Investigation of CD26, a potential SARS2-CoV-2 receptor, as a biomarker of age and pathology

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Abstract

Objective: In some individuals, corona virus SARS-CoV-2 infection leads to a variety of serious inflammatory symptoms, including blood clotting and acute respiratory distress. Death due to COVID-19 shows a steep rise in relation to age. Comorbidities such as type 2 diabetes mellitus (T2DM), hypertension, and cardiovascular disease also increase susceptibility. It has been reported that T-cell regulatory dipeptidyl peptidase 4 (DPP4; CD26) binds to the external spike (S) glycoprotein of SARS-CoV-2 as a receptor, for the viral entry into the host cell. CD26 is expressed on many cells, including T and NK cells of the immune system, as a membrane-anchored form. A soluble form (sCD26) is also found in the blood plasma and cerebrospinal fluid.

Approach and results: To investigate a possible relationship between soluble CD26 levels, age and pathology, serum samples were collected from control, T2DM and age-related dementia (ARD) subjects. A significant reduction of serum sCD26 levels was seen in relation to age. ARD and T2DM were also associated with lower levels of sCD26. The analysis of blood smears revealed different cellular morphologies: in controls, CD26 was expressed around the neutrophil membrane, whereas in T2DM, excessive sCD26 was found around the mononucleated cells. ARD subjects had abnormal fragmented platelets and haemolysis due to low levels of sCD26.

Conclusions: These findings may explain the heterogeneity of SARS-CoV-2 infection, high serum sCD26 levels may protect from viral infection by competitively inhibiting the virus binding to cellular CD26, whereas low sCD26 levels could increase the risk of infection. Measuring serum sCD26 level may help to identify individuals at high risk for the COVID-19 infection.

Keywords: Corona virus COVID-19, dipeptidyl peptidase 4 (DPP4), SARS-CoV-2 infection, acute respiratory distress, Type 2 diabetes mellitus (T2DM), age-related dementia (ARD), Covid-19 biomarker.
Introduction

A novel coronavirus SARS-CoV-2 is the cause of the latest pandemic, characterised with severe acute respiratory syndrome (ARS) and high mortality due to both a direct cytotoxic viral effect and a severe systemic inflammation [1-3]. SARS-CoV-2 is a new member of the Coronaviridae family, beta-coronavirus genus of the B lineage, and is closely related to the severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and several bat coronaviruses [4, 5]. COVID-19 disease, caused by SARS-CoV-2, has a considerably worse prognosis in older and obese people, some ethnic populations and with comorbidities such as diabetes mellitus, hypertension, cardiovascular disease and chronic lung disease [6-9].

All coronaviruses (CoV) utilise a portion of the spike protein called the receptor-binding domain (RBD) to recognise and attach to the surface of human cells, allowing the viral particle to gain entry through the plasma membrane [10, 11]. SARS–CoV-2 uses angiotensin-converting enzyme-2 (ACE2), the same receptor as SARS–CoV, to infect humans [12]. However, there is recent evidence that the outer membrane spike glycoprotein S1 of SARS–CoV-2 binds to DPP4/CD26 when entering into the cells of the respiratory tract [10, 11, 13, 14]. Differential expression of ACE2 and DPP4/CD26 as receptors of spike glycoproteins may help to explain the heterogeneity of clinical features in people infected with different corona viruses [15, 16]. For cell entry, SARS-CoV-2 S protein also requires a protease, such as Furin, Trypsin, human airway trypsin-like protease, cathepsin-L or the transmembrane protease serine 2 (TMPRSS2) [16-19]. ACE2 or CD26 may be activated by proteolytic cleavage by TMPRSS2 or cathepsin L in late endosomes in a pH-dependent manner but the virus can also be activated by trypsin-like proteases at the cell surface in a pH-independent manner [19, 20].

Dipeptidyl peptidase 4 (DPP4), also known as cluster of differentiation 26 (CD26), is a serine exopeptidase, a multifunctional type-II transmembrane glycoprotein that presents in a dimeric form on the cell surface. CD26 is multifunctional, highly conserved among mammals and plays a major role in glucose metabolism [16]. It preferentially cleaves dipeptides from hormones and chemokines after a proline
amino acid residue, thereby regulating their bioactivity [17, 18]. CD26, being a proteolytic enzyme that is expressed in most cell types (particularly in the immune cells, T-cells and NK cells), exists in both membrane-anchored (mCD26) and soluble form (sCD26) [19]. sCD26 is thought to be shed from the membrane into plasma, and still maintains its enzymatic activity. sCD26 is also found in cerebrospinal fluid (CSF), exerting its multifunctional effects through various signaling pathways that induce and regulate inflammatory and immunological processes [20]. In humans, mCD26 is primarily expressed on the lungs epithelial cells, endothelial membranes of the pancreas, small intestine, liver, kidney, prostate, placenta, and on activated leukocytes [21, 22]. Variations in sCD26 levels in serum have been reported as clinically relevant in several pathophysiological conditions including in type-2 diabetes mellitus (T2DM) and virus infections [20]. sCD26 has the capacity to degrade incretins, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) (both involved in stimulating insulin secretion in a glucose dependent manner), as well as the brain natriuretic peptide (BNP), and the chemokine stromal derived factor-1α (SDF-1α), involved in cardiovsular function [23, 24].

