate that the particles present in the EV sample were within a reasonable size range for EXs.

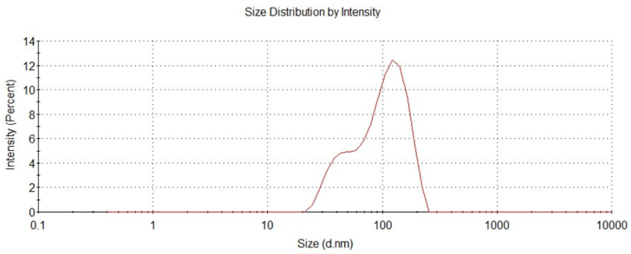

Fig. 6. DLS characterization plot for EXs isolated using 1 x UC which has an average diameter of 170 nm.

IV. CONCLUSION AND RECOMMENDATIONS

MSC-EXs hold extensive potential in the medical field. However, to obtain relevant and reliable results, standardization of isolation techniques is required. Evaluating the inclusion of the additional UC step contributes towards determining a standardized process and allows researchers to better understand the relevance of certain techniques and protocols.

From the results obtained it has been determined that the washing UC step can provide improved purity but reduced yield. There are instances in research where purity may be desired over yield and vice versa. Therefore, this experiment has revealed that the inclusion of an additional UC step is entirely dependent on whether a higher purity or a higher yield is required. For example, a high yield is often more desirable in diagnostics while a high purity is a concern for therapeutic applications [39]. It is suggested that careful consideration be made regarding the inclusion of an additional UC step to ensure the results are accurate, reliable, and reproducible.

As researchers strive to develop applications for EXs, effective isolation techniques are becoming a priority, however, current isolation techniques have limitations. As highlighted in this paper, utilizing UC currently requires that a decision be made between high purity and high yield. However, in future applications it is more desirable to obtain both high purity and high yield. Therefore, further improvement of isolation techniques or the development of novel isolation techniques is required.

Due to time constraints only an individual round of testing was conducted. It is therefore recommended that additional rounds of testing be conducted using the same methodologies provided to validate the results obtained. The PPRF lab intends to further examine and determine the accuracy and reliability of these findings.

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Exploring the Relationship Between Eating Disorders and Fitspiration Influencers

M. Mehra[1], J. Godley[1*]

1 Department of Sociology, University of Calgary, Calgary, AB, T2N 1N4, Canada

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Abstract— This paper explores the new trend of "fitspiration" and the prevalence of eating disorders and mental health struggles among a sample of Fitness Influencers on Instagram. The rise in fitspiration on social media has allowed individuals to display their bodies and their workouts and send messages to the public with the hopes of inspiring others. This new era of fitspiration has deemed the new ideal body type for young women as strong, fit, and muscular, fueled through discipline and hard work. This new trend is often portrayed as a healthy alternative to thinspiration, being overly thin, as it encourages working out, and "building a body" that you love; actively rebelling against the "skinny body" ideal. However, this research challenges the idea that fitspiration is a healthy alternative to thinspiration, arguing that the same expectations and fundamental values are present in both. We conduct a content analysis of Instagram posts from 50 female fitness-influencer individuals. Each influencer was categorized according to whether they mentioned having a past eating disorder or mentioned and/or advocated for mental health or mentioned neither. Results reveal stark differences in content between these three categories. We find that fitness influencers with past eating disorders display more eating disorder language, higher body dissatisfaction, and more workout content than influencers from the other two categories. These results indicate the potential spread of unhealthy messages regarding fitness and body image on fitspiration Instagram pages, ultimately posing harm to users. The new trend of fitspiration has potentially created new body image standards which, although disguised as a healthy alternative to thinspiration, may be harmful to women.

I. INTRODUCTION

We live in a visual, aesthetically focused world where body image has been a prevalent topic, especially during the rise of social media. Social media has created an interesting dynamic regarding individuals' agency. In the past, individuals had agency over what they viewed, such as which magazines they read; now, due to the use of algorithms, individuals have lost part of their control over the content to which they are exposed. This is problematic because Instagram is widely used among, youth, teens and young adults when brains are still developing. Social media has also brought attention to the harmful nature of being exposed to unattainable body standards. To push back against this, we have seen a shift in body image ideals especially among women, moving from an overly thin ideal with visible bones, to one that features defined muscles, and represents strength. However, the irony and paradox is that this phenomenon, known as fitspiration, may have created new body image standards for women to achieve. These messages are communicated through fitness influencers, who encourage their followers to strive for such a physique. The issue is that these messages of encouragement can accidentally disguise more harmful messaging that follows the same themes as thinspiration content. This research explores this very phenomenon, investigating whether female fitness influencers' public posts reflect their eating disorder and mental health histories.

II. DEFINITIONS

Influencers are defined as persons who regularly create and share social media content (Alikilic & Ozkan, 2018). Influencers are often classified according to their number of followers (Schaffer, 2020; Steele, 2017). Micro influencers are social media users whose number of followers is between 1,000 and 100,000 and have an average 25%–50% engagement rate per post, while macro influencers are users who have between 100,000 and 1 million followers and have an average 5%–25% engagement rate per post (Christodoulaki, 2018). Micro influencers often operate in a niche area and communicate one-on-one with their followers (Meric Bor & Erten, 2019). Businesses aiming to reach a broad demographic audience therefore usually work with macro influencers (Kaya, 2018), often through professional collaborations (Influicity, 2018). A fitness influencer is someone who operates within the niche of the fitness industry. This may include posting information on workouts, diets, meals, cooking and so on. The potentially problematic aspect of this is that "literally anyone can [be a fitness influencer]," (ISSA, 2022). This is how misinformation, problematic routines and harmful information/messages are spread.

