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Skeletal Muscle and the Maintenance of Vitamin D Status

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Abstract: Vitamin D, unlike the micronutrients, vitamins A, E and K, is largely obtained, not from food, but by the action of solar UV light on its precursor, 7-dehydrocholesterol, in skin. With the decline in UV light intensity in winter, most skin production of vitamin D occurs in summer. Because no defined storage organ or tissue has been found for vitamin D, it has been assumed that adequate vitamin D status in winter can only be maintained by oral supplementation. Skeletal muscle cells have now been shown to incorporate the vitamin D-binding protein (DBP) from blood into the cell cytoplasm where it binds to cytoplasmic actin. This intracellular DBP provides an array of specific binding sites for 25-hydroxyvitamin D (25(OH)D) which diffuses into the cell from the extracellular fluid. When intracellular DBP undergoes proteolytic breakdown, the bound 25(OH)D is then released and diffuses back into blood. This uptake and release of 25(OH)D by muscle, accounts for the very long half-life of this metabolite in the circulation. As 25(OH)D concentration in blood declines in winter, its cycling in and out of muscle cells appears to be upregulated. Parathyroid hormone is the most likely factor enhancing the repeated cycling of 25(OH)D between skeletal muscle and blood. This mechanism appears to have evolved to maintain adequate vitamin D status in winter.

Key words: vitamin D; muscle; parathyroid hormone; vitamin D-binding protein

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35 The concentration in blood serum or plasma of 25-hydroxyvitamin D [25(OH)D], the most
36 plentiful vitamin D metabolite, has become established as the definitive indicator of vitamin D
37 status [1,2]. When the concentration falls below a generally agreed level, (usually 50 nmol/L),
38 vitamin D status is said to be insufficient or deficient, in the same way that status is defined for
39 other small molecules derived from the environment, such as vitamins A, E and K. However,
40 there are three substantial differences between this measurement of vitamin D status and
41 those of these fat-soluble micronutrients. The first is that 25(OH)D concentration in blood
42 varies with season. Because it is derived from vitamin D produced in skin by the action of solar
43 ultraviolet radiation on 7-dehydrocholesterol, the 25(OH)D levels rise during the months of
44 summer and fall during winter, particularly in those people who live far from the equator [3,4].
45 Are the lower values in winter really an indication of deficiency or insufficiency of vitamin D, if
46 this is a universal feature of populations?
47
48 The second difference with vitamins A, E and K, is that there is no apparent storage organ or
49 tissue for vitamin D or 25(OH)D. Although vitamin D is found in adipose tissue, suggesting that
50 this is a storage site [5,6], it can only be released when stored fatty acids are mobilized to
51 supply energy [7,8,9]. Thus, sequestered vitamin D in adipocytes cannot be a functional store,
52 ready to be transported to the liver and converted to 25(OH)D, when circulating levels of this
53 metabolite decline.
54
55 The third unique feature is that 25(OH)D has a very long residence time in blood. The half-life is
56 very variable between 15 and 50 days [10] with a mean value in a recent study of 89 days [11].
57 In contrast, other hormonal steroids in blood, including the vitamin D hormone,
58 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), are cleared in minutes or hours after entering the
59 circulation [12,13]. An explanation for this long residence time of 25(OH)D is not readily
60 apparent, particularly because the vitamin D binding protein (DBP) in blood, to which 25(OH)D
61 is tightly bound, has a comparatively short half-life of only 1-3 days and is continuously being
62 replenished from synthesis and secretion by the liver [14,15]. Therefore, for 25(OH)D to be
63 retained in blood for such a long time, it either has to transfer from one DBP molecule to

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64 another in the circulation or else it repeatedly passes to and from some extravascular site,
65 binding to a new DBP molecule with each cycle. The DBP concentration in blood is in vast
66 excess to that of 25(OH)D with only 1-5% of the protein molecules having a 25(OH)D molecule
67 bound to the single, high affinity, specific binding site [16].
68
69 The discovery that maternal 25(OH)D in rats was transported across the placenta and
70 accumulated in the skeletal muscle of fetuses, suggested that skeletal muscle might have a
71 functional role in conserving this vitamin D metabolite [17]. Although the concentration of
72 25(OH)D in muscle of sheep and cattle is only about 0.1-0.3 μg/100g [18], muscle represents
73 30-38% of body mass in humans [19] and thus total 25(OH)D in total skeletal muscle could be
74 comparable to that in the circulation (Table 1).
75
76 **Table 1**. Comparison of total body 25(OH)D in blood plasma and skeletal muscle
77

