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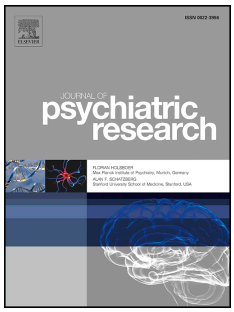

Increased brain vitamin D receptor expression and decreased expression of cathelicidin antimicrobial peptide in individuals who died by suicide

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Increased Brain Vitamin D Receptor Expression and Decreased Expression of Cathelicidin Antimicrobial Peptide in Individuals Who Died by Suicide

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Increased Brain Vitamin D Receptor Expression and Decreased Expression of Cathelicidin, an Antimicrobial Peptide, in Individuals Who Died by Suicide

Abstract

Vitamin D deficiency is associated with immune dysregulation, increased vulnerability to infections, depression, and suicidal behavior. One mediator of vitamin D-dependent immune regulation and antimicrobial defense is the cathelicidin antimicrobial peptide (LL37), encoded by the cathelicidin-related antimicrobial peptide (*CRAMP)* gene. We compared the mRNA expression of the *CRAMP* gene, the vitamin D receptor (*VDR*) gene, as well as the *CYP27B1* and *CYP24A1* genes (involved in vitamin D metabolism) in the dorsolateral prefrontal cortex (dlPFC) and anterior cingulate cortex (ACC) between depressed individuals who died by suicide (n = 15) and matched (age, gender, and post-mortem interval) non-psychiatric controls (n = 15). Gene expression was measured through qRT-PCR with TaqMan® primers and probes, with GAPDH and β-actin genes as endogenous controls. Statistical analyses included *t*-tests and Pearson correlations. *CRAMP* mRNA expression was downregulated and *VDR* mRNA expression was upregulated in both dlPFC and ACC in suicides relative to controls, with no significant differences in expression of *CYP24A1* and *CYP27B1*. To our knowledge, this is the first study on brain cathelicidin expression in the human brain in relationship to suicide. Increased *VDR* and decreased *CRAMP* expression are consistent with previously reported associations between vitamin D deficiency, immune dysregulation, and suicidal behavior, and should lead to future studies uncovering novel interactive targets for suicide prevention.

Keywords: Cathelicidin-Related Antimicrobial Peptide (*CRAMP*); Suicide; Vitamin D; Vitamin D Receptor

Introduction

Suicide, an intentional act of ending one's life, is the 10th leading cause of death in the United States (Curtin et al., 2016; Stone et al., 2018). In 2016, there were nearly 45,000 suicides, and costs related to suicidal behavior and self-injury were estimated to be around $69 billion (Murphy et al., 2018). Since 1999, the suicide rate has increased by 30% in the US (Stone et al., 2018), which is in contrast to the national goal established by the American Foundation for Suicide Prevention and the National Action Alliance of Suicide Prevention of reducing the annual suicide rate by 20% by 2025 (Office of the Surgeon and National Action Alliance for Suicide, 2012). Such statistics and apparent deficiencies in suicide prevention strategies make suicide a national health concern that needs novel comprehensive approaches for effective prevention, risk management, and improved prognostic outcomes (WHO, 2014).

Suicide is a complex behavior with diverse etiopathogenic mechanisms ranging from distal factors such as family history (Brent and Melhem, 2008; Brodsky et al., 2008) and early-life adversity (Turecki et al., 2014) to more proximal factors such as symptoms of psychopathology, including anhedonia and hopelessness (Beck et al., 2006; Kovacs and Garrison, 1985; Sudol and Mann, 2017) and stressful life events (Turecki et al., 2014). Distal factors act in the context of dynamic gene-environment interactions to set up vulnerable (intermediate) brain phenotypes that, in the presence of proximal triggers, can lead to suicide attempts (Turecki and Brent, 2016). There is a great need to identify underlying neurobiological mechanisms and intermediate brain phenotypes for suicidal behavior, which may lead to improved prediction and targeted interventions in specific subgroups of people at risk (Brundin et al., 2017; Zalsman et al., 2016).

In recent years, inflammation has been increasingly implicated in suicidal behavior, independent of its effects on underlying mental illness such as depression (Holmes et al., 2018; O'Donovan et al., 2013; Postolache et al., 2016; Torres-Platas et al., 2014). The inflammatory hypothesis of suicide proposes a prolonged low-grade immune activation potentially contributory to suicidal ideation and behavior, as evidenced by certain molecular and cellular biomarkers of inflammation (Janelidze et al., 2011). Although there is considerable heterogeneity among specific molecular biomarkers, the pleotropic cytokine interleukin (IL)-6 has been most robustly associated with suicidal behavior in a meta-analysis (Gananca et al., 2016). In another meta-analysis, peripheral levels of the proinflammatory cytokine IL-1β and IL-6 were found to be significantly increased in patients with psychiatric disorder and history of suicidal ideation and behavior as compared to non-suicidal psychiatric patients (Black and Miller, 2015). Other molecular changes include decreases in IL-2 and increases in acute phase reactants including C-reactive protein (CRP) and certain proinflammatory cytokines including tumor necrosis factor (TNF) (Brundin et al., 2017; Postolache et al., 2016). Cellular biomarkers such as increased blood granulocyte counts (Keaton et al., 2019) and microglia and astrocyte activation in the anterior cingulate cortex (Brisch et al., 2017; Steiner et al., 2008; Torres-Platas et al., 2014) have also been associated with suicidal behavior. In post-mortem studies, Tonelli et al. (2008) and Pandey et al. (2012) first reported increased cytokine mRNA expression (Tonelli et al., 2008) and mRNA & protein levels (Pandey et al., 2012) respectively, in the prefrontal cortex of depressed individuals who died by suicide as compared to non-psychiatric controls. Recently, Pandey et al., (2019) have reported an increased association of suicide with mRNA and protein expression of certain toll-like receptors (TLRs) that are induced by molecular markers of infection (also known as pathogen-associated molecular patterns, PAMPs) and damage-