III. LITERATURE REVIEW

A. Social Media & Mental Health

Social media operates through algorithms which manipulate what people see based on past viewing patterns. This targeted information may be harmful for mental health. As stated in the Wall Street Journal article "Facebook Knows Instagram is Toxic for Teen Girls," the network of social media sites is aware of the damage and potential risk. Meta CEO Mark Zuckerberg is aware of the manipulative and targeted nature of his apps (Facebook, Instagram, WhatsApp, Messenger, etc.) and the negative environment fostered from such apps. It has been shown that individuals using social media are more likely to compare themselves to idealistic images that influencers post (Chae, 2018). Online social comparison creates especially damaging effects for women, including decreased mood and increased body dissatisfaction (Jennings, LeBlanc, Kitsch, Lancaster & Allen, 2020). It has been shown that this correlation between social media and negative body image is mediated by the individual's internalized thoughts and messages; the higher one's negative internalized level, the more likely they are to experience negative body image and body dissatisfaction (Bell, 2016). This effect is amplified in people with disordered eating history and impacts the emotional outcomes associated with body focused media (Jennings et al., 2020).

When looking at clinical samples of individuals with current or past eating disorders, "more frequent use of image-centric social media is associated with more frequent exposure to both thinspiration and fitspiration," (Griffiths et al., 2018). The more one uses social media, the more images they will come across involving thinspiration and fitspiration. The exposure to these images is associated with more frequent physical appearance comparisons and greater symptom severity (Griffiths et al., 2018). Further, viewing thin-ideal images in print and online increases negative affect and disordered eating behaviors (DEBs) in women who engage in DEBs at least once per week (Christensen, Forbush, Cushing, Lejuez, Fleming & Romine, 2021). This research points out that there is not a direct relationship between social media use and eating disorders; instead, the relationship is mediated by the exposure of thinspiration or fitspiration content and body comparisons (Griffiths et al., 2018). The targeted nature of the social media algorithm amplifies and perpetuates a cycle of dissatisfaction, leading to poor mental health.

The manipulative nature of social media does not stop with the large corporations that run the platforms. Social media has been plagued with the reputation of being fake, due to manipulating one's appearance by editing photos, smiling when having a bad day, etc. Specific to fitspiration, influencers gain trust and friendship of their followers by using body-shape focused, visual content and targeted communication techniques (Pilgrim & Bohnet-Joschko, 2019). Followers then identify with the ideas demonstrated by influencers, creating a relationship of dependency on social media outlets (Pilgrim & Bohnet-Joschko, 2019). Essentially fitspiration influencers demonstrate to their followers that "only those who create a body shaped through control and discipline are healthy and beautiful and can be happy," (Pilgrim & Bohnet-Joschko, 2019).

B. Compulsive Exercise

Among fitness influencers, exercise is often regimented, routine and the focus of their content. However, excessive and regimented exercise has been shown to be an indicator and precursor to eating disorder behavior (Davis, Blackmore, Katzman, & Fox, 2005) and has been recognised as a major factor in the etiology, development and maintenance of eating disorders (Meyer, Taranis, Goodwin & Haycraft, 2011). Further, it has been found that compulsive exercise is often one of the last symptoms to resolve during eating disorder treatment (Fietz et al., 2014). Compulsive exercise is described as a routine-like pattern of exercise, often performed despite possible negative consequences, and includes intense feelings of guilt at any missed exercise session (Meyer, Taranis, Goodwin & Haycraft, 2011; Fietz, Touyz & Hay, 2014; Goodwin, Haycraft & Meyer, 2014; Hefner, Dorros, Jourdain, Liu, Tortomasi, Greene, Brandom, Ellet & Bowles, 2016).

A study examining the association between consumption of various types of social media and reports of disordered eating along with compulsive exercise found that the use of phone applications and microblogs featuring nutrition and eating habits and exercise are positively associated (Hefner, Jourdain, Liu, Tortomasi, Greene, Brandom, Ellet & Bowles, 2016). These results indicate that the more time individuals spend on social media viewing fitspiration content, the more likely they are to also report disordered eating and symptoms of compulsive exercise (Hefner, Jourdain, Liu, Tortomasi, Greene, Brandom, Ellet & Bowles, 2016).

Compulsive exercise has been established to have links to mental health issues such as depression and anxiety (Feitz et al., 2014; Weinstein, Maayan & Weinstein, 2015). It has also been shown to be consistently associated with higher levels of perfectionism and rigidity (Meyer, Taranis, Goodwin & Haycraft, 2011), as well as restraint/restriction in anorexia (Dalle-Grave, Calugi & Marchesini, 2008). These are key components to eating disorder behavior (Meyer et al., 2011). When specifically looking at clinical populations of adolescent females with anorexia, it was revealed that not exercising or giving into the urge of compulsive exercise elevated measures of eating disorder behavior, anxiety, depression, and obsessive-compulsive tendencies (Noetal, Miskovic-Wealthy, Crosby, Hay, Madden & Touyz, 2016). This may have been due to both the positive and negative reinforcement encountered when exercising, leading to a compulsive need to engage in the activity. Positive reinforcement is experienced by the regulation of weight and shape (Meyer, Taranis, Goodwin & Haycraft, 2011), while negative reinforcement is experienced through withdrawal symptoms such as anxiety, guilt, depression and irritability (Bamber, Cockerill, & Carroll, 2000; Scharmer et al.; Szabo, Frenkl, & Caputo, 1997). Additionally, there seems to be an addictive nature to compulsive exercise. Adolescents with anorexia who score higher on addictive personality measures report higher levels of commitment to exercise, linking the area to addiction disorders (De Luca, Simonato, Mooney, Bersani, Corazza, Corazza, & NPS Unit, 2017). Researchers advocate that there is a need to inform healthcare professionals about the risks associated with compulsive exercise as well as develop innovative prevention responses to safeguard the public (De Luca et al., 2017). Overall, past research regarding excessive and compulsive exercise highlights the phenomenon as one that is problematic, cyclic and heavily linked to mental health as well as eating disorder behavior, only amplified by the presence of social media.