The emergent global spread of SARS-CoV-2 and its grave impact on public health demands an immediate, coordinated effort in biomedical research to increase understanding of the pathogenesis of the virus and its entry into the host’s cells. The high fatality rate imposes an urgent need for rapid identification of effective treatments including novel therapeutics, antivirals and vaccines. We screened mCD26 and sCD26 expression to investigate its role in innate immunity and inflammation [25]. To achieve this, we included samples from T2DM [24] and age-related dementia (ARD) subjects who are considered at high risk for COVID-19 and share some pathological features [26, 27]. Via analysis of CD26 involvement in various clinical and pathological conditions that are also characteristic of COVID-19, we provide evidence for sCD26 implication in viral infections, such as SARS-CoV-2.

Materials and methods

Ethics and participants
Ethics and research and development (R&D) approvals were granted by the National Research Ethics Committee of the East of England. Cambridge Health Authorities Joint Ethics Committee granted ethical approval for use of human brain tissue and serum samples (Project ref no: REC:15/WM/0379). Written consents were obtained from controls, adults with diabetes participants and subjects with age related dementia (ARD) with the capacity to consent. Verbal assent was obtained from participants with ARD lacking capacity to provide written assent, and this was provided instead by an appointed consultee, in accordance with the Mental Capacity Act of the UK (2005). Information on older controls (n=50) and ARDs (n=50) has already been disclosed in a previous publication [28].

**Paraffin-embedded sections of human brain**

Human brain sections of young and aged postmortem CNS-diseased, were provided by the Cambridge Brain Bank under the project reference number REC:15/WM/0379.

**Blood and serum/plasma collection**

Whole blood, serum and plasma samples from human younger controls (YC: n=50, aged between 30 and 55 years), and older controls (OC: n=50, aged between 56-85 years), middle age type II diabetes (T2DM, non-demented, n=50, aged between 30-55 years) (please note that none of the control subjects had a clinical diagnosis of dementia), and age related dementia (ARD) (OD, n=50, aged between 56-85 years) were collected for DNA and protein analysis. All blood samples were collected for serum in BD vacutainer SST advance tubes (containing inert gel barrier and clot activator coating). Serum and plasma were separated immediately by centrifugation at 2465 g for 6 minutes at 4°C, aliquoted, and stored at –80°C until analysis.

**Blood slide preparation:**

A drop of blood placed on a slide was spread using a spreader as described previously [29]. The slide was air-dried and fixed in 100% methanol and stored at 4°C. Further investigations, including immunohistochemistry, were performed on stored fixed slides as described later.

**Animals**
Three months old C57/Bl6 mice were purchased from Charles River and bred for the experiments at University of Cambridge Bioscience facility (UBSS, at John van Geest Centre for Brain repair animal facility). All experiments were performed according to the “Animal (Scientific Procedures) Act 1986” and the “Guidance of the Operation of ASPA 2014”, and were approved by the Ethical Committee of the UK Home Office (PPL no 70/7920).

**Mice Tissue preparation**

All animals were housed under standard conditions (12h light-dark cycle, 20°C ambient temperature) with free access to food and water. Animals undergoing perfusion were anaesthetised with raising concentration of CO₂ followed by terminal injection of Euthatal (0.3 ml per 100g of body weight). Collection of fresh tissues was performed after raising the concentration of CO₂ followed by cervical dislocation. For histochemical analyses, animals were anaesthetised with pento-barbitone and flash-perfused transcardially with 0.9% saline followed with 4% (v/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (pH 7.4). Brains were removed, post-fixed for 4 h in the same fixative, and then cryoprotected with 30% sucrose in 0.1 M phosphate buffer saline (PBS). Brains and other tissues (spleen, and duodenum) were sectioned by microtome as described previously [28]. For **in situ** hybridization unfixed tissues were carefully dissected from C57/Bl6 mice from various brain regions and different organs including spleen, lung, duodenum, and embryos (embryonic day 18, ED 18) and snap frozen in dry ice until analyzed by **in-situ** hybridization.

**In situ hybridisation (ISH)**

Brain tissues and other organs were prepared for **in situ** hybridization and probed as described previously [30]. To synthesize DIG-labeled RNA probes, the target CD26 cDNA was amplified by PCR using primers designed on the basis of the mouse CD26 cDNA sequence used in RT-PCR. The primers used for DIG labeling were with:

**CD26-insF**: TAATACGACTCATAACAAGAAATATCCTCTACTATTAG
**CD26-insR**: ATTTAGGTGACACTATAGAGAAATCCACTTCCAACATCGAC

sequenced and homology checked by BLAST search (NCBI database). In vitro transcription reactions were performed using dig-UTP RNA labeling mix and SP6 or T7 RNA polymerase (Roche, Mannheim, Germany).
*In situ* hybridisation with DIG labeled probe was carried out on spleen, brain, and other embryonic tissues sections fixed in 4% PFA for 10 min, permeabilized for 10 min in (0.1M PBS with 0.5% Triton X-100), and acetylated by 10 min incubation in triethanolamine solution. Pre-hybridisation was performed in hybridisation buffer for 3 hours at 62°C and then hybridised with DIG-labeled probes (100 ng/ml) in the same buffer overnight at 62°C. Stringent washing was performed in 0.2x SSC for 1 h at 62°C. For the detection of DIG-labeled hybrids, the slides were equilibrated in maleic acid buffer (MAB, 0.1M maleic acid and 0.15M NaCl, pH 7.5), incubated for 1 h at room temperature (RT) with 1% blocking reagent made in MAB (blocking buffer), and then for 1 h with alkaline phosphatase-conjugated anti-DIG antibodies (Roche, Mannheim, Germany) diluted 1: 5000 in blocking buffer. The slides were washed twice for 30 min in MAB and incubated overnight in color development buffer [2.4 mg levamisole (Sigma), 45 µL 4-nitroblue tetrazolium (Sigma), and 35µL 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) in 10 ml of a buffer made of 0.1M Trizma base, 0.1M NaCl and 0.005M MgCl2, pH 9.5]. The reaction was stopped in neutralizing buffer (0.01M Trizma base and 0.001M EDTA, pH 8) and sections were mounted in PBS–glycerol and a coverslip applied over the section on the slide. Non-specific binding was analysed using sense probes.

