

1 **Recent developments in D₂O tracer approaches to measure rates of substrate**
2 **turnover: implications for proteins, lipids and nucleic acid research**

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11 **Abbreviated Title: D₂O in the measurement of substrate turnover**

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30 **Abstract**

31 **Purpose of Review**

32 Methods that inform on dynamic metabolism that can be applied to clinical
33 populations to understand disease progression and responses to therapeutic
34 interventions are of great importance. This review perspective will highlight recent
35 advances, development and applications of the multivalent stable isotope tracer D_2O
36 to the study of substrate metabolism with particular reference to protein, lipids and
37 nucleic acids, and how these methods can be readily applied within clinical and
38 pharmaceutical research.

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40 **Recent Findings**

41 Advances in the application of D_2O techniques now permit the simultaneous dynamic
42 measurement of a range of substrates (i.e protein, lipid and nucleic acids, along with
43 the potential for 'OMIC's methodologies) with minimal invasiveness- further creating
44 opportunities for long-term 'free living' measures that can be used in clinical settings.
45 These techniques have recently been applied to ageing populations and further in
46 cancer patients revealing altered muscle protein metabolism. Additionally the efficacy
47 of numerous drugs in improving lipoprotein profiles and controlling cellular
48 proliferation in leukemia have been revealed.

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50 **Summary**

51 D_2O provides opportunities to create a more holistic picture of *in vivo* metabolic
52 phenotypes, providing a unique platform for development in clinical applications and
53 the emerging field of personalized medicine.

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55 **Key words (3-5)** deuterium oxide, D_2O , stable isotope, skeletal muscle, metabolism

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56 **Abstract: 188**

57 **Introduction**

58 The ability to determine the metabolic regulation of diseases, ageing and trauma at
59 the whole body or organ level has been a significant driver in scientific research. One
60 of the major challenges to achieving this is how to capture the dynamic nature of
61 metabolic processes *in vivo*, in humans. Stable isotopes are the research tool
62 making this possible since they permit quantification of protein, lipid and nucleic acid
63 metabolism, which has traditionally been performed through the use of substrate-
64 specific tracers (e.g. ¹³C/¹⁵N amino acid tracers, ¹³C palmitate, and ²H glucose) [1].
65 Recently, experimental use of the D₂O tracer, which can be considered “non-
66 substrate specific” (i.e. incorporating into all major macromolecules), has undergone
67 a resurgence (Figure 1) [2]. Here, we consider how D₂O is revolutionising the study
68 of *in vivo* dynamic metabolism; we describe the basis of D₂O implementation,
69 focusing on its use in humans and recent technical advances that extend the utility of
70 this tracer to study human substrate metabolism *in vivo*, in particular its rapidly
71 progressing translation to a clinical setting.

72

73 **Application of deuterium oxide as a stable isotope tracer**

74 D₂O was one of the first isotope tracers to be used in metabolic research soon after
75 it's discovery by Harold Urey in 1932, the seminal works of Schoenheimer,
76 Rittenberg and Ussing demonstrated incorporation of deuterium from D₂O into many
77 metabolic pools [1]. Once introduced into cellular pools D₂O equilibrates throughout
78 all body water and is incorporated into metabolites via condensation/hydrolysis
79 reactions involving water; crucially, this occurs in a constant and predictable manner
80 (Figure 1). Using appropriate D₂O dosages, permits the measurement of a huge
81 range of metabolic processes, from the synthesis of deuterated precursors and their
82 subsequent incorporation into polymers can be made e.g. deuterated alanine into
83 protein, glucose into glycogen, fatty acids into triglycerides and ribose moieties into
84 nucleic acids (RNA/DNA) (Table 1) [2]. D₂O has a slow elimination rate from human

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85 body water ($t_{1/2}$ 9-11 days) and so steady or pseudo-steady state enrichments can be
86 easily maintained by regular daily or weekly top-ups, providing the unique potential
87 for measurements of metabolism to be performed over hours, days, weeks or even
88 months [3–6]. Further, by collection of regular saliva (or urine) samples, body water
89 enrichment can be easily monitored throughout, tracking with precursor labeling,
90 enabling subjects to undertake their usual habitual activity and dietary regimes.

91 These unique properties of D_2O has made the popularity of its application,
92 particularly to human research, increase exponentially over recent years. A major
93 advantage of using D_2O over substrate-specific tracers is the ease of administration,
94 being orally consumed negating the need for sterile I.V infusions and a controlled
95 laboratory environment, such that subjects can be studied 'free-living' over long
96 periods [3,6]. This provides a unique opportunity to metabolically phenotype a
97 greater range of populations particularly in a clinical setting where access has been
98 restricted or contraindicated with I.V tracers (i.e. in care homes, adolescents etc.).
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100 **Recent progress in using D_2O to study protein synthesis in humans**

101 Although the application of D_2O to the measurement of protein turnover dates back to
102 the work of Hans Ussing in 1941, it is only in the past decade that its validity for
103 measuring muscle protein turnover has been established and subsequently applied
104 in humans [3–7]. Given the importance of skeletal muscle as a metabolic tissue in
105 health and disease, it is unsurprising the application of these techniques has initially
106 been focused on the measurement of muscle protein synthesis (MPS). Moreover, the
107 accessibility of skeletal muscle for biopsy coupled to the slow turnover of the body
108 water pool makes D_2O ideally suited for application to the study of this slowly turning
109 over metabolic pool. One of the first attempts to measure the rate of MPS in humans
110 maintained body water around 2% over a 6-week period (by ingesting 150 ml D_2O
111 (70 atom percent (AP)) per day during week 1 then 100 ml D_2O daily thereafter). In
112 comparing a young sedentary and an older group undertaking an aerobic training