C. The Shift

"Recent studies have documented a shift in the cultural ideal of physical attractiveness with women subscribing to a visibly toned ideal that emphasizes health and fitness," (Robinson et al., 2017). On social media, this shift is demonstrated through a shift from thinspiration posts to more fitspiration posts. Thinspiration posts tend to showcase more bones and mental illness (Alberga, Withnell & Von Ranson, 2018) whereas, 'fitspiration' promotes health and well-being through the promotion of healthy eating, exercise and self-care. The overall fitspiration philosophy is one which emphasizes strength and empowerment. In particular, fitspiration promotes health and fitness, rather than thinness and weight loss. As such, fitspiration has been positioned as a healthy alternative to the internet-based trend known as ‘thinspiration’ (amalgamation of thin and inspiration),” (Tiggerman & Zaccardo, 2018).

While some state that fitspiration inspires others to get fit (Wortham, 2017) and is seen as a positive alternative to thinspiration, more studies have shown that exposure to fitspiration images lead to increased body dissatisfaction and that fitness idealized images do not motivate participants to engage in higher levels of exercise (Robinson et al., 2017). Women in one study reported a weekly average of 8.4 thinspiration exposures and 9.5 fitspiration exposures, with both thinspiration and fitspiration images lowering body satisfaction (Griffiths & Stenfanovski, 2019). What is striking here is that the exposure was nearly identical in both categories, indicating that being exposed to either fitspiration or thinspiration will result in lower body satisfaction (Griffiths & Stenfanovski, 2019). Both thinspiration and fitspiration reinforce negative body image issues (Alberga, Withnell & Von Ranson, 2018).

Despite the philosophy behind fitspiration, women are still held to body image ideals. The majority of the [fitspiration] women [on social media] contain only one body type: thin and toned (Alberga et al., 2018; Tiggerman & Zaccardo, 2018). This is reinforced through messages and comments on social media platforms that “encourage appearance related body image standards, behaviors and weight management behaviors more frequently than health related standards, and behaviors,” (Simpson & Mazzeo, 2017). Messages include fit praise, with emphasis on toned, defined muscles and thin praise, suggesting that women are supposed to be thin but also fit (Simpson & Mazzeo, 2017). In addition, fitspiration images tend to be of women exercising or in exercise gear (that is tight and revealing), overlain with inspirational quotes such as “strong beats skinny every time,” implying that just being skinny is not good, you must be strong as well (Holland & Tiggemann, 2017).

In addition, both thinspiration and fitspiration sites contain potentially dangerous thematic content in terms of emphasizing dietary restriction and harmful messages about women’s body ideals (Boepple & Thompson, 2016). Many sites promoting fitspiration and thinspiration include guilt-inducing messages regarding weight, fat stigmatization, and dieting/restriction (Boepply & Thompson, 2016). Furthermore, the frequency of dieting and fat-shaming messages does not differ between thinspiration and fitspiration posts (DiBisceglie & Arigo, 2021). Sites supposedly devoted to healthy pursuits (fitness) may also contain thematically similar content to thinspiration websites (Boepply & Thompson, 2016). Problematically, these images may oversell the attainability of this ‘ideal’ body, potentially setting many women up for failure (Robinson et al., 2017). The saturation of athletic ideal images further makes this ideal body seem both attainable and normative, desensitizing women from their generally unobtainable nature (Sabiston & Chandler, 2009).

There are also troubling findings regarding female fitness influencers themselves. Women who post fitspiration images score higher on drive for thinness, bulimia, drive for muscularity and compulsive exercise (Holland & Tiggemann, 2017). In one study, a fifth (17.5%) of these women were at risk for diagnosis of a clinical eating disorder (Holland & Tiggemann, 2017). Despite influencers’ healthy intentions, women who post fitspiration on Instagram are more likely than other Instagram users to exhibit disordered exercise and eating behaviors (Holland & Tiggemann, 2017). Works have been extended to highlight the effects felt by fitstagtrammers and followers when viewing fitspiration posts. Both fitstagrammers and followers noted that they experience both positive and negative feelings in response to fitspiration posts, with women and the followers experiencing more negative feelings (DiBisceglie & Arigo, 2021).

D. Current Situation

Previous research has established that thinspiration posts are harmful to viewer’s mental health. Research has also explored the interaction between fitspiration posts and their audience, connecting viewing with phenomena such as compulsive exercise and eating disorders. However, past research has not looked at the fitness influencers themselves, and their history/relationship with eating disorders, compulsive exercise and mental health. This research seeks to compare those with a public history of eating disorders, mental health struggles or other issues regarding body image. Many fitness influencers have no professional qualifications, yet many choose to share their fitness journey to help others. Though there are many male fitness influencers, this study specifically focuses on individuals who identify as women in social media due to the high variability and drastic shifts in body ideals in the female physique throughout the past decades. An important element to mention regarding women, although not looked at specifically in this study, is that past findings indicate that racial, ethnic and cultural influences may also affect body image ideals and the types of exercise, diet and behaviours that female influencers share. Numerous research studies have shown that African American women tend to have higher self esteem and a more positive body image than White women (Botta, 2000; Molloy, et al., 1998; Schooler, et al., 2004).

Our research analyzes posts that are labeled and found under a fitspiration term that is supposedly positive and meant to promote health. We challenge the view that fitspiration is a healthy alternative to thinspiration, and hypothesize that fitspiration content from individuals with a history of eating disorders will look different than content from influencers that have no history. This paper will specifically explore three areas: (1) the content of influencers’ posts, (2) whether influencers have a history of eating disorders and/or struggles with mental health, and (3) does the content of the posts by influencers that do have an eating disorder history differ from the posts by influencers who have no eating disorder history?