**Solid phase enzyme linked immunosorbent assay (ELISA)**

The soluble CD26 plasma concentration was measured with the human DPPIV/CD26 DuoSet ELISA development System kit (R&D Systems, Catalog number DY1180) according to the manufacturer's instructions. Briefly, for the detection of sCD26, in 96-wells culture plates were incubated overnight with monoclonal antibody (mAb) anti-CD26 capture antibody (1µg/ml) (R&D system, cat number 842127). The following day the plates were washed three times with washing buffer (0.05% Tween 20 in 0.1M phosphate buffer saline (PBS) pH7.4) and blocked in blocking solution (1% BSA and 0.05% Tween 20 in 0.1M PBS) for 2 hours at RT. After blocking, the plates were washed three times with washing buffer and loaded with 10µl plasma into 90µl blocking solution and incubated 4 hours at RT. All experiments were performed in quadruplicate unless otherwise specified. A recombinant human protein (R&D, Cat number 842129) was diluted in assay buffer in a twofold serial dilution and used for the standard curve with a concentration range of 1000, 500, 250, 125, 62, 31, 15, and
0 pg/ml. After 4 hours of incubation the samples were removed and the plates were washed three times for 5 min with washing buffer before incubation for 2 hours at RT for detection with a biotinylated rabbit monoclonal anti-human CD26 (cat number 842128, R&D) antibody (1μg/ml) diluted in blocking buffer. After three further washing steps, the plates were incubated with anti-rabbit HRP-conjugated secondary antibody (1:4000) for 1 hour followed by three washes. 100μl of 1-Step ULTRA tetramethylbenzidine (TMB-ELISA, ThermoScientific) was added for ~30 minutes at room temperature. Finally, 100μl of 2M H₂SO₄ was added to quench the reaction. Colorimetric quantification was performed with an Infinite m200 plate reader (Tecan) at 450/540 nm.

**Antibodies**

The following primary antibodies were used: mouse monoclonal anti-CD26 (mAb M-A261, Invitrogen) and rabbit polyclonal (pAb) anti-CD26 antibody (Abcam Ab28340). Other antibodies used in this study were rabbit pAb anti-ApoE (Abcam Ab85311), anti-CD3 (PA5-32318) Thermo Fisher scientific, anti-CD42b (Abcam Ab104704), anti-rabbit polyclonal glycophorin (Abcam 196568), anti-DMT1(Abcam Ab123085), Anti-FPN (Abcam Ab85370), anti-GFAP (Abcam Ab48050), anti-CD68 (Sigma-Aldrich, AMAB98073), and anti-CD11b (Thermo Fisher, mAbM1/70). The following secondary antibodies were used: i.e. biotinylated goat anti-rabbit-Ig and biotinylated horse anti-mouse (both from Vector Laboratories, 1:250 for IHC); Alexa Fluor 568-labelled donkey anti-mouse-Ig, Alexa Fluor 488-labelled donkey anti-rabbit-Ig, and Alexa Fluor 568-labelled donkey anti-goat-Ig (all from Invitrogen, 1:1000 for immunofluorescence).

**Immunofluorescence (IF)**

Brain and other sections were blocked using blocking buffer (0.1 M PBS, 0.3% Triton X100, 10% normal donkey serum) for 1 hour at room temperature, then incubated overnight at 4°C with primary antibody diluted in blocking buffer. Alexa Fluor-conjugated secondary antibodies were used for detection and samples counterstained with 4′6-diamidino-2-phenylindole (DAPI, Sigma). Sections were then mounted on glass slides with coverslips using Fluoro Save (Calbiochem).
Microscopy

Bright field images were taken and quantified using Lucia imaging software and a Leica FW 4000 upright microscope equipped with a SPOT digital camera. Fluorescence images were obtained using a Leica DM6000 wide field fluorescence microscope equipped with a Leica FX350 camera with x20 and x40 objectives. Images were taken through several z-sections and de-convolved using Leica software. A Leica TCS SP2 confocal laser-scanning microscope was used with x40 and x63 objectives to acquire high-resolution images.

Image and statistics analysis

Data were analyzed by the paired Student t test (two-tailed) for two group comparison, or by ANOVA test for multiple comparison testing. Values in the figures are expressed as mean ± SEM. A one–way ANOVA was used for comparison of data among control, T2DM and ARD and conducted with IBM-SPSS statistic 19 software. Significance was analyzed using Graphpad and p values ≤ 0.001 were considered significant and are indicated in the corresponding figures and figure legends.

Results

Soluble CD26 serum protein levels are reduced in dementia, diabetes and in older people

To explore the level of sCD26, serum samples from young and old controls [age matched type-2 diabetes mellitus (T2DM: n=50, 30-55 years; non-demented) and age related dementia (ARD: n=50, aged between 56-85 years) subjects were analysed by sandwich ELISA using Human DPP4/CD26 (R&D system, catalog number: DY1180). All samples were analysed on the same day, using same standard to reduce the day to day variation.