associated molecular patterns (DAMPs), in the prefrontal cortex of suicide cases relative to psychiatric and non-psychiatric controls (Pandey et al., 2019). It is important to ascertain that inflammation had been associated with suicidal behavior beyond its association with mental illness as demonstrated by statistical adjustments for psychiatric symptoms, especially in longitudinal paradigms, and by using psychiatric, rather than healthy controls (Brundin et al., 2017). Similarly, molecular cascades activated by inflammation, such as the kynurenine pathway, leading to molecular signaling that has been previously associated with suicidal behavior, have been linked with suicidal behavior beyond mediation, at least in part, by mental illness and its severity. This has been dissected either by study design, or by statistical adjustment for symptom severity (Brundin et al., 2016; Erhardt et al., 2013; Sublette et al., 2011). Considering the role in molecular signaling and interactions with multiple downstream cellular effectors of the immune response, several reports provide a mechanistic link between suicidal behavior and common immune-mediated conditions such as infections (Gjervig Hansen et al., 2019; Lund-Sorensen et al., 2016), including infections with *Toxoplasma gondii* (Arling et al., 2009; Pedersen et al., 2012; Sutterland et al., 2019; Zhang et al., 2012), cytomegalovirus (CMV) (Burgdorf et al., 2019), and influenza (Okusaga et al., 2011), as well as allergy (Postolache et al., 2008; Qin et al., 2011), aeroallergen exposure (Postolache et al., 2004; Qin et al., 2013; Stickley et al., 2017), autoimmune disorders (Benros et al., 2013; Chwastiak et al., 2002; Feinstein, 2002; Xie et al., 2012), traumatic brain injury (TBI) (Brenner et al., 2013; Madsen et al., 2018; Teasdale and Engberg, 2001), and psychological stress (Garate et al., 2013; Pittenger and Duman, 2008). Among these studies, several have identified a privileged link with suicidal behavior by finding associations with immune mediated conditions in individuals without a prior existing diagnosis of mental illness (Qin et al., 2011) or resisting adjustment for a

diagnosis of mental illness (Pedersen et al., 2012) or for severity of symptoms of mental ilness (Zhang et al., 2012).

Considering the inflammatory hypothesis of suicide, it would be expected that conditions associated with immune-dysregulation contribute to the risk of suicide. For example, vitamin D deficiency could be associated with suicidal behavior via immune dysregulation (Chun et al., 2014; Fletcher et al., 2019; Harrison et al., 2019; Hewison, 2012a; Laird et al., 2014), and indirectly through autoimmunity, and increased vulnerability to infections (Bacchetta et al., 2014; Fabri et al., 2011). Indeed, very low levels of serum 25-hydroxyvitamin D (calcidiol or 25-hydroxycholecalciferol; 25(OH)D), in the deficient range, have been reported to be associated with an increased risk for suicidal behavior (Grudet et al., 2014; Park et al., 2016; Umhau et al., 2013). Umhau et al. (2013) reported that although mean serum 25(OH)D levels were not significantly different between those who died by suicide and controls, those in the lowest octile of season-adjusted 25(OH)D (<15.5 ng/mL) had a higher risk of suicide than the rest (Umhau et al., 2013). Grudet et al. (2014) found low 25(OH)D levels in individuals with history of suicide attempt relative to both healthy as well as depressed non-suicidal controls. Furthermore, 25(OH)D levels were negatively associated with blood levels of proinflammatory cytokines (Grudet et al., 2014).

Traditionally known for its role in skeletal homeostasis (DeLuca, 1982), vitamin D is now well recognized as an important immunomodulator and neuroprotective agent (Adams and Hewison, 2008; Liu et al., 2007; Munger et al., 2004). The active form, calcitriol (1,25-dihydroxyvitamin D, $1,25(OH)_2D$), binds to the vitamin D receptor (VDR) and forms a complex with retinoid X receptor (Goltzman et al., 2018; Issa et al., 1998). This complex then translocates to the nucleus, where it can bind to the promoter region of targeted genes and can interact with

other transcription factors leading to repression or activation of transcription (Goltzman et al., 2018; Issa et al., 1998). Several studies have suggested that 1,25$(OH)_2$D may increase the levels of anti-inflammatory cytokines such as IL-10 and decrease proinflammatory cytokines such as IL-1ß, IL-12, IL-17, interferon (IFN)-γ and TNF (Baeke et al., 2010; D'Ambrosio et al., 1998; Heine et al., 2008; Staeva-Vieira and Freedman, 2002; Tang et al., 2009). In addition, a potentially important, yet understudied, mechanism by which vitamin D exerts its regulatory and anti-infectious effects is the induction of antimicrobial peptides, which are potent and broad spectrum agents that fend off viruses, bacteria, fungi, and protozoa (De Smet and Contreras, 2005; Zasloff, 2019). In humans, 1,25$(OH)_2$D increases the production of cathelicidin LL37 (Gombart et al., 2005), a C-terminal cleavage product of 18 kDa protein (hCAP-18) (Sorensen et al., 1997; Sorensen et al., 2001) and an anti-microbial peptide encoded by the cathelicidin-related antimicrobial peptide (*CRAMP)* gene, that activates innate mechanisms to fight intracellular infection, especially in combination with activation of macrophages by pathogens (Liu et al., 2006).