IV. METHOD

This study used Instagram as the primary source of data for analysis. Due to Instagram’s highly visual but also contextual nature, this platform allowed us to analyze images as well as text. A new Instagram profile was created in order to generate a sample that was unaffected by prior searches, algorithms, likes, etc. First, we created a list of influencers from which to choose our sample. The list creation process encompassed two phases, each 1 week in duration: (1) selection by searching the hashtag #fitnessmotivation, and (2) selection by the “feed”. Each day at the same time, between 7pm-8pm

[2] Collections sizes differed because more screenshots of profiles were taken than were needed
[3] Most likely due to the Instagram algorithms, linking and presenting like content

MST, the first 50-70 posts that had the hashtag #fitnessmotivation were collected[2]. The time frame was selected due to the popularity of posting at this time in western locations (US and Canada). Our sample later expanded to include other nations such as Europe and Australia[3].

In phase one when searching the hashtag #fitnessmotivation, a grid is presented with photos that are linked to that specific hashtag. In phase two, a grid with photos is also presented, however, these images are selected by the Instagram algorithm based on past searches, likes, etc. Each profile associated with the first 50-70 images from each search phase was investigated and either deemed viable to be included in the sample pool or not. In this study, Influencer accounts were selected based on the following criteria: female, at least 1k followers, profile must focus on fitness and be a personal account[4]. It was decided that having a minimum of 1k followers was acceptable for the study in order to capture the "regular" people trying to make it as fitness influencers. It gives us a look at the not-so-famous influencers trying to attract a larger audience.

After completing both phases in creating our large pool of influencers, duplicates were removed, and profiles not in English were excluded from the list due to our inability to fully capture the message in a translation. Individuals focusing on yoga or spirituality were also excluded, as they do not represent the same idea/definition of fitspiration or fitness influencer we are trying to capture. In addition, verified individuals were also excluded. Instagram verification is how you prove that your Instagram account is the authentic presence of a notable public figure, celebrity or global brand. As mentioned above, we are attempting to capture the 'normal' people who are influencing. Our pooled list contained XXX profile. A random sample of 50 profiles was selected from this list, to comprise our sample. Once we had our list of 50 who were included in our sample, the 6 most recent posts (image and text) from each of these profiles were saved as PDFs or screenshots and analyzed in NVivo. In order to analyze the posts, content analysis guidelines were produced with different criteria for coding the profile bio, the images and the text[5]. To establish interrater reliability two pilot runs were conducted on 3 profiles with two researchers conducting the analysis and comparing results.

Figure 1. Content Analysis Nodes

Bio	Image	Text
Balance Fitness Journey Lifestyle Nutrition	Bones Visible[6] Clothing • Casual • Going out • Lingerie • Swimsuit • Workout Comparison Focus • Abs • Lower Body • Upper Body Food Full Body Location • Bathroom/Locker room • Bedroom • Gym • Outdoors Makeup/hair done Others in photo Posed, Flexed Revealing clothing Selfie Supplements Suspected photoshop Representative of text	Eating Disorder Language (word frequency) • Big • Bulk/Gains/Grow • Calories/Macros • Cut • Diet • Fat • Lean • Small • Strong • Thin • Toned • Weight General Language • Advertisement • Balance • Body Satisfaction • Clothing • Discipline • Goals • Guilt • Journey/Progress • Listen to your body • Motivation • Workout

The words identified as eating disorder language were based on past studies also using a content analysis to investigate Instagram posts (Alberga et al, 2018). Eating disorder language was coded if the following words were mentioned in the text: big, bulk, gains, grow, calories, macros, cut diet fat, lean, small, strong, thin, toned, weight. General language nodes focus on the overall message of the post. Image nodes were coded if the picture represented or contained any of the options listed. The bio at the top of the profile was also analyzed and it was recorded if the profile mentioned any of the words listed. The last step of this process was to see if the influencer had a history of mental health struggles or an eating disorder diagnosis. Other information collected from the influencer's bio at the top of their profile was also recorded. This included geographical location, any professional fitness qualifications, whether they were a visible minority or not, and the number of followers.

V. RESULTS

The sample of 50 individuals was from the US (31), Canada (11), the EU (4), Australia (3), and the UAE (1). In terms of ethnicity, 36 were "white" and 14 were "visible minority." The number of followers were split into three categories: 22 profiles had <10k followers; 17 profiles had 10-50k; and 11 had over 50k. In terms of qualifications, the majority of individuals (20) had no professional certificates or experience in personal training. Fourteen individuals were considered personal trainers or had a form of certification in fitness. Ten individuals considered themselves bodybuilders and competed in competitions. Three individuals were brand affiliates, one with Lululemon and two with supplement companies. Two individuals were athletes competing in competitions. Lastly, one

[4] Not a company, brand, etc.

[5] Note that follower comments and anything in the comments section of the post was not analyzed.

[6] Note that 'bones visible' was in no reference to being healthy or unhealthy, it was more about the presence of ribs bones, collar bones, etc. being visible. This is not to say that the influencer was unhealthy, simply that having visible bones identifies with a certain idealized body type that endorses thinner bodies.

individual was a certified dietician.