Levels of sCD26 were lower in older subjects as well as in patients with comorbidities such as T2DM and ARD. The serum protein analysis by ELISA showed that the mean level of sCD26 in control subjects was higher than seen in their older counterparts (901.3 pg/ml, vs 664.2 pg/ml, $R^2 = 0.6$, p value ≤0.0001) in YC and OC respectively (Table 1). In younger T2DMs the mean level was (402.9 pg/ml,
compared to the mean level in ARD of 216.8 pg/ml, $R^2 = 0.7$, p value ≤0.0001) (Figure 1A). A significant reduction of serum sCD26 levels was seen in ARD and in T2DM subjects compared with age-matched controls (Figure 1A, Table 1).

**CD26 mRNA expression pattern identified in immune cells**

Using *in situ* hybridization (ISH) with a DIG labeled sense and anti-sense CD26 probes, we visualized the cellular location of CD26 mRNA in freshly frozen human tonsil, mouse heart, pancreas, embryonic and adult spleen, duodenum, liver, lung and placenta, as shown previously (see methods) [29]. There was no non-specific binding with sense probe (Figure 1B), whereas with the anti-sense probe, a predominant staining in the outer darker T cells region of the lymphoid follicle was observed with much less staining in the inner area of the germinal center (Figure 1C). Higher mRNA expression was seen in the embryonic tissues (in the endothelial layer of the heart, pancreas and spleen) (Figure 1 D-F). The highest levels were seen in the placenta, in trophoblast cells, in the spleen, in the duodenum, particularly in the intestinal epithelial cells (Figure 1 K, G, H & I). The membrane bound mRNA levels was also visible in the lung epithelial cells (Figure 1J). However, very limited mRNA was seen in the mouse brain tissues (Figure 1 L), except in blood vessels, sub ventricular zone (SVZ) and choroid plexus (CP) epithelial cells (Figure 1 M-O). Together, these findings indicated that CD26 mRNA is expressed in T cells, epithelial and endothelial cells of many different organs.

**CD26 protein is expressed in the choroid plexus epithelial cells and meningeal blood vessels**

COVID-19 infection affects epithelial and endothelial lining of the lung and meningeal macrophages as well as other cells of the immune system [31, 32]. For validation of CD26 protein levels in the brain and other immune cells, tissue sections were stained by immunofluorescence (IFC) using anti-CD26 mouse monoclonal (MAB) antibody (M-A261, Invitrogen) and analyzed using confocal microscopy. A clear signal was seen in the human spleen white pulp, very close to the central canal and the T cell area surrounding the lymphoid follicles (Figure 2 A-C). CD26 and CD3
(a marker expresses in T cells) colocalised in the outer darker region of the germinal centre containing large and medium sized T lymphocytes (Figure 2 C).

There was no visible mRNA expression of CD26 in the adult human or mouse brain, but unexpectedly high levels of proteins were observed in the human CP epithelial cells and meningeal blood vessels (Figure 1 M & O). CD26 protein expression was present in the mouse embryonic CP (Figure 2 D), human CP epithelial cells and macrophages that colocalised with Apolipoprotein-E (ApoE) (Figure 2E). CD26 protein was seen in the perivascular macrophages and colocalised with CD68 (a known peripheral macrophage marker) in lateral ventricle (Figure 2F). It was visible in the epithelial layer of a blood vessels (Figure 2 H). When human brain sections (n=6) from ARD subjects were stained with CD26 and an astrocyte marker with GFAP antibody, sCD26 protein was detected in the wall of blood vessels: presumably sCD26 which entered the brain parenchyma from blood vessels via astrocytes (Figure 2 G-H). CD26 was detected in the maningial macrophages colocalised with CD11b, a monocytes/microglial marker (Figure 2I).

Membrane bound CD26 protein binds with divalent metal proteins in the duodenum

Although SARS-CoV-2 may primarily enter through the lung’s epithelial cells, the small bowel may also be an important entry point or an interaction site [33]. Our in-situ data (Figure 1H) revealed prominent CD26 expression in the small intestine (duodenum and jejunum). We immunostained mouse gut tissues with CD26 antibody and other known duodenal membrane binding protein markers, divalent metal protein 1 (DMT1) and ferroportin (FPN) to assess the expression further [34, 35]. The most distinct feature of the small intestine is the mucosal lining, with brush border villi and crypts, lined with columnar cells. CD26 staining was seen in the columnar cells within the crypts, particularly in the apical surface and co-localised with metal binding protein DMT1 at the apical surface (Figure 2J-K). Large populations of T-lymphocytes positive for CD26 co-localised with another metal binding protein (FPN) at the basolateral site and in the central core of lamina propria containing blood vessels (Figure 2L). Throughout the small intestine, confluent lymphoid tissues known as Peyer’s patches were visible and CD26 positive (Figure 2L).
CD26 and Coagulopathy