Human cathelicidin antimicrobial peptide LL-37, along with α-defensins and β-defensins, constitute one of the evolutionarily ancient and highly effective innate host defenses against pathogens (Hancock et al., 2016; Zasloff, 2019). Generally, antimicrobial peptides (AMPs) are short, cationic, and amphipathic peptides that affect integrity of bacterial membranes through depolarization (Anderson et al., 2004), puncture (Bucki et al., 2010), activating degradation, and redistribution of lipids in the lipid bilayer (Bandurska et al., 2015; Basanez et al., 2002). In addition, cathelicidin peptide has been shown to decrease inflammation by limiting activation of dendritic cells (Kandler et al., 2006) and decreasing TNF production in M1 and M2 macrophages (Brown et al., 2011). Although the understanding of the role of antimicrobial peptides in

immunology has been increasing, there is, to our knowledge, limited research that investigates their potential participation in brain structure and function. In animal models, glial cells and astrocytes express the cathelicidin gene (*CRAMP)* (Brandenburg et al., 2008), where it plays an important role in innate immunity against pathogens causing bacterial meningitis. Cathelicidin LL37 protein has also been found in the cerebrospinal fluid (CSF) of patients with acute bacterial meningitis (Brandenburg et al., 2008) and in cerebral abscesses (Hassel et al., 2018). One study by Lee et al. (2015) reported cathelicidin LL37 protein expression in substantia nigra and sensory cortex of post-mortem human brain, which was relatively upregulated in the brains of patients with Alzheimer's disease as compared to healthy controls (Lee et al., 2015). In addition, lipopolysaccharide (LPS) and IFN-γ induced expression of cathelicidin LL37 in human astrocytes and microglia in cultured cell lines (Lee et al., 2015). However, there are no available published studies of its expression in the dorsolateral prefrontal cortex (dlPFC) and anterior cingulate cortex (ACC) of the human brain, including investigations in individuals with suicide or other suicidal behaviors. Since both suicide and vitamin D deficiency have been linked to inflammation (Brundin et al., 2017; Grudet et al., 2014; Laird et al., 2014; Postolache et al., 2016), it is worth exploring the role of inflammation in general, and the potential interaction of cathelicidin and vitamin D deficiency with suicidal behavior.

In this study, we estimated cathelicidin activity via *CRAMP* mRNA levels and brain vitamin D biology by measuring *VDR*, *CYP27B1* (cytochrome P450 family 27 subfamily B member 1), and *CYP24A1* (cytochrome P450 family 24 subfamily A member 1) mRNA expression. Specifically, we hypothesized that *CRAMP* mRNA is expressed in the dlPFC and ACC of human brain and that *CRAMP* mRNA is downregulated while *VDR*, *CYP27B1*, and

CYP24A1 are upregulated in individuals with depression who died by suicide as compared to those who died by other causes.

Methods

Human postmortem brain studies

Participants

The study was determined as exempt by the Institutional Review Board of the University of Alabama at Birmingham. Brain tissues were obtained from the Quebec Suicide Brain Bank as described in detail in previous studies (Lopez et al., 2014; Smalheiser et al., 2012). The activities of the Quebec Suicide Brain Bank were approved by the Douglas Hospital McGill University IRB. Family members/informants signed written informed consents. The study was performed in dlPFC (Brodmann area 46) and ACC (Broadmann areas 24, 32, and 33) obtained from the right hemisphere of 15 non-psychiatric controls (further referred to as controls) and 15 depressed individuals who died by suicide (further referred to as cases). Selection of dlPFC and ACC of the right hemisphere was based on previous studies implicating these brain regions in suicidal behavior (Dwivedi, 2012; Fiori and Turecki, 2012; Torres-Platas et al., 2014; Torres-Platas et al., 2011). The methods of suicide included hanging, jumping from height, poisoning (carbon monoxide), and overdosing (drug), as shown in **Table 1**. Normal controls died by cardiac arrest, vehicle accident, or accidental drug overdose. dlPFC and ACC were identified and dissected from respective neuroanatomical regions by using reference neuroanatomical maps. Gyri and sulci were used to landmark specific frontal cortical areas.

Psychiatric diagnoses of the subjects were made by psychological autopsy based on DSM IV criteria, using structured clinical interview for DSM-IV (SCID-i) (First and Gibbon,

2004), as described in detail in previous studies (Lopez et al., 2014; Smalheiser et al., 2012). Both cases and controls were characterized by the same psychological autopsy methods, therefore avoiding the occurrence of systematic biases.