When looking for evidence of an eating disorder or mental health, the profiles were coded as having an eating disorder if it was explicitly mentioned in a post on their profile. For example, if an individual had posted a transformation photo showing them in the hospital with a feeding tube to show their recovery, or if they had posted text saying they had struggled with an eating disorder, they were coded as an influencer with an eating disorder history. Profiles were coded under "mental health" if they mentioned some form of struggle with mental health. For example, if they stated that they struggled/struggle with anxiety, depression, and/or experienced suicidal thoughts they were coded as an influencer with a mental health history. If neither eating disorders nor mental health were mentioned on their profile, individuals were coded as "other". Overall, out of a sample of 50 fitness influencers, 11 had previously struggled with an eating disorder, 8 individuals mentioned a struggle with mental health and 31 individuals mentioned neither . It is important to note that although they were not coded as either having an eating disorder or struggling with mental health, 15 of the 31 individuals in the 'other' category mentioned some sort of harmful relationship with food, body image, weight loss.

Our main interest regarding the results were differences across our descriptive categories: (1) history of eating disorder, (2) mention of mental health struggles (3) other. As presented in the charts below, in terms of eating disorder language , 70% of posts from influencers with past eating disorders used eating disorder language in their posts, while only 25% of posts from influencers with mental health struggles used eating disorder language. Eating disorder language examples included references to wanting to "lean out" or "lose fat." One individual even mentioned the struggle of being a bodybuilder and watching her body change, "You get used to the smallest version of yourself in the mirror that you thought day and night to create. And then within weeks you have to say goodbye to the lines that had become natural to you as they fade away." Others mentioned things such as the rigidity in their eating schedules and the kinds of food they eat. For example one influencer stated, "Food, scheduled eating is consistent for me. It helps me make sure my diet is perfect." What was unexpected was that 52% of posts from individuals with neither contained eating disorder language. This indicates that whether individuals had history of an eating disorder or whether they mentioned mental health mattered (Table1).

Table 1. Eating Disorder Language References by Description

Nodes	Description Eating Disorder (n=11)	Description Mental Health (n=8)	Description Other (n=31)	Total (n=50)	Percent
Big	0	0	0	0	0.00
Bulk/Gains/Grow	10	1	29	40	0.13
Calories, Macros	11	2	19	32	0.11
Cut	3	0	5	8	0.03
Diet	2	0	5	7	0.02
Fat	2	0	2	4	0.01
Lean	5	1	2	8	0.03
Small	1	0	0	1	0.00
Strong	5	6	18	29	0.10
Thin	0	0	0	0	0.00
Toned	3	1	4	8	0.03
Weight	4	1	12	17	0.06
Total	46	12	96	154	0.51
Percent	0.70	0.25	0.52		

As shown in Table 2, eating disorder language across follower groups (<10k, 10-50k, and >50k) were fairly even, ranging from 29%-40%. This demonstrates that the number of followers did not impact the content that fitspiration influencers were posting.

Table 2. Eating Disorder Language References by Followers

Nodes	Followers <10k (n=22)	Followers 10-50k (n=17)	Followers >50k (n=11)	Total (n=50)	Percent
Big	0	0	0	0	0.00
Bulk/Gains/Grow	18	16	6	40	0.13
Calories, Macros	12	14	6	32	0.11
Cut	3	3	2	8	0.03
Diet	1	5	1	7	0.02
Fat	1	1	2	4	0.01
Lean	1	6	1	8	0.03
Small	0	1	0	1	0.00
Strong	16	7	6	29	0.10
Thin	0	0	0	0	0.00
Toned	4	2	2	8	0.03
Weight	10	5	2	17	0.06
Total	66	60	28	154	
Percent	0.33	0.28	0.39		

Table 3 shows the overall message of the posts. Only 53% of individuals with past eating disorders used generally positive language (balance, journey, progress, listen to your body, motivation). This is contrasted with those who struggled with mental health issues/advocate for mental health as 71% of their posts used positive language. Forty four percent of posts from individuals in the "other" category contained positive language. These results make sense as the individuals advocating for mental health want their language to align with what they stand for.

Table 3. Positive Language References

	Eating Disorder (n=11)	Mental Health (n=8)	Other (n=31)	Total (n=50)
Balance	4	5	13	22
Journey, Progress	11	12	34	57
Listening to body	10	4	4	18
Motivation	10	13	30	53
Total	35	34	81	150
Percent	0.53	0.71	0.44	

When it came to the content of the profiles, 71% of content from

[7] Qualitative notes were taken regarding the relationship with food, weight loss, body image, etc. in this group of individuals
[8] ED represents "eating disorder"
[9] This could include workout videos, form tips[9], how to structure a workout, etc.

those with a history of eating disorders was workout related[9], whereas only 67% of content from those with mental health struggles and 60% of those with neither contained workout content, as demonstrated in the chart below (Table 4). This indicates that these profiles were posting on other things. Some examples of popular content included posing and "showing off" in front of the camera, posting inspirational messages, spending time with friends or being outdoors.

It was revealed that those in the "other" category had the most advertising content, as 30% of their posts contained some form of advertisement (for supplements, clothing, etc.). This is contrasted with the eating disorder category where 20% of posts contained advertisement and even less for the mental health group, as 8% of those posts contained advertising. This aligns with the results regarding mention of clothing[10]. Clothing was mentioned in 14% of posts from individuals in the "other" category, only 9% of posts from individuals with past eating disorders mentioned clothing and only 4% of posts from individuals who mentioned mental health contain clothing references.

In terms of specific messages of posts, it was revealed that individuals who mention mental health also mention discipline the most, with 10% of posts mentioning discipline. Individuals with the history of an eating disorder mentioned discipline in 8% of their posts and those in the other category mentioned discipline in 7% of their posts. In addition, individuals who mention mental health also mention goals the most, as 8% of their posts referenced setting, reaching and achieving goals. Goals were mentioned in 6% of posts from individuals with a history of eating disorders and 7% in posts from individuals in the "other" category. References to guilt were the highest in the eating disorder group, with 5% of posts mentioning guilt. 4% of posts from individuals who mention mental health involved guilt and 2% of posts from individuals in the other category referenced guilt (Table 4).