	Blood Plasma Volume (L)	Muscle Mass (kg)
Total for 70 kg human	2.7 – 4.3 L[1]	21 - 26.6 kg[2]
25(OH)D concentration	20 μg/L[3]	1 – 3 μg/kg[4]
Total body 25(OH)D	54 – 86 μg	21 – 80 μg

78 [1]Reference [20]
79 [2]Assuming body mass of 70 kg [19]
80 [3]Minimum adequate status 25(OH)D concentration
81 [4]Assuming human muscle has similar 25(OH)D concentrations to sheep and cattle [18]
82
83 In addition, studies in adolescent children [21] found that plasma 25(OH)D concentration was
84 positively correlated with total body lean mass, the main component of which is skeletal
85 muscle. Furthermore, there are now several published findings of a positive relationship
86 between the intensity of physical exercise and the concentration of 25(OH)D in blood [e.g.
87 22,23], particularly in winter when there would be little exposure to solar UV light during
88 outdoor exercise [21]. Muscle biopsies from sheep grazing outdoors in winter showed a
89 significantly higher concentration of 25(OH)D than biopsies from sheep outdoors at the end of
90 summer (Table 2).
91
92

93 **Table 2**. Plasma and muscle concentrations of 25(OH)D in outdoor grazing sheep
94 at latitude 33.9° S. Mean values ± SEM.
95

Season	Plasma 25(OH)D_3	Muscle 25(OH)D
End of summer values (n = 5)	10.67 ± 1.65 ng/ml	0.27 ± 0.04 µg/100g tissue
End of winter values (n = 5)	5.36 ± 0.71 ng/ml*	0.47 ± 0.12 µg/100g tissue*

96 *significantly different from summer values by t-test $p<0.05$

97

98 The key discovery that pointed to a role for muscle in maintaining vitamin D status came from
99 studying the properties of muscle cells *in vitro* [24]. The cell membrane was found to contain
100 the proteins megalin and cubilin. These proteins, like those in the renal tubule cells, transport
101 DBP from the extracellular fluid into the cell cytoplasm. Some of the internalized DBP could be
102 bound to actin in actomyosin, via its specific actin-binding site, but much of the remainder is
103 bound to actin dispersed throughout the cytoplasm. Both megalin and DBP have also been
104 found in human muscle biopsies [25]

105

106 *In vivo* experiments with ^{131}I-labelled DBP in rabbits, revealed that DBP had a short residence
107 time in skeletal muscle and soon underwent proteolysis [14]. DBP is a member of the
108 albuminoid family of proteins. It is synthesized and secreted by the liver, with similar
109 characteristics to blood albumin [26]. Because albumin in the extracellular fluid in skeletal
110 muscle is only about one third the concentration in plasma [27], the extracellular DBP
111 concentration will likewise be about 1.5 to 2 µmol/L compared to the blood concentration of 5-
112 6 µmol/L [28]. Thus, much of the 25(OH)D in the extracellular fluid will have dissociated from
113 the low concentration of DBP and would be able to diffuse into muscle cells where it would
114 bind to the internalized DBP in the cell cytoplasm. In comparison with control cells such as
115 osteoblasts, 25(OH)D is retained within myocytes for considerably longer (Fig. 1). When
116 cytoplasmic DBP undergoes proteolysis, the bound 25(OH)D is released and would diffuse from
117 the myocyte and return to the circulation, once again being bound to the plentiful DBP. This
118 repeated uptake and release of 25(OH)D by the total mass of skeletal muscle cells accounts for
119 the apparent long residence time of 25(OH)D in blood plasma.