One of the emerging hallmarks of COVID-19 is a coagulopathy, termed as “sepsis-induced coagulopathy” (SIC) with elevated D-dimer and fibrinogen levels [36]. It is related to an infection-induced systemic inflammatory response with endothelial dysfunction and microthrombosis often leading to organ failure [36-38]. Several observational studies have characterized monocytes during SARS-CoV-2 infection [39]. We investigated cellular expression of CD26 in blood smears from different age groups (n = 20, age between 30-85 years) and imaged using confocal microscopy. The blood smears from young and old subjects were co-labeled with CD26 monoclonal antibody and either with an erythrocyte plasma membrane marker (glycophorin) or a platelet marker (CD42b). Glycophorin was expressed on the surface of red blood cells (RBCs) (Figure 3A-C) with CD26 seen around the RBCs (could be on erythrocytes spikes) (Figure 3A-B). There was stronger CD26 expression around the RBCs membranes in the controls (Figure 3A & G) and in T2DM compared to in ARD (Figure 3B & C). CD26 was expressed on the surface of mononucleated cells (MNCs) around the outer membrane (Figure 3D-E), and a web-like appearance that co-localised with integrin CD42b protein was observed (Figure 3F). The control subjects had stronger CD26 expression around the neutrophil membrane (Figure 3D, G & J), whereas in T2DM there was excessive sCD26 around the MNCs and haemolysis in red blood cells (RBCs) (Figure 3H & K). The young and old control subjects expressed much more CD26 in MNC (Figure 3D, G & J, L), whereas younger T2DM and older ARD subjects carried abnormal fragmented platelets stained with CD42b (Figure 3 H-I). Additionally, scattered sCD26 was observed extra-corporally of RBCs and within MNCs in the T2DM samples (Figure 3 H-K). A classic dysmorphology (bi-lobed neutrophils) was frequently seen in blood of T2DM (Figure 3H & K) whereas normal neutrophils positive for CD26 were found in controls (Figure 3 D-G)

In ARD subjects (with dementia), RBCs appeared abnormally shaped (macrocytic with damaged erythrocytes spikes) and platelets found were fragmented (Figure 3 F-I). These findings suggest that further investigation of CD26 involvement in lymphocyte regulation is warranted, especially in relation to the coagulation cascade.
Discussion

COVID-19 caused by SARS-CoV-2 is now a pandemic [2, 3, 40]. SARS-CoV-2 pathology is similar to that of SARS-CoV and MERS-CoV induced viral pneumonia [41]. Coronavirus has specific immune response and immune escape characteristics and can cause severe pathogenic mechanisms through inflammation leading to a variety of symptoms like acute respiratory distress syndrome, gastro-intestinal disorder, renal failure, vascular bleeds, stroke, T-cell specific immune responses and cytokine storm [4, 42, 43]. Cytokine storm is hypercytokinemia that increases the volume of pro-inflammatory cytokines in the serum (e.g. IL-1β, IL-6, IL-12, INF-γ) and chemokines (CXCL10 and CCL2), and is correlated with pulmonary inflammation and extensive lung involvement as seen in COVID-19 patients [44]. MERS-CoV infection was also described to provoke increased concentration of cytokines (IL-15, IL-17, TNFα and INF-γ) [45]. It is reported that victims infected with SARS-CoV-2 also demonstrate high levels of IL-1β, TNFα, INF-γ, CXCL10 and CCL2 which may be attributed to the activated response of Th1(T helper) cells [46]. CXCL10 binds to CXCR3, a G-protein-coupled receptor, which is known to be expressed on activated T lymphocytes, natural killer (NK) cells and some epithelial and endothelial cells. CXCL10-CXCR3 acts in an autocrine fashion on the oxidative burst and chemotaxis in the inflamed neutrophils, leading to fulminant pulmonary inflammation [47].

Recently, sequence and modeling analysis of S glycoproteins of MERS-CoV, SARS-CoV-2 and further co-purification with the MERS-CoV S1 domain, identified that CD26 functions as a cellular receptor for MERS-CoV and SARS-CoV-2 [16, 48]. Another recent study reported a correlation between DPP4 and ACE2, suggesting that both membrane proteins are relevant in the pathogenesis of virus entry [49]. The co-expression of ACE2 and DPP4/CD26 as receptors of spike glycoproteins suggests that different human coronaviruses (CoVs) may target similar cell types across different human tissues, and this may also explain the presence of similar clinical features in patients infected with different CoVs. In addition, it was shown that DPP4 acted for CoV co-receptor, thus suggesting a potential similar mechanism of entry for SARS-CoV-2. There is a connection between the SARS-CoV, SARS-CoV-2, ACE2 and the
rationale for soluble ACE2 as a potential therapy by using ACE2+–small extracellular vesicles (sEVs) [50]. We suggest that soluble CD26 circulate via platelets and T cells carrying vesicles as shown in the normal blood smear (Figure 3). It could be therapeutic approaches to use soluble CD26 as a decoy receptor which could inhibit the entry of the virus through cell surface [16, 50, 51]. In this study, we analysed the expression pattern in healthy subjects and subjects who are high risk for COVID-19. Our findings now need to be validated with COVID-19 patients.

The role of CD26 in immune regulation has been extensively characterized, with findings elucidating its linkage with signaling pathways and structures involved in T-lymphocyte activation as well as antigen presenting dendritic T-cell interaction [52, 53]. In this study, we show that CD26 is expressed in T-cells, to extrafollicular area of tonsil and at T-cell areas in the germinal center of the spleen and is also co-localised with CD3 [54]. The outer darker region of the germinal center of tonsil, showed that it contained large and medium sized lymphocytes as described before [54]. Spleen may be one of the organs directly attacked by the virus in some patients who died from COVID-19. T and B lymphocyte in the spleen decreased in varying degrees, lymphoid follicles are atrophied, decreased or absent [55]. T-cells secrete cytokines and chemokines in immune cells as reported in MHC gene cluster [56]. These findings suggest that the binding of sCD26 to the spike (S) glycoprotein may also depend on T-cell population.