RNA isolation

Total RNA was isolated from frozen tissue using the TRIzol method (Invitrogen, Grand Island, NY, USA) (Rio et al., 2010). Briefly, ~30 mg of frozen tissue was immediately transferred to a pre-chilled 1.5 ml Eppendorf tube. Initially, 500 µl TRIzol was added to the tube and homogenized with a mechanical tissue homogenizer with repeated strokes until the homogenate looked apparently free of tissue clumps. Then, additional TRIzol was added to the homogenate to make the final volume 1 ml. The sample was pipetted gently to mix up the homogenate and incubated at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. Afterward, 200 µl of chloroform was added to the homogenate for phase separation and incubated at room temperature for 3 more minutes followed by high-speed centrifugation at 13,000 RPM for 15 minutes at 4 °C. The aqueous phase was carefully transferred to a fresh tube and an equal volume of isopropanol and 1 µg of glycogen (Roche Life Science, Indianapolis, IN, USA) were added. Alcohol precipitation of RNA was carried out overnight at –20 °C and the precipitated RNA was then washed with 70% alcohol. Finally, RNA was resuspended in nuclease-free water using a volume that was based on the size of the pellet. The yield of RNA was determined by measuring the O.D. at 260/280 nm. Samples with a ratio below 1.8 were rejected. The integrity of RNA was checked by the Agilent 2100 Bioanalyzer. Only samples with an RNA integrity number (RIN) >8 were used.

qRT-PCR

The mRNA expression levels of *CRAMP*, *VDR*, *CYP27B1* and *CYP24A1* in the dlPFC and ACC were compared between cases (n = 15) and non-psychiatric controls (n = 15). TaqMan® primers and probes were used, with GAPDH and β-actin genes as endogenous controls. Briefly, mRNA levels were determined using a two-step qPCR. One µg of total RNA was reverse transcribed using 50 ng random hexamers, 2 mM dNTP mix, 10 U ribonuclease inhibitor, and 200 U MMLV-reverse transcriptase enzyme in a final reaction volume of 20 µL. The primer/probe sets for all target genes and endogenous controls were obtained from ABI (Foster City, CA, USA) as the TaqMan Gene Expression Assay kit (*CRAMP*: Hs00189038_m1; *VDR*: Hs00172113_m1; *CYP27B1*: Hs01096154_m1; *CYP24A1*: Hs00167999_m1). To determine the linear range and sensitivity of the kits, a standard curve was generated using serial 10-fold dilutions of pooled cDNA derived from at least 5 normal control subjects amplified in duplicates by qPCR reactions. Only those PCR reactions showing efficiencies above 95% were considered acceptable. All genes tested had similar efficiencies as the endogenous controls and were run in parallel with the endogenous controls. The PCR reaction was carried out in a final volume of 20 µl, containing 5 µl of cDNA diluted 1:10 with DEPC water, 1x TaqMan primer/probe mix and 1x TaqMan® Universal PCR Master Mix (ABI). For each primer/probe tested, the PCR reaction also included a non-reverse transcription negative control to confirm the absence of genomic DNA, and a non-template negative control to check for primer-dimer formation. All experiments were performed in duplicate as follows: denaturation at 95 °C for 10 min followed by 40 cycles of a two-step program (denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min on the Mx3005p. The amounts of target genes expressed were normalized to the geometric mean of β-actin and GAPDH. Fold changes between subject

groups were measured using the 2-ΔΔCT method, where ΔΔCT = (CT target - CT normalizer) sample - (CT target - CT endogenous gene) control (Livak and Schmittgen, 2001).

Statistical analysis

Data were analyzed with SPSS (version 23; IBM, Armonk, NY, USA). Comparison between cases and controls was performed by using independent-sample *t* tests. Correlations between mRNAs with covariates were determined using Pearson product-moment correlation analyses. *P* values ≤ 0.05 were considered statistically significant.

Results

Demographic characteristics: The demographic characteristics of cases and controls are provided in **Table 1**. Mean age of cases and controls was 36.66 ± 3.28 years and 39.00 ± 3.80 years, respectively. Of all the individuals who died by suicide, 2 showed positive antidepressant toxicology. There were no significant differences in age ($t = 0.46$, df = 28, $p = 0.64$), PMI ($t = 0.05$, df = 28, $p = 0.96$), brain pH ($t = 1.21$, df = 28, $p = 0.77$), or RIN ($t = 0.30$, df = 28, $p = 0.24$) between suicide cases and normal controls (**Table 1**).

mRNA expression: The expression of *CRAMP*, *VDR*, *CYP27B1*, and *CYP24A1* were determined in two brain areas, dlPFC and ACC, by qRT-PCR. It was observed that the mRNA level of *CRAMP* was significantly downregulated in both dlPFC ($t = 2.59$, df = 28, $p = 0.015$) and ACC ($t = 4.19$, df = 28, $p < 0.001$) of individuals who died by suicide compared to normal controls. On the other hand, mRNA level of *VDR* was significantly upregulated in both these brain areas of cases (dlPFC: $t = 2.54$, df = 28, $p = 0.017$; ACC: $t = 2.85$, df = 28, $p = 0.008$). For both *CRAMP* and *VDR*, the degree of change was slightly greater in ACC (*CRAMP*: 0.40-fold; VDR: 2.46-fold) compared with dlPFC (*CRAMP*: 0.54-fold; *VDR*: 2.11-fold). Expression levels

of *CYP27B1* and *CYP24A1* were not significantly different between cases and normal controls either in the dlPFC (*CYP24B1*: $t = 0.007$, df = 28, $p = 0.99$; *CYP27A1*: $t = 0.08$, df = 28, $p = 0.93$) or the ACC (*CYP24B1*: $t = 0.46$, df = 28, $p = 0.65$; *CYP27A1*: $t = 0.55$, df = 28, $p = 0.59$).