120

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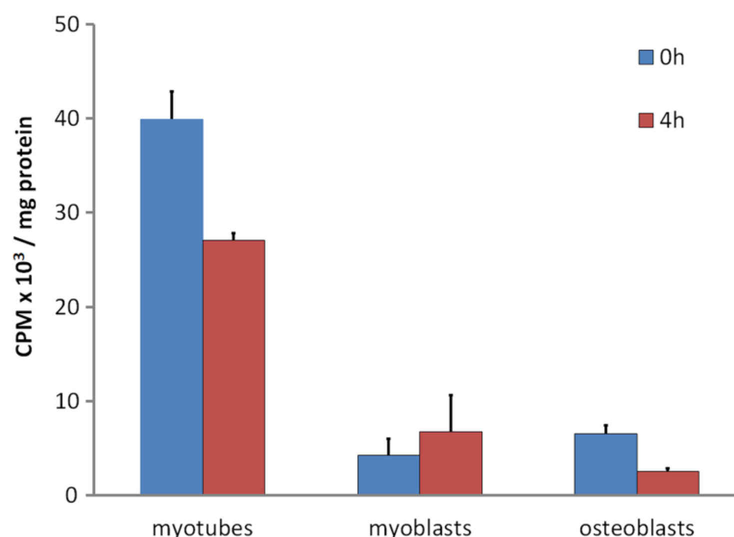
121 **Figure 1.**



122

123 **Fig 1. Retention of tritiated 25(OH)D3 in C2 myotubes, C2 myoblasts and MG63 osteoblasts.**
124 Cells were incubated for 16 h with 25-[26,27-3H]hydroxyvitamin D3 (Perkin Elmer, MA, USA) in
125 DMEM supplemented with serum replacement (Sigma-Aldrich, MO, USA) followed by 3x
126 washes with ice cold PBS. At this point, 0 h, or 4 h later, cells were harvested, lysed and
127 assayed for protein by bicinchoninic acid assay (Thermo-Scientific, IL, USA) or radioactivity
128 counted by liquid scintillation counting (see [24]). Data as means ± SEM (n = 3 wells/group)
129

130 An alternative explanation for the long half-life of 25(OH)D in blood could be that there is
131 continuous entry of newly synthesized 25(OH)D, perhaps from parent vitamin D trapped in
132 adipose tissue. This possible steady input could be replacing a steady loss of 25(OH)D from
133 blood, and thus account for an apparently long half-life. However, the long residence time in
134 blood of tritium-labelled 25(OH)D in both mice [24] and humans [10] demonstrates that it is
135 persistence of the same molecules in blood that is the real explanation. This phenomenon can
136 only be explained by recycling of 25(OH)D from some extravascular region and skeletal muscle
137 is the only candidate for that region.
138

139 Nevertheless, experiments investigating the uptake and retention of 25(OH)D in cultured
140 muscle cells in vitro, have shown that raising the concentration of DBP in the culture medium
141 blocks the uptake of 25(OH)D into the cells (Table 3). The higher the concentration of DBP, the
142 lower the concentration of unbound 25(OH)D. This observation fits with the interpretation that
143 it is unbound 25(OH)D that enters muscle cells, rather than that which might be carried in,

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144 bound to DBP, via the megalin/cubilin protein internalization process. The fact that only 1-5% of
145 DBP in the circulation is transporting a specifically bound 25(OH)D molecule, also indicates that
146 transport on DBP cannot be the mechanism for the intracellular accumulation of 25(OH)D.
147
148 **Table 3**. Uptake of tritium-labelled 25(OH)D_3 by differentiated myotubes after 4 h in the
149 presence of varying concentrations of DBP.
150

DBP concentration	0	1nM	10nM	100nM
[^{3}H]25(OH)D cpm/mg cell protein	1284±147	1233±67	778±76*	402±20*