Although SARS-CoV-2 may primarily enter the cells of the lungs, the small bowel may also be an important site of entry or interaction site, as the enterocytes are rich in angiotensin converting enzyme (ACE)-2 receptors [33]. Duodenal mucosa expresses mCD26 particularly on the apical site of the brush border epithelial cells where T-cells present and bind with many divalent metals including Fe\(^{2+}\), Ca\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\) and Hg\(^{2+}\) [57]. Particularly, Iron (Fe\(^{2+}\)) play an important role in inflammation and causes disbalance and hyperferritinaemia in Covid-19 [58]. Most iron absorption occurs in the duodenum via the divalent metal transporter1 (DMT1)-mediated uptake and ferroportin-1 (FPN1)-mediated export across the apical and basolateral membranes, respectively [34, 35]. We have shown that CD26, a cysteine-rich protein, can bind with divalent metal binding proteins (DMT1) and ferroportin (FPN) [28, 34, 35]. Much of this research focus has centered on the ectodomain of the spike protein. The ectodomain is anchored to a transmembrane region, followed by a cytoplasmic tail.
Recently, it was reported that a sequence similarity exists between the cysteine-rich cytoplasmic tail of the coronavirus spike protein with the cysteine-rich domain in hepcidin protein, which is a key regulator of iron homeostasis in humans and other vertebrates has been reported [59-61]. SARS-CoV-2 spike (S) glycoprotein can bind DMT1 on the apical site of brush border epithelial cells in the gut mucosa, and could mediate membrane fusion and virus entry into the cells. High levels of sCD26 can therefore potentially compete and prevent viral entry through gut mucosa and exert beneficial immunoprotective effects via elevation of GIP, a peptide that is produced in gastrointestinal K cells and further proteolysed by CD26 [62]. As a consequence, the small bowel may serve as a viral entry site or as a potentiating organ, magnifying the systemic inflammatory response, small bowel being the largest lymphoid organ of the body. A significant proportion of COVID-19 patients with gastrointestinal symptoms clearly support the involvement of the small bowel in SARS-Cov-2 [33].

One of the emerging hallmarks of COVID-19 is a coagulopathy, termed as “sepsis-induced coagulopathy” (SIC) with elevated D-dimer and fibrinogen levels [36]. It was shown that platelet activation and platelet-leukocyte interactions participate in the pathophysiology of viral infections, including dengue, HIV, and influenza [63]. Recently, a hypercoagulability state has been reported as a major pathologic event in COVID-19, and thromboembolic complications are listed among life-threatening complications of the disease [64]. Platelets are chief effector cells of haemostasis and pathological thrombosis [65]. In this paper, we demonstrated that increased platelet activation and platelet-monocyte aggregate formation are observed in diabetic subjects (Figure 3 E &H), and much more prominent in ARD (Figure 3 F & I) but not in controls (Figure 3 D &G).

Recent clinical evidence has revealed T2DM and cardiovascular risk factors increases susceptibility to COVID-19 infection [66]. The elderly, and those with underlying medical conditions particularly obesity, diabetes, age related dementia are the pre-existing diseases associated with the greatest risk and death in Covid-19 pandemic [26, 27, 67]. We therefore chose type 2 diabetes mellitus (T2DM), age related dementia (ARD) and age matched young and old controls who are high risk for COVID-19 infection. They all could thus have common risk factors that affect different organs, particularly the epithelial membrane responsible for the viral entry. We found that CD26 is expressed in the epithelial membrane of the choroid plexus.
(CP) and in the endothelium of blood vessels in the brain. The CP is a secretory tissue responsible for producing both the CSF in the vertebrate brain and many innate immune molecules to protect the brain from infection by surveilling the blood brain barrier (BBB) [29, 68]. CD26 protein is present in the CP epithelial membrane and in the outer layer of brain pia mater particularly in the meningeal macrophages, where it is colocalised with other brain spectrins, known as fordin and ankyrin [29, 69]. Similarly, CD26 is expressed in erythrocyte membranes (also having erythrocyte spikes) and is colocalised with membrane proteins, glycophorin and integrin α2β3 [70]. Since SARS-CoV-2 spike (S) glycoprotein binds to erythrocytes, causing clotting defects in the small blood vessels [37, 71], we analysed blood smears from young, diabetic (T2DM) and older subjects (ARD). In young controls, the higher levels of sCD26 around neutrophils, and in platelets was seen, whereas in immunocompromised T2DM subjects many fractured platelets and clumped RBCs with haemolysis were visible, alongside a web-like expression of integrin proteins was present surrounding the neutrophil and granulocytes (also visible in ARD subjects). Furthermore, in ARD subjects macrocytic, broken RBCs and fragmented platelets were present. These findings demonstrated the impaired coagulopathy in older subjects and those with T2DM and dementia, and complement our previous findings of another immunoprotective molecules such as TREM2, suggesting that CD26 may also be required for the haemopoietic cellular growth and protection [72]. Levels of serum CD26 are ~ 3 times higher in pregnant women at ~ 3μg/ml (RR-C unpublished). CD26 mRNA expression is elevated in placental trophoblasts, where it may induce regulatory T-cell (Treg) differentiation and protect the foetus from external infections [73]. These findings suggest that newborn babies and young children may be protected from COVID-19 due to high levels of sCD26. Another study reported female HIV positive but disease free sex-workers in Nairobi also showed higher levels of serum sCD26 and thereby presumably protecting them from all other viral infections [74]. Our findings suggest that T-cell regulatory CD26 is expressed in different organs from tonsil, spleen, gut mucosa to brain (particularly in CP and in BBB) and sCD26 may play a crucial role as a decoy receptor which could inhibit the entry of the virus through cell surface, allowing more time for neutralizing antibodies to be raised against the SARS-CoV-2 spike protein.
Conclusions

We have shown sCD26 expresses in the serum, and mCD26 in the T-cells (in tonsil and spleen), in CP, duodenum and erythrocytes. We observed a significant reduction of serum sCD26 levels with age and in T2DM subjects compared to age-matched controls. Thus, we suggest that measuring sCD26 level in the serum of young and old control subjects and comparing them with SARS-CoV-2 virus carrying subjects could be explored as a possible test for inflammatory stages in COVID-19: high serum sCD26 level could protect from viral infection by blocking the receptor from virus entry, whereas low sCD26 level may be associated with a higher risk of infection. A therapeutic approach may be to use soluble CD26 as a decoy receptor which could competitively inhibit the entry of the virus through cell surface.