Effects of confounding variables: Age, PMI, brain pH, or RIN had no significant impact on expression of *CRAMP*, *VDR*, *CYP27B1*, and *CYP24A1* genes in dlPFC and ACC, when compared between cases and controls. Similarly, age, PMI, or RIN had no significant correlation with expression levels of any of the genes when cases and controls were combined for analysis. Only brain pH had a significant negative correlation with any gene expression variables, specifically with *VDR* gene expression ($p = 0.045$), when cases and controls were combined for analysis (**Table 2**). Of 15 individuals who died by suicide, two had positive antidepressant toxicology at the time of death. However, mean gene expression levels were not significantly different between those who showed positive antidepressant toxicology and those who did not (data not shown).

Discussion

To our knowledge, this is the first study that has specifically focused on *CRAMP* and *VDR* in suicide. We observed decreased expression of the *CRAMP* gene and increased expression of the *VDR* gene in the brains of individuals with depression who died by suicide relative to non-psychiatric controls. In addition, we did not find any significant differences in the expression of *CYP27B1* (1-alpha-hydroxylase) or *CYP24A1* (24-hydroxylase) genes, which encode key enzymes involved in the synthesis and degradation, respectively, of $1,25(OH)_2D$. Although this is a novel post-mortem study in suicide, Jiang et al. (2013) have previously

reported increased expression of *CYP27B1, CYP24A1* and *VDR* as well as higher hippocampal $1,25(OH)_2D$ levels in rats showing depressive-like symptoms after exposure to chronic unpredictable mild stress (Jiang et al., 2013).

Vitamin D receptors (VDR) are widely distributed in the brain and found in neurons (Bolde et al., 2020), astrocytes (Landel et al., 2018), oligodendrogliocytes (Baas et al., 2000), and microglia (Eyles et al., 2005), which can be activated by $1,25(OH)_2D$ to induce proliferation, differentiation, neuroplasticity, as well as neuroprotection through anti-inflammatory effects (Garcion et al., 2002). The exact mechanisms for regulation of the *VDR* gene remain to be fully elucidated. Current evidence suggests roles for diverse environmental, genetic, and epigenetic factors (Saccone et al., 2015). One key regulator of the *VDR* gene is $1,25(OH)_2D$ itself, which is produced by sequential hydroxylation of vitamin D in the liver (25-hydroxylation) and kidney (1-hydroxylation). The prohormone precursor of $1,25(OH)_2D$, cholecalciferol (vitamin D), is made in the skin through UVB exposure and obtained from the diet. $1,25(OH)_2D$ binds to the VDR/retinoid X receptor heterodimer, which then binds to vitamin D response elements around the *VDR* gene to induce its transcription through a transcriptional autoregulation mechanism (Zella et al., 2006; Zella et al., 2010). Epigenetic modifications such as histone modification (Kim et al., 2005) and hypermethylation of the *VDR* gene promoter (Marik et al., 2010), as well as microRNA (miR125b) regulation of *VDR* gene expression (Mohri et al., 2009) have also been observed. Yet, not only can vitamin D deficiency be predictively linked with activation of the immune system (Kruit and Zanen, 2016; Mellenthin et al., 2014; Murr et al., 2012), but also the activation of immune mechanisms actively lowers vitamin D levels and alters *VDR* expression (Coleman et al., 2016; Kim et al., 2013; Silvagno et al., 2010). Proinflammatory states such as autoimmune disorders (Ayuso et al., 2017; Kim et al., 2013), infections (Coughlan et al., 2012;

Liu et al., 2012), inflammation (Agrawal et al., 2012), and tumors (Sertznig et al., 2009; Silvagno et al., 2010) are associated with changes in *VDR* expression, often influenced by vitamin D levels. For example, LPS, a glycolipid that is produced and secreted by gram-negative bacteria, modulates *VDR* expression differently based on the presence of vitamin D deficiency (Gambhir et al., 2011; Pramanik et al., 2004). Pramanik et al. (2004) showed that LPS downregulated 1,25$(OH)_2$D-induced *VDR* protein expression in THP-1 cells, a human blood monocytic cell line. In addition, although both LPS and 1,25$(OH)_2$D independently stimulate *VDR* mRNA expression, VDR protein levels are not increased after LPS stimulation, suggesting a simultaneous LPS-mediated inhibition at the translational or post-translational level (Pramanik et al., 2004). Similarly, Coleman et al. (2016) reported that both 1,25$(OH)_2$D and LPS stimulate *VDR* transcription in peripheral blood mononuclear cells (PBMCs) of vitamin D-replete healthy older adults (age > 50 years), but there was a negative correlation between serum 25(OH)D levels and LPS-induced *VDR* mRNA expression levels (Coleman et al., 2016). This might be explained by the differential regulation of the vitamin D pathway in immune cells where LPS has been shown to stimulate the constitutive expression of 1α-hydroxylase that converts 25(OH)D to 1,25$(OH)_2$D (Fritsche et al., 2003), resulting in lower serum 25(OH)D levels, especially in laboratory blood samples deprived of skin sources of vitamin D.