Table 4. Percent of General Language by Description

	Eating Disorder (n=11)	Mental Health (n=8)	Other (n=31)
Advertisement	0.20	0.08	0.30
Clothing	0.09	0.04	0.14
Discipline	0.08	0.1	0.07
Goals	0.06	0.08	0.07
Guilt	0.05	0.04	0.02
Workout	0.21	0.29	0.14

As shown in Table 5, there were differences in messages surrounding body satisfaction and dissatisfaction. For those with a history of eating disorders 8% of posts mentioned something about being critical or unhappy with their body's current state. This was double the number of references than both those who mentioned mental health struggles and the "other" category, where only 4% of posts mentioned body dissatisfaction. It is also important to note that there were more evaluations of one's body among those with past eating disorders. In addition, there were more messages of being satisfied with one's body across all categories. However, what stands out is that there were twice as many references to body dissatisfaction in the ED group as opposed to the 2 other groups.

Table 5. Body Satisfaction Language

	Eating isorder (n=11)	Mental Health (n=8)	Other (n=31)
Satisfied	0.14	0.08	0.08
Dissatisfied	0.08	0.04	0.04

When it came to images, there were certain features that stood out; 42% of posts from individuals with a history of an eating disorder had bones visible, whereas only 23% of posts from individuals who advocate mental health had bones visible (Table 6). Forty four percent of posts from individuals with neither demonstrated visible bones in their images. This same image criteria were also analyzed in terms of number of followers. It was revealed that there was a positive correlation between the number of followers and the percentage of images that had bones visible. Twenty eight percent of images from influencers with <10k followers had bones visible; 45% of images from influencers with 10-50k followers had bones visible; and 56% of images from individuals with >50k followers had bones visible (Table 7).

Table 6. Image Node Percentages by Description

	Eating Disorder (n=11)	Mental Health (n=8)	Other (n=31)	Total Percentage
Bones Visible	0.42	0.23	0.44	0.40
Casual	0.12	0.15	0.17	0.16
Going Out	0.02	0.02	0.02	0.02
Lingerie	0.02	0	0.02	0.02
Swimsuit	0.06	0.06	0.10	0.08
Workout	0.71	0.67	0.60	0.63
Comparison Photos	0.06	0	0.02	0.02
Abs	0.20	0.23	0.30	0.27
Lower Body	0.45	0.40	0.47	0.45
Upper Body	0.36	0.35	0.30	0.32
Food	0.03	0	0.02	0.02
Full Body	0.74	0.79	0.78	0.77
Bathroom/Locker Room	0.21	0.15	0.12	0.14
Bedroom	0.14	0.21	0.14	0.15
Gym	0.47	0.40	0.34	0.38
Outdoors	0.05	0.13	0.20	0.16
Makeup/Hair done	0.56	0.50	0.51	0.52
Others in Photo	0.09	0.06	0.11	0.10
Posed/Flexing	0.59	0.58	0.74	0.68
Revealing Clothing	0.36	0.40	0.46	0.43
Selfie	0.42	0.23	0.34	0.34
Supplements	0.06	0.06	0.04	0.05

[10] Many of the advertisements were for gym/workout clothing.

Table 7. Image Node Percentages by Followers

	Followers <10k (n=22)	Followers 10-50k (n=17)	Followers >50k (n=11)	Total Percentage (n=50)
Bones Visible	0.28	0.45	0.56	0.4
Casual	0.35	0.17	0.11	0.16
Going Out	0.03	0.02	0.03	0.02
Lingerie	0.02	0.02	0.03	0.02
Swimsuit	0.09	0.11	0.12	0.08
Workout	1.29	0.63	0.62	0.63
Comparison Photos	0.03	0.04	0.02	0.02
Abs	0.39	0.32	0.32	0.27
Lower Body	0.91	0.51	0.36	0.45
Upper Body	0.59	0.39	0.27	0.32
Food	0.03	0.02	0.03	0.02
Full Body	1.48	0.82	0.76	0.77
Bathroom/ Locker Room	0.27	0.2	0.08	0.14
Bedroom	0.27	0.15	0.18	0.15
Gym	0.76	0.37	0.39	0.38
Outdoors	0.32	0.18	0.12	0.16
Makeup/Hair Done	1.02	0.5	0.56	0.52
Others in Photo	0.26	0.11	0.03	0.1
Posed/Flexed	1.35	0.75	0.59	0.68
Revealing Clothing	0.61	0.49	0.58	0.43
Selfie	0.67	0.44	0.2	0.34
Supplements	0.06	0.06	0.08	0.05
Total	11.68	6.86	6.36	6.3

As demonstrated above, images showed less differences than the texts, however some observations stood out. For example, influencers with a history of an eating disorder had images focused on their abs 20% of the time. This is contrasted with those in the mental health group where 23% of their images focused on their abs and 30% of images from the other group focused on their abs. Across categories the most images were taken in the gym: 47% of images from the eating disorder category, 40% of images from the mental health category and 34% of images in the "other" category. In terms of hair and makeup being done, individuals with a history of eating disorders had the highest percentage of having hair and makeup done in their photos, with 56% of images showing this. Both those in the mental health and "other" category only had their hair and makeup done in 50% of their images. Posing and flexing were similar across eating disorder and mental health groups, with 59% of posts from individuals with eating disorder history and 58% of images from individuals who mention mental health. This is contrasted with results yielded from the "other" category, as it was shown that 74% of these images demonstrated posing/flexing. Lastly, individuals with a history of an eating disorder were more likely to post selfies. 42% of images from individuals with past eating disorders were selfies, whereas only 23% of images from the mental health category were selfies and 34% from the "other" category.