151 * significantly different from 1nM DBP $p<0.001$
152
153 The accumulation of tritium-labelled 25(OH)D over 16 hours by cultures of differentiated
154 mouse skeletal myotube cells (the differentiated cell type derived from myoblasts in culture,
155 equivalent to myocytes *in vivo*), compared to the very low uptake by undifferentiated
156 myoblasts and osteoblasts [29], demonstrated that some internal 25(OH)D specific binding sites
157 were present in the myotubes and not in the other two control cell types (Fig. 2). As the culture
158 medium during the time of these incubations did not contain DBP the specific binding inside
159 myotubes would have been to DBP that had been internalized from the culture medium while
160 the myotubes were differentiating from myoblasts. When myotubes were cultured with
161 fluorescently labelled DBP for up to 24 h, and then observed by confocal microscopy, the
162 fluorescent protein was clearly seen within the cells (Fig. 3). Some of the fluorescence was in
163 linear streaks along the long axis of the myotubes, suggesting that some of the labelled DBP
164 was bound to actin in the actomyosin contractile elements. But the fluorescent pattern was
165 also distributed generally within the cell, indicating that much of the DBP was bound to the
166 abundant actin, known to be distributed throughout the myotube cytoplasm (30).
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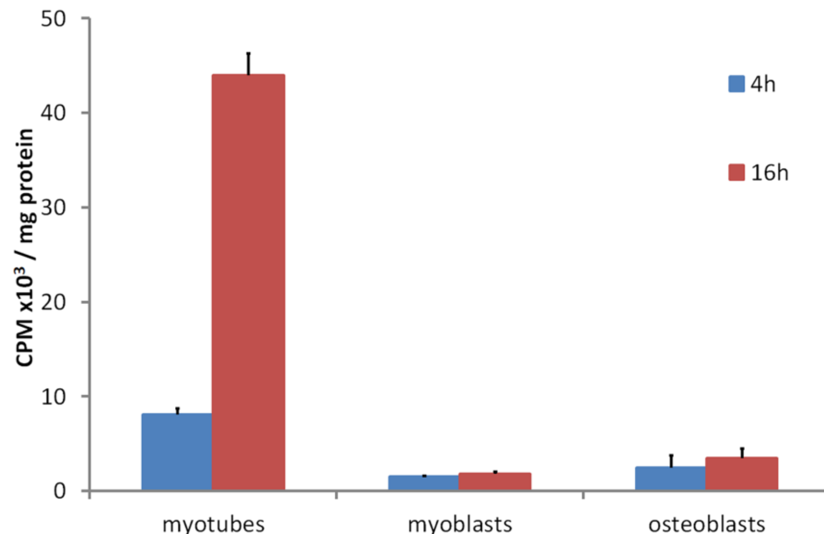
173 **Figure 2**.



174

175 **Fig 2. Time dependent ^{3}H-25(OH)D uptake in C2 myotubes and myoblasts and MG63**
176 **osteoblasts**. Cells were incubated for 4 or 16 h with [26,27-^{3}H]25-hydroxyvitamin D_3 in DMEM
177 supplemented with serum replacement followed by 3x washes with ice cold PBS. At each time
178 point, cells were harvested, lysed and assayed for protein by bicinchoninic acid assay or
179 radioactivity counted by liquid scintillation counting (see [24]). Data shown as means ± SEM (n =
180 3 wells/group)

181

182 It now appears that the megalin/cubilin protein internalization mechanism in myocytes is not
183 limited to DBP because other extracellular proteins, such as albumin, have also been
184 demonstrated to be incorporated into myotube cells in culture by this mechanism (unpublished
185 results). The function of uptake of other extracellular proteins by skeletal muscle cells *in vivo* is
186 a matter of speculation. Because skeletal muscle has a high rate of protein turnover,
187 particularly when undergoing regular physical exercise [31] the internalized proteins, after
188 proteolysis, could be supplying essential amino acids for protein resynthesis. It is therefore
189 conceivable that because DBP in myotube cultures is capable of retaining 25(OH)D for many
190 hours (Fig. 1) [24], that the DBP bound to cytoplasmic actin, is protected from the proteolysis
191 that other internalized proteins undergo. Nevertheless, when the cytoplasmic DBP is eventually
192 broken down, the retained 25(OH)D would be released and could then diffuse out of the cell
193 and back into the circulation.