Proinflammatory cytokines, in particular TNF, also modulate *VDR* mRNA expression in a manner similar to LPS (Ziv et al., 2016). In addition, Boontanrart et al. (2016) showed that microglia, when activated by IFN-γ or LPS, not only express proinflammatory cytokines, chemokines, and effector molecules, but also increase the expression of *VDR* and *CYP27B1* (1α-hydroxylase enzyme that converts 25(OH)D to activated 1,25$(OH)_2$D) (Boontanrart et al., 2016). Thus, when activated microglia are exposed to 25(OH)D, the expression of proinflammatory

cytokines is decreased and expression of anti-inflammatory cytokine IL-10 is increased through the influence of $1,25(OH)_2D$ signaling on cytokine gene expression (Boontanrart et al., 2016). Unfortunately, we did not collect data on VDR protein, blood 25(OH)D levels, or inflammation markers. Therefore, no conclusions regarding the source of increased *VDR* mRNA levels in this study can be reached. However, considering the fact that suicide is associated with a proinflammatory state and vitamin D deficiency, it is plausible that upregulation of *VDR* mRNA levels in dlPFC and ACC of depressed individuals who died by suicide might be due, in part, to vitamin D deficiency (Grudet et al., 2014; Umhau et al., 2013) and dysregulated inflammation (Brundin et al., 2017; Hewison, 2012b; Lagishetty et al., 2011; Postolache et al., 2016; Schwalfenberg, 2011). This may provide an opportunity for interventions with either vitamin D, calcitriol, or anti-inflammatory therapy to mitigate the risk of suicide, to be tested in future studies (Tariq et al., 2011).

It is worth mentioning here that, apart from suicidal behavior, major depressive disorder, a significant risk factor for suicidal behavior, has also been associated with low vitamin D levels (Anglin et al., 2013; de Oliveira et al., 2018; Lee et al., 2011; Milaneschi et al., 2010; Spedding, 2014; von Kanel et al., 2015), cathelicidin (Kozlowska et al., 2017), and dysregulated inflammation (Haapakoski et al., 2015; Howren et al., 2009; Köhler et al., 2017; Kohler et al., 2014; Schiepers et al., 2005). For example, an inverse association between prenatal log 25(OH)D levels and post-partum depressive symptoms was found in a prospective study of 91 pregnant African American women, which was moderated by IL-6 and IL-6/IL-10 ratio (Accortt et al., 2016). Similarly, there was an increase in blood IL-6 and TNF and a marked decrease in 25(OH)D in individuals with both depression and Alzheimer's disease (AD) as compared to healthy controls and AD patients without depression (Banerjee et al., 2017). Since all of the

cases in our study who died by suicide were also diagnosed with major depressive disorder, it is uncertain whether the findings of this study are specific to suicide or depression. More research having psychiatric controls is needed to further enhance our understanding of inflammatory mechanisms and vitamin D signaling in both depression and suicide.

The other two key findings of our study were: 1) the identification of *CRAMP* mRNA expression in brain regions with a major role in behavioral regulation and dysregulation; and 2) the downregulation of *CRAMP* mRNA in the dlPFC and ACC of individuals with depression who died by suicide. Human LL-37, a C-terminal cleavage product of the 18 kDa protein (hCAP-18) encoded by *CRAMP* (Sorensen et al., 1997; Sorensen et al., 2001), is a part of the innate immune system and has mainly been studied in relation to infections and autoimmune diseases (Bandurska et al., 2015). *CRAMP* is expressed in multiple cell types including epithelial cells (Hase et al., 2002), keratinocytes (Frohm et al., 1997), microglia, and astrocytes (Brandenburg et al., 2008). Kozłowska et al. (2017) reported that elderly depressed patients had higher serum LL-37 protein levels than healthy subjects (Kozlowska et al., 2017), but this was only detected in a small sub-sample of patients. In addition, it is unclear whether there is a strong correlation between serum and CNS levels of LL-37. Although previous studies detected the expression of *CRAMP* in human brain (Lee et al., 2015; Xu et al., 2018), this is the first study that has measured changes in *CRAMP* expression in post-mortem human brain samples of individuals who died by suicide.

The mechanisms involved in regulation of *CRAMP* expression have not been fully elucidated. It is expressed constitutively in epithelia while expression in immune cells is induced by various factors such as TLRs (Liu et al., 2006), TNF (Kim et al., 2009), LPS, calcipotriol (a synthetic derivative of $1,25(OH)_2D$) (Kim et al., 2009), phenylbutyrate (Mily et al., 2013), and