There were no major differences in clothing between groups, as most had a similar proportion of workout, going out, casual clothing as well as lingerie and swimsuits. Nor were there major differences between the area of the body that was focused on between groups. Each had similar proportions of posts focusing on lower body, abs and upper body. In addition, all groups had a similar proportion of posts that pictured posing, flexing and revealing clothing. This makes sense as much of social media highlights "the best" photos, where people ensure they look their best when being displayed to the world. One coding criteria, suspected photoshop, was removed from final results due to the difficult nature of identifying it and not being able to establish interrater reliability.

Further investigation was conducted in areas such as qualifications, ethnicity and image representation. In terms of qualifications, we specifically looked at bodybuilding across our three main categories. There was a difference, as 36% of individuals with past eating disorders went into body building, whereas only 12% who mention mental health and 16% of "other" individuals. We also looked at ethnicity. Results regarding ethnicity were in line with past findings, demonstrating that more white individuals struggled with eating disorders and mental health than visible minorities. As shown in Table 8, there was a difference in the percentage of images that were representative of the text. Influencers with past eating disorders and those who mention mental health had roughly the same amount of images representative of the associated text in the posts. However, those in the "other" category had a much lower percentage of images that were representative.

Table 8. Images Representative of associated text by description

	Image representative of text	Percentage
Eating Disorder (n=11)	45	0.68
Mental Health (n=8)	31	0.65
Other (n=31)	92	0.50

VI. DISCUSSION

To summarize the results, eating disorder language was highest in individuals who had a history of an eating disorder. Positive language (balance, journey, progress, listening to your body) was highest among those who mentioned and advocate for mental health. Workout related content was high across all categories but was the highest among individuals with past eating disorders and lower among those in the "other" category. This could possibly be due to other motivations of those in the "other" group, such as advertising, as this group had the highest percentage of advertising in their posts. Those in the mental health group had the highest percentage of posts referencing discipline, however the mention of goals and guilt were similar across categories. Strikingly, those with past eating disorders mentioned body dissatisfaction or being critical of their bodies twice as often as those in the mental health and "other" group.

Images showed fewer differences across the categories, indicating that images posted between categories were very similar, however the differences in the text were more impactful. Those in the mental health category had bones visible the least amount of the time. However, we also saw an increase in the number of posts containing visible bones as the follower count increased. There were no real patterns when it came to the focus of the image, as they all hovered around the same percentage. Those with past eating disorders took the most pictures in a gym setting and had their hair/makeup done more than the other two groups. Those in the "other" category were pictured posing and flexing more than those in the mental health and eating disorder groups.

Other important observations included that a much higher percentage of individuals who experienced an eating disorder participated in bodybuilding, potentially indicating a correlation between bodybuilding, and eating disorder recovery.

A. Implications

These results indicate that fitspiration posts from individuals with past eating disorders send different, almost hidden and covert, messages than posts from individuals who mention mental health and those with no history of an eating disorder or mention of mental health. The messages are hidden within the text portion of the post, as images are similar across all categories. This could indicate that the text is more powerful than the image, as the text provides a context in which the image is interpreted.

There are more negative associations with body image, used as a reason to work out among individuals with past eating disorders. This is supported by Christensen, Forbush, Cushing, Lejuez, Fleming & Romine, 2021 as they mention that negative body image often mediates the relationship between social media and eating disorders. When looking at the population of bodybuilders it could be argued that there is evidence of eating disorder symptomatology, disguised by the label of an athlete competing in a sport. Due to the fact that there was a higher percentage of individuals with past eating disorders that got involved in bodybuilding, one could reasonably make this conclusion. Past research highlights regimented routines are a key factor to eating disorder etiology (Davis, Blackmore, Katzman, & Fox, 2005; Meyer, Taranis, Goodwin & Haycraft, 2011). These are key elements of bodybuilding such as food, workouts, sleep, that are tracked in order to achieve a physical, visual result. As supported by Meyer, Taranis, Goodwin & Haycraft, 2011; Fietz, Touyz & Hay, 2014; Goodwin, Haycraft & Meyer, 2014; Hefner, Dorros, Jourdain, Liu, Tortomasi, Greene, Brandom, Ellet & Bowles, 2016, this is done regardless of negative consequences and mirroring eating disorder behavior. These results indicate a possible relationship between eating disorders and regimented fitness routines. Ultimately, we see that this does not represent the original motive for fitspiration. Instead, we see profiles cast as encouraging fitness and health but tainted by past experiences.

To contrast, those who advocate for mental health seem to better represent the original idea and motive of fitspiration; highlighting health rather than aesthetics. This is demonstrated through the use of more positive language than the other groups and less bones visible in their images. However, there is still more to investigate here. More research should be done in this area, regarding the relationship between past mental health struggles, such as depression, and getting involved in fitness. It has been shown that fitness can improve mental health as long as individuals do not fall into compulsive exercise behavior as stated by Davis, Blackmore, Katzman, and Fox (2005). It is also possible that individuals who have had mental health struggles do not put as much emphasis or focus on physical appearance results, contrasting the results from the eating disorder group. As demonstrated by the results, individuals in this category mentioned more language around a balanced lifestyle, progressing along their journey to good mental health and listening to your body. This creates a different narrative for their audience and themselves regarding what fitness means to them and what they deem as the "results" from working out. Ultimately the mental health group looked for improvement or "results" in areas other than physical appearance.

As mentioned previously, there is more to explore in this area. In this study, what stood out was the "other" category. This category did not follow any pattern and consistently had numbers that often fell in between the eating disorder and mental health groups. It should be noted that 15 out of the 31 influencers in the "other" category had or continue to have a troubled relationship with food, weight and/or body image. This could have impacted the content, as this was not considered when coding our descriptive categories. Further, this could indicate that there are undiagnosed, unadmitted issues these individuals are struggling with. This category and individuals with undiagnosed struggles need to be investigated in the world of fitspiration to identify how their past and current experiences are impacting their posts to a public audience.