Data Availability: All data analysed in this paper are already included in the manuscript, including a table.

Clinical perspectives

- The rapid global spread of SARS-CoV-2 and its grave impact on public health immediately demands prompt and efficient coordinated effort in biomedical research to increase the understanding of the pathogenesis of the virus and its impact upon the host’s cells. Type-2 diabetes mellitus (T2DM) and cardiovascular risk factors increase the susceptibility to COVID-19 infection. Variations in soluble CD26 levels in serum have been reported as clinically relevant in several pathophysiological conditions including T2DM and virus infections, where CD26 may play a critical role in the innate immunity and T-cell regulation.

- We investigated the membrane bound (mCD26) and soluble (sCD26) expression to uncover CD26 role in the innate immunity and inflammation. For this, we analysed samples from T2DM and age-related dementia (ARD) subjects who are considered at high risk for COVID-19. We show sCD26 is expressed in serum, T-cells (in tonsil and spleen) whereas mCD26 in choroid plexus, duodenum and erythrocytes.

- We suggest that measuring sCD26 level in the serum should be explored as a possible test for inflammatory stages in COVID-19: high serum sCD26 level could protect from viral infection by blocking the receptor from virus entry, whereas low sCD26 level may be associated with a higher risk of infection. It
could be a therapeutic approaches to use soluble CD26 as a decoy receptor which could competitively inhibit the entry of the virus through cell surface.

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Compliance with ethical standards

Ethical approval all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest

The authors declare that they have no conflicts of interest. CamKolInv is a non-profitable organization.

Author’s contributions

AAR, JH and SC performed biochemical analysis, critically evaluated the results. AAR performed tissue analysis and confocal microscopy. SZ evaluated clinical findings, and EL-M evaluated clinical samples and edited the manuscript. RR-C and JT contributed to the hypothesis development and edited the manuscript. RR-C performed study design, supervised the project, critically evaluated the results and
wrote the manuscript. All authors carefully read, edited and approved the final version of the manuscript.

**Abbreviations**

ACE2: angiotensin-converting enzyme-2, ApoE: Apolipoprotein-E, ARD: age-related dementia, ARS: acute respiratory syndrome, BBB: blood brain barrier, BNP: brain natriuretic peptide, CD26: cluster of differentiation 26, mCD26: membrane-anchored CD26, sCD26: soluble form CD26, CD42b: cluster of differentiation 42b, CP: choroid plexus, CSF: cerebrospinal fluid, DMT1: divalent metal protein 1, DPP4: dipeptidyl peptidase 4, ELISA: enzyme linked immunosorbent assay, FPN: Ferroportin, GIP: glucose-dependent insulinotropic polypeptide, IF: Immunofluorescence, IHC: immunohistochemistry, ISH: in-situ hybridization, RBD: receptor-binding domain, MAB: maleic acid buffer, PBS: phosphate buffer saline, MAB: mouse monoclonal antibody, MERS-CoV: Middle East respiratory syndrome coronavirus, MNC: mononucleated cell, OC: older control, PFA: paraformaldehyde, R&D: research and development, RT: room temperature, SARS-CoV: severe acute respiratory syndrome coronavirus, SARS-CoV-2: severe acute respiratory syndrome coronavirus-2, SDF-1α: stromal derived factor-1α, SIC: sepsis-induced coagulopathy, T2DM: type 2 diabetes mellitus, TMB: tetramethylbenzidine, Treg: regulatory T-cell, YC: younger control.

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Figure Legends:

Figure 1: Soluble CD26 serum protein levels are reduced in dementia, diabetes and in older people

A: The ELISA analysis performed with human serum showed that CD26 levels were highest in the young controls (YC), followed by old controls (OC), significantly decreased in young diabetics (T2DM) and lowest in the ARD (OD). (YC vs T2DM, and OC vs OD, p ≤ 0.0001; YC vs OC, p ≤ 0.001 (p ≤0.001*** and p ≤0.001 showed
with ****). All serum samples were analysed at the same time to reduce the day to day variation. CD26 mRNA expression was detected by in situ hybridization using sense and antisense DIG labelled CD26 probe. A sense probe was used to investigate non-specific binding in tonsil section, no non-specific binding was observed (B). Localization of DPP4 enzyme activity in tonsils, a predominant staining of CD26 mRNA was observed in the outer darker extra follicular T cells region of tonsil with much less staining in the inner germinal centers (C), in the endothelium of heart of embryonic day 18 (D), endothelial layer of pancreas (E), embryonic spleen (F), in adult spleen (G). In adult mouse duodenum CD26 expressed in the epithelial brush border (H), around the hepatocytes in the liver (I), in the lung epithelial cells (J) and in the placental trophoblast cells (K, cellular localization of mRNA indicated by arrow in all panels). There was no mRNA visible in the mouse brain cells (L), whereas present in the brain parenchymal blood vessels (M), in sub ventricular zone (SVZ) (N) and in the choroid plexus (CP) (O). Together, these findings indicated that CD26 mRNA is expressed in T cells, epithelial and endothelial cells of many different organs. scale bar: B-F, H-J= 50 μm, K to O, = 100μm).