endoplasmic reticulum stress (Park et al., 2011). We found that, despite upregulation of *VDR* mRNA, *CRAMP* mRNA was downregulated. In this regard, Kim et al. (2009) reported that *LL-37* mRNA and protein expression was upregulated in keratinocytes following exposure to UVB radiation and treatment with calcipotriol, LPS or TNF. However, when calcipotriol was applied to keratinocytes already exposed to UVB, LPS, or TNF, *LL-37* mRNA and protein expression was suppressed (Kim et al., 2009). Therefore, it can be postulated that $1,25(OH)_2D$ stimulates cathelicidin *LL37* expression under non-inflammatory conditions while it suppresses the expression under inflammatory conditions. Recently, Wang et al. (2018) reported that *TNF* was upregulated in the prefrontal cortex of individuals with depression who died by suicide (Wang et al., 2018). Consistent with the results of these studies, it is possible that upregulated *VDR* mRNA expression in the prefrontal cortex decreases the expression of *CRAMP* in the presence of increased TNF. The alternative explanation is that vitamin D deficiency (low 25(OH)D levels) leads to underexpression of *CRAMP* and, secondarily, receptivity to reactivation of latent infections (Biswas et al., 2017) as well as more intense and prolonged inflammation. Although it is generally presumed that the relationship between vitamin D and inflammation is unidirectional, i,e, vitamin D deficiency leads to increased inflammation, there may well be a bidirectional, and possibly cascading, relationship between the two; i.e., low serum 25(OH)D levels can also result from underlying inflammation, in addition to nutritional deficiency or reduced sunlight exposure (Autier et al., 2014; Mangin et al., 2014). For example, low serum 25(OH)D levels have been reported in sarcoidosis (Berlin et al., 2014; Sage et al., 2011), an autoimmune disease known to have increased macrophagic expression of 1α-hydroxylase (Adams and Gacad, 1985; Adams and Hewison, 2012; Barbour et al., 1981) as well as increased serum $1,25(OH)_2D$ levels (Insogna et al., 1988; Zimmerman et al., 1983). Similarly, low serum

25(OH)D and increased or normal 1,25$(OH)_2$D levels have been found in Crohn's disease (Abreu et al., 2004; Joseph et al., 2009) and systemic lupus erythematosus (SLE) (Amital et al., 2010; Muller et al., 1995). Since studies of vitamin D in depression and suicide have measured serum 25(OH)D only, it remains to be explored whether such discrepancy between serum 25(OH)D and 1,25$(OH)_2$D exists in depression and suicide. It is interesting to note here that despite modest evidence of low serum 25(OH)D levels associated with depression (Almeida et al., 2015; Anglin et al., 2013; de Oliveira et al., 2018; Spedding, 2014), randomized controlled trials (RCTs) of vitamin D supplementation have reported mixed results in improving depressive symptoms (Erhard et al., 2017; Gowda et al., 2015; Shaffer et al., 2014; Vellekkatt and Menon, 2019). This discrepancy may also point to the possibility that low serum 25(OH)D levels in depression reflect increased extra-renal conversion of 25(OH)D to 1,25$(OH)_2$D, and hence a marker of immune activation rather than true deficiency (Autier et al., 2014; Mangin et al., 2014). Thus more research studies, having longitudinal and, perhaps, interventional designs with measurements of both serum 25(OH)D and 1,25$(OH)_2$D, are needed to uncover complex interplay among vitamin D, cathelicidin, and inflammatory pathways.

One major limitation of our study is that we did not perform a western blot analysis to measure protein levels of VDR, CRAMP/LL37, CYP27B1, or CYP24A1, necessitating more research to identify regulatory mechanisms involved in *VDR* and *LL-37* expression at translational or post-translational levels. In addition, our study had a small sample size and no data were available for blood or CSF 25(OH)D or 1,25$(OH)_2$D levels, or peripheral inflammation markers.

Although this study is a preliminary observation that needs replication, it provides a novel molecular target, i.e. cathelicidin, as interfacing between vitamin D deficiency and deficits

in immune regulation and infection control, potentially contributing to mood disorders and suicide.

In conclusion, the findings of elevated *VDR* and lower *CRAMP* mRNA expression in the brains of individuals with depression who died by suicide supports the growing body of evidence that distinct inflammatory mechanisms may be involved in depression and suicide and may be modulated by vitamin D metabolites. More research is needed to understand whether association of severe hypovitaminosis D with suicide is independent of its association with depression and whether vitamin D deficiency reflects merely a nutritional deficiency that can be corrected by supplementation, or is an indicator of perturbations of complex inflammatory mechanisms, involving cathelicidin-related innate immune dysfunction, that will require different anti-inflammatory strategies to resolve.

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Table 1: Demographic and clinical characteristics of non-psychiatric controls and individuals who died by suicide

	Non-psychiatric Controls	Cases Suicide	Statistical Analysis
Number of Subjects	15	15	N/A
Psychiatric diagnosis	None	MDD	N/A
Age (years)	36.66 ± 3.28	39.00 ± 3.80	$t = 0.46$, df = 28, $p = 0.65$
Gender **Males**	15	13	N/A
Females	0	2	N/A
Postmortem interval (h)	34.66 ± 5.05	35.00 ± 4.09	$t = 0.05$, df = 28, $p = 0.96$
Brain pH	6.51 ± 0.05	6.60 ± 0.05	$t = 1.2$, df = 28, $p = 0.24$
RIN	7.90 ± 0.13	7.96 ± 0.16	$t = 0.30$, df = 28, $p = 0.77$
Cause of death	9 Cardiac arrest/4 accidental death/1 drug overdose	10 Hanging/2 jump from height/1 carbon monoxide poisoning/2 drug overdose	N/A
Antidepressant positive	0	2	N/A

Abbreviations: MDD, major depressive disorder; N/A, not applicable; RIN: RNA integrity number