B. Major Themes

There were three major themes discovered while examining posts: aesthetic focus, compulsive exercise enmeshed with body image standards, and evidence of tension during shifts in body image ideals; these themes were found across each of the three categories. Although individuals and their profiles were promoting fitness, many were looking for aesthetic evidence and results from working out. One individual, a part of the "other" category, captioned her photo "I went through many phases where I felt like crap and hated how I looked, even when I was working out because I felt like nothing was changing." Another individual, with a history of an eating disorder, posted a picture of her posing saying, "I'm bringing my best physique this year." In addition, multiple individuals across all categories discussed their struggle with body dysmorphia, struggling to appreciate what they see in the mirror despite working out. Lastly, one individual involved in bodybuilding and with a history of an eating disorder mentioned, "the look on stage is NOT healthy to maintain - don't let people trick you into thinking that it is." Each of these examples demonstrates how heavily the fitspiration world is focused on physical appearance, at many times viewing their bodies in a more negative light.

Throughout the analysis process there was evidence of compulsive exercise being linked to personal characteristics. Taking time off from the gym was linked with qualities of laziness and feelings of guilt, while qualities such as perseverance and discipline were linked with going to the gym. For example, one individual, with a history of an eating disorder, mentioned "I need to do more. I need to work harder. I need to be a better friend, family member, and partner. I need to serve my audience more. I need to feel like I am enough. The never ending cycle of needing to do more is exhausting." Another, who mentioned past struggles with mental health, made multiple references to the mantra "No excuses" or "No Days Off." Further, when one individual, part of the "other" category had to take time off to heal from an injury there were interpretations of guilt mentioned in her post as she apologized to her audience for not making it to the gym for a few weeks. In addition, one individual, with previous mental health struggles, mentioned how she was working out despite doctor's orders and what was in her best interest, "I'm between a rock and a hard place because it's not in my best interest to lift super heavy… when you love to push your body… it's hard to grasp the idea of taming that." All these captions disregard the importance of listening to your body and demonstrate a problematic relationship with exercise, linking it to personal qualities. One individual, with precious mental health struggles, captioned a photo with "proud but never satisfied" and another, with a history of an eating disorder, states "I'm starting to become proud [of my body]." Both these women appeared very fit, flexing their defined muscles, and were relatively thin. This idea creates a narrative for young women that they should always strive to be better than what they are visually and physically, and that staying the same is not good enough or valuable. This again, reinforces the pressure and applies one's worth to going to the gym, working out, and seeing results.

The third theme that was prevalent throughout posts from individuals, was the struggle women feel with the changing of body ideals, specifically highlighting the tension between moving from a thin ideal to a more curvy but strong ideal. One individual, with a past eating disorder, demonstrated how fitspiration body ideals still align with past thinspiration body ideals, as one of her workout videos was titled "how to make your waist smaller." In the same vein, across all categories, influencers were pictured showing off their defined abs, indicating that one can be strong and have muscles but must have a low enough body fat for your body to have certain proportions and muscles to be visible. Another individual, who was classified in the "other" category, conveyed the struggle of gaining muscle after being conditioned to be "thin and light" for so many years. Further, she discusses being proud of her body but discounts herself by mentioning how the presence of "unwanted body fat" makes her feel negatively. These examples capture the irony of fitspiration. The intended motive of fitspiration was to rebel against the thin ideal for women, however, it has become an advocate for low body fat percentages to highlight muscular results that represent hard work, perseverance and discipline and that one is the opposite of lazy.

C. Limitations

Several limitations impact our study, including the Instagram algorithm. What is shown on the feed page is based on previous engagement on the app. We realize that this played an influential role in our selection process, making the sample not truly random. However, this was an essential element to capture, as this mimics the Instagram user's experience. For example, if an individual looks up posts about fitness on the app, the app will direct other posts involving fitness to her feed the next time she uses the app. Therefore, we selected our population for analysis using the "feed" feature.

In addition to the Instagram algorithm, another limitation was the unknown nature of diagnoses. Those who mentioned an explicit diagnosis or explicit struggle with mental illness were categorized accordingly. However, individuals who did not mention a specific condition were categorized as "other". This study did not account for individuals who had struggled in the past but did not mention it on their profile. As stated previously, this is a plausible reason for why we see results in the "other" category consistently between the eating disorder and mental health group.

VII. CONCLUSION

This research demonstrates that fitness influencers with a history of eating disorders use different language and convey different messages than those with past mental health struggles and those with no history. Those with a history of eating disorders used more eating disorder coded language and demonstrated double the amount of body dissatisfaction messaging than the other two groups. Despite its intention, not all fitspiration messaging is healthy and therefore is not necessarily a healthy alternative to thinspiration messaging. It has also been revealed that fitspiration influencers who advocate for mental health demonstrate a more idealistic representation of fitspiration material, as it was intended. There is still more research needed in this area. Future research needs to consider the intersecting scenarios of gender, culture, ethnicity, and socioeconomic status. Any one of these characteristics can impact or be impacted by social media and body standards, causing unique distress or unique success for an individual. Future research also needs to focus on the individuals with undiagnosed struggles. We cannot discount the fact that many people struggle with weight, food, body image, etc. but did not receive an official diagnosis or were not deemed "sick enough" to receive help. Future research should examine whether eating disorders/mental health issues are being disguised in the fitness and fitspiration arena? In our research we have seen that the messaging is different, but we do not know if these individuals continue to struggle. One could infer from the results that there is still evidence of eating disorder behavior and thought patterns, however without interviewing individuals this is difficult to ascertain. We conclude that as the presence and scale of social media continues to grow, its effects need to be investigated in order to reveal the covert dynamics and influence taking place.

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