194

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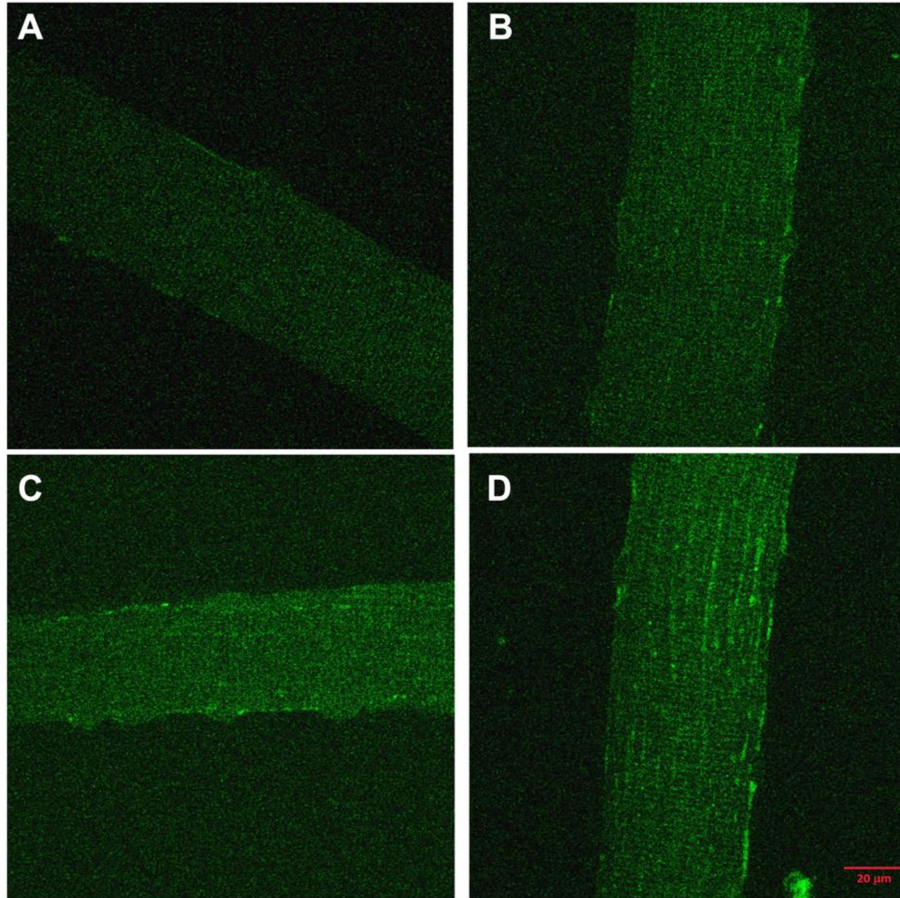
196 **Figure 3**.



197

198 **Fig 3. The uptake of Alexafluor488- labelled DBP into primary muscle fibers**. Myofibers were
199 isolated from the flexor digitorum brevis of euthanized Balb/c mice and incubated in
200 quadruplicate wells on poly-L-lysine-coated glass coverslips. Fibers were incubated for 0, 4, 12
201 or 24 hours with 1 µM of Alexa Fluor488-DBP (labelling kit from Molecular Probes) in DMEM
202 supplemented with 20% serum replacement. Incubations were set up to finish at the same
203 time. Cells were then fixed in paraformaldehyde for 20 minutes. Fluorescent images were
204 captured with a Zeiss LSM 510 Meta Spectral confocal microscope, using optimized saturation
205 settings for the 24-hour time point. The fluorescence intensity can be seen increasing from A) 0
206 hours, B) 4 hours, C) 12 hours and D) 24 hours.

207

208 Because the concentration of 25(OH)D in skeletal muscle of sheep (Table 2) was significantly
209 higher in winter than in summer, while the plasma concentration of 25(OH)D was decreasing, it
210 is likely that some endocrine factor was modifying the storage capacity of muscle according to
211 changes in vitamin D status. The circulating levels of at least two hormones rise in winter and
212 decline in summer. These are thyroid stimulating hormone (TSH) [32] and parathyroid hormone