Table 2: Correlation analysis

		dlPFC				ACC			
		CRAMP	***VDR***	***CYP27B1***	***CYP24A1***	***CRAMP***	***VDR***	***CYP27B1***	***CYP24A1***
Age	Pearson Correlation	.142	-.213	-.321	.181	.034	-.231	.088	-.061
	Sig. (2-tailed)	.453	.258	.084	.340	.859	.220	.645	.750
PMI	Pearson Correlation	-.360	-.176	.160	.241	-.100	.136	-.010	.315
	Sig. (2-tailed)	.051	.351	.398	.200	.597	.475	.958	.090
RIN	Pearson Correlation	.254	.302	.089	-.155	-.011	.006	.305	-.026
	Sig. (2-tailed)	.176	.105	.641	.415	.954	.975	.101	.892
pH	Pearson Correlation	.077	**-.369***	-.013	-.006	.116	-.248	.104	.352
	Sig. (2-tailed)	.685	.045	.946	.977	.542	.187	.584	.057

Abbreviations: ACC, anterior cingulate cortex; dlPFC, dorsolateral prefrontal cortex; PMI, postmortem Interval; RIN, RNA integrity number. "*" denotes statistical significance ($p < 0.05$)

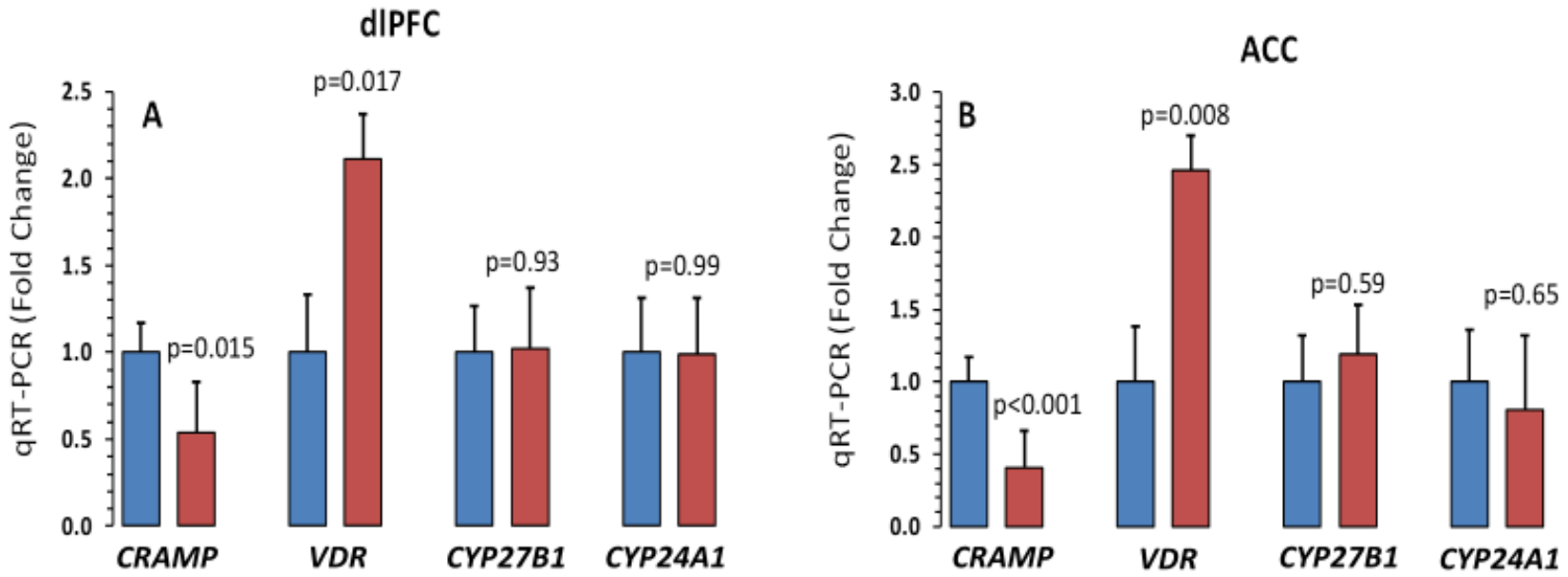

Figure 1: mRNA expression of *CRAMP*, *VDR*, *CYP27B1*, and *CYP24A1* in the dorsolateral prefrontal cortex (dlPFC) (**A**) and anterior cingulate cortex (ACC) (**B**) of cases (n = 15) (red) and non-psychiatric controls (n = 15) (blue). mRNA levels of these genes were determined by qRT-PCR using TaqMan primers and probes as indicated in methods. Data were calculated as fold-change. Data are the mean + SEM.

Author Statement

T.T.P and Y.D were senior investigators on the study and were involved in concept, data analysis and interpretation of the project. Y.D. designed and supervised the performance of laboratory work, and quality control, and performed the statistical analysis. GT provided the tissue and information regarding their group allocation. F.A, T.T.P and E.E.L drafted the manuscript. C.A.L, G.T, Y.D, L.A.B, E.S and J.W.S were involved in results interpretation and critical revision of manuscript. All authors have made intellectual contributions to the article, edited versions of the manuscript, and approved the final submission for publication.

Declaration of interest:

Authors declare no conflict of interest.