Figure 2: CD26 protein is expressed in the choroid plexus epithelial cells, meningeal blood vessels and bind with divalent metal proteins in the duodenum

Double immunofluorescence (IFC) staining was performed in the human spleen using mouse monoclonal (Mab) anti-CD26 (Green) and rabbit polyclonal (Pab) anti-CD3 (red), and DAPI (4’,6-diamino-2-phenylindole) for nuclei (Blue) (A–C). CD26 immunoreactivity was seen in the human spleen in white pulp, very close to the central canal and in T cell area surrounding the lymphoid follicles (A–B). CD26 and CD3 (a marker expresses in T cells) co-localised in the outer darker region of the germinal center containing large and medium sized T lymphocytes and both proteins co-localised (C). Embryonic mouse choroid plexus (CP) was stained with CD26 (green) and DAPI (Blue) and imaged using confocal microscopy. Very high CD26 expression was present in the mouse embryonic CP epithelium (D). Human normal brain section particularly CP was stained with CD26 (green) and ApoE (red), both proteins were present in the CP epithelial membrane and co-localised in the macrophages (E). A mouse brain section from lateral ventricle stained with CD26 (red) and a macrophages marker CD68 (green), CD26 positive macrophages were visible at the wall of ventricle and in the CP, both protein colocalised (F). An ARD brain section from cortex, close to a blood vessel was stained with CD26 (green) and astrocyte marker (GFAP, red), showed that CD26 protein carried by RBCs and macrophages entering through damaged blood vessels (BV), surrounded by GFAP positive astrocytes (G). Another blood vessel in the brain stained with CD26 (green) visible in the epithelium (H). A human brain section from frontal cortex stained with CD26 (red) and a microglial marker CD11b (green) co-localised in the meningeal macrophages in the Pial surface of cortex (I). Cellular localization of CD26 protein indicated with white arrow in all panels).

Duodenum sections from adult mouse was stained with CD26 (green) and DAPI (blue), visible in the columnar cells within the crypts (J), and co-localised with metal binding protein DMT1 at the apical surface (K). Large populations of T-lymphocytes positive for CD26 co-localised with another metal binding protein ferroportin (FPN) at the basolateral site and in the central core of lamina propria containing blood vessels (L). Scale bar in A-F =40μm, G-L =25μm.
Figure 3: CD26 is expressed on the surface of mononucleated cells (MNCs) around the outer membrane, and a web-like appearance that co-localised with integrin observed

The blood smears from normal, T2DM and ARD were stained with CD26 (green) and either glycophoryn (red) or CD42b (red) and imaged using confocal microscopy. In normal blood smears glycophoryn was expressed on the surface of red blood cells (RBCs) (A) and CD26 seen around the RBCs in T2DM and ARD (B-C). Blood smear showed normal mononucleated cells (MNC) surrounded by CD26 (green) and CD42b (red) in normal platelets (red) (D), in T2DM (E) and in ARD (F). In T2DM there was excessive sCD26 around the MNCs and some clumped platelets (red) in the periphery (H-K). A demented (ARD) subjects showed a web-like expression of CD26 proteins (green) was present surrounding the neutrophil and granulocytes (F), and fractured platelets (red) around the MNC with visible nets of integrin (I). One old control blood smear stained either with CD26 (green) and either with glycophoryn or CD42b (red). CD26 was present around the MNC (G) and some platelets colocalised with CD26 and CD42b (J). Cellular localization of CD26 protein indicated with white arrow in all panels). CD26 level was highest in the controls, lower in T2DM and lowest in the ARD (L). Scale bar in A-K=10µm. *** P= <0.001, **** P= <0.0001.

Table 1

| Serum CD26 (pg/ml) | Young Control | Young Diabetic | Old Control | Old Demented |
|--------------------|---------------|---------------|-------------|--------------|
| Average (pg/ml)    | 901.26        | 402.88        | 664.22      | 216.88       |
| Two-way ANOVA      | Ordinary      |               |             |              |
| Alpha              | 0.05          |               |             |              |
| Source of Variation| % of total variation | P value | P value summary | Significant |

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|                         | SS   | DF | MS  | F (DFn, DFd) | P value |
|-------------------------|------|----|-----|-------------|---------|
| Row Factor              | 970863 | 49 | 19814 | F (49, 147) = 0.9051 | P = 0.6493 |
| Column Factor           | 13450000 | 3 | 4483000 | F (3, 147) = 204.8 | P < 0.0001 |
| Residual                | 3218000 | 147 | 21890 |              |         |

### Tukey's multiple comparisons test

| Group 1                          | Group 2                          | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
|---------------------------------|----------------------------------|------------|-----------------|--------------|---------|-----------------|
| Young Control vs. Young Diabetic |                                  | 498.4 (pg/ml) | 421.5 to 575.3 (pg/ml) | Yes          |         |                 |
| Young Control vs. Old Control    |                                  | 237 (pg/ml) | 160.2 to 313.9 (pg/ml) | Yes          | ****    | < 0.0001        |
| Young Control vs. Old Demented   |                                  | 684.4 (pg/ml) | 607.5 to 761.3 (pg/ml) | Yes          | ****    | < 0.0001        |
| Young Diabetic vs. Old Control   |                                  | -261.3 (pg/ml) | -338.2 to -184.4 (pg/ml) | Yes          | ****    | < 0.0001        |
| Young Diabetic vs. Old Demented  |                                  | 186 (pg/ml) | 109.1 to 262.9 (pg/ml) | Yes          | ****    | < 0.0001        |
| Old Control vs.                  |                                  | 447.3 (pg/ml) | 370.4 to       | Yes          | ****    | < 0.0001        |