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213 (PTH) [33]. There is no information to link TSH or thyroid hormone with changes in the uptake
214 or release of 25(OH)D by skeletal muscle. However, the small increase in PTH concentration in
215 blood plasma when 25(OH)D levels fall to 50-60 nM/L [33] make it a candidate as a regulator of
216 muscle uptake and release of 25(OH)D.
217
218 Receptors for PTH have been demonstrated on mouse muscle fibers and on differentiated
219 myotubes in culture, but receptors are not found on undifferentiated myoblast cells [34].
220 Incubation of myotubes with low concentrations of PTH (0.1-1 pM) for 3 hours, diminished the
221 subsequent uptake of ^{3}H-25(OH)D over 16 hours. Conversely, the release back into the medium
222 of ^{3}H-25(OH)D, already accumulated by myotubes, was enhanced by addition of low
223 concentrations of PTH to the incubation medium [34]. Although these results demonstrate an
224 effect of PTH on the transport of 25(OH)D into and out of myotubes, they paradoxically suggest
225 that an increased exposure of muscle cells to PTH would decrease their ability to conserve
226 25(OH)D. The rise in PTH concentration in blood as the 25(OH)D levels fall in winter suggests
227 that if PTH is the hormone regulating muscle uptake and release of 25(OH)D, then that
228 enhanced uptake and delayed release of ^{3}H-25(OH)D should have been seen with cells *in vitro*.
229 However, in those experiments [34], the incubation medium with added PTH did not contain
230 any DBP. Hence the action of PTH in those studies was compatible with the hypothesis that its
231 effect on muscle cells would be to enhance both the breakdown of cytoplasmic DBP and
232 perhaps its rate of uptake from extracellular fluid. The effect *in vivo* of enhanced DBP uptake
233 and breakdown in muscle would be to increase the rate of cycling of 25(OH)D by muscle cells
234 and thus prolong the residence time of 25(OH)D in the circulation. In the disease state of
235 hyperparathyroidism, the greatly elevated concentration of PTH in blood, results in increased
236 loss of 25(OH)D, mediated by the increased renal production of 1,25(OH)$_2$D which activates the
237 hepatic uptake and destruction of 25(OH)D [10]. In contrast, the very small increase in PTH in
238 blood with the seasonal decline in 25(OH)D concentration, does not provoke increased
239 secretion of 1,25(OH)$_2$D from the kidney. Nevertheless, that concentration of PTH observed
240 with the seasonal fall in vitamin D status has been shown to activate the muscle PTH receptor
241 on myocytes *in vitro* [34].

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242

243 **Conclusion**

244 This active cycling of 25(OH)D into and out of skeletal muscle cells explains the long residence
245 time of 25(OH)D in blood. Perhaps then, if ways could be found to optimize this process some
246 of the diseases linked epidemiologically with low vitamin D status could be minimized. Low
247 circulating levels of vitamins A, E and K can be related directly to the specific pathology in their
248 function caused by deficiency of these micronutrients. Because vitamin D has been classified as
249 a similar micronutrient, attempts have been made to obtain evidence for a direct link between
250 low concentrations of 25(OH)D in blood and a range of diseases such as diabetes, and various
251 cardiovascular and oncology diseases, where there are epidemiological suggestions that low
252 vitamin D status is a causative factor in their etiology. Although the level of 25(OH)D in blood
253 may indicate the adequacy of vitamin D supply to meet requirements for production of
254 1,25$(OH)_2$D, it is not a direct indicator of the function of that hormone. Because the many
255 regulatory functions of 1,25$(OH)_2$D also involve other endocrine and gene expression variables,
256 an epidemiological link between low vitamin D status and a disease process is not necessarily a
257 cause and effect relationship. This is well illustrated by the difficulty of demonstrating health
258 benefits of vitamin D supplementation in many clinical trials [35]. Nevertheless, if ways could be
259 found of optimizing the efficiency of the muscle conservation mechanism for 25(OH)D, perhaps
260 by a pharmacological agent or some exercise regime, that would ensure that vitamin D status
261 would also be optimized by a process that has evolved to adapt to seasonal changes in vitamin
262 D supply.

263

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265 C.G-T; validation, D.R.F., R.S.M., M.S.R.; investigation, M.S.R., M.A., D.A.P., C.G-T.; writing—
266 original draft preparation, D.R.F., M.S.R., R.S.M; writing—review and editing, M.A., T.C.B-S;
267 visualization, M.S.R., D.A.P.; supervision, R.S.M, D.R.F., M.S.R., C.G-T.; project administration,
268 R.S.M., M.S.R., D.R.F.; funding acquisition, R.S.M., D.R.F., M.A. All authors have read and agreed
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279

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