113 program, the investigators showed greater MPS in the old group, demonstrating the
114 utility of D₂O for investigating the mechanisms of long-term “anabolic interventions”
115 [2]. By further refining these principles with highly sensitive gas chromatography
116 pyrolysis isotope ratio mass spectrometry (GC-pyr-IRMS), substantial improvements
117 in the analytical limit of detection (LOD) and resolution were made, leading to the
118 possibility of reduced D₂O dosing i.e. a single bolus 150ml 70AP, permitting MPS
119 measurement over 8 days [6]. This is especially important when one considers the
120 potential issue of nausea associated with consumption of increased volumes of D₂O.
121 Using these refined techniques we proved the concept that exercise-induced
122 increases in myofibrillar, collagen and sarcoplasmic fractional synthetic rates could
123 be quantified over as little as 2 days, with measures of MPS over 3 hours (in
124 response to amino acid feeding) also possible, simply by increasing the D₂O dose
125 [7]. Importantly, hourly MPS rates were identical to those we and others had shown
126 in prior acute studies using stable-isotopically labeled AA and in direct comparison
127 with substrate-specific AA tracers, D₂O yielded quantitatively similar increases in
128 MPS with feeding [7].

129
130 Following these initial measures of MPS with D₂O, a series of studies have
131 demonstrated its wide applicability for the study of both short-term (4-7 days) as well
132 as longer-term (4-8 wks) interventions [3–6]. Predominantly, these so far have been
133 used to demonstrate what has been coined “integrated” responses of MPS to a
134 range of anabolic stimuli including; resistance exercise, high intensity interval training
135 [8], aerobic exercise and long-term (4-wks) sprint interval training [9]. Further, we
136 have demonstrated that there is significant hypertrophy and structural remodeling in
137 the early stages of resistance exercise (~3-wks) supported by integrated increases in
138 MPS [10,11]. Interestingly as training continued (up to 6-wks), increased MPS was
139 attenuated despite progressive intensity [10], reflecting an adaptive waning to the
140 anabolic stimulus. These studies have provided an integrated understanding of the

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149 role of protein turnover in regulating established physiological adaptation to exercise
150 [2].

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152 D₂O has also been recently used in a more clinical context. Advanced ageing is
153 associated with a continual and progressive decline in skeletal muscle mass, quality
154 and function [12]. While the etiology of this in humans remains poorly defined, it is
155 clear that older individuals display blunted acute responses to anabolic stimuli such
156 as feeding (particular amino acids and RE – so called “anabolic resistance” [12,13].

157 Using D₂O to compare long-term MPS between young and older individuals, we were
158 the first to demonstrate that blunted acute responses of MPS to exercise also
159 manifest as long-term deficits in MPS [10]. This was not necessarily predictable
160 given the lack of linkage between acute MPS responses to exercise and resulting
161 muscle hypertrophy. Moreover, recently, it was shown that studying nutrition as well
162 as exercise interventions was also feasible; in this study, the authors showed that
163 adding supplemental leucine to meals could increase integrated MPS in older

164 individuals [4]. These studies demonstrate that D₂O applications have the potential to
165 inform on integrated and temporal responses to nutrition and exercise interventions

166 in a “mechanistic fashion”. It is also of great interest that D₂O has shown utility in a
167 clinical setting in being applied to measure MPS in patients with upper GI cancer.
168 Using a single bolus approach over 4 days immediately prior to surgery, patients
169 losing weight had higher rates of MPS (0.073 %/h) when compared to weight stable
170 and controls (0.058 %/h), possibly indicating greater protein turnover rates, although
171 to lose muscle mass over time, protein breakdown would have to exceed MPS [14].

172 Nonetheless, this study does show the feasibility of applying D₂O in clinical
173 populations; future work will expand the use of this tracer and seek both mechanistic
174 insight of disease/ageing processes in addition to nutritional, exercise or
175 pharmacological interventions. Moreover, with the very recent introduction of

176 dynamic proteomic techniques alongside the use of D₂O, it is now possible to

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177 measure the turnover of a large number of individual proteins [9] rather than studying
178 bulk myofibrillar or collagen protein fractions, as outlined by the recent "Virtual
179 Biopsy" technique [15]. These developments have opened up a whole new stream of
180 measures to aid in the mechanistic understanding of human ageing and disease.

181
182 **Recent progress in using D₂O to study fat and lipid metabolism**

183 Lipid metabolism has been the mainstay application of D₂O for ~80 years. Great
184 technical and methodological refinement over the past 70 years punctuated by the
185 seminal works of Jungas, Previs and Brunengraber and Hellerstein and Parks have
186 engendered an array of D₂O based lipid assays [16]. For example, by measuring the
187 amount of deuterium incorporated from water into newly synthesized fatty acids,
188 glycerol-3-phosphate and/or cholesterol combined with mathematical modeling
189 techniques, D₂O has the unique potential for measuring rates of *de novo* lipogenesis
190 (DNL), triglyceride synthesis (and turnover) and sterol biosynthesis simultaneously.
191 The details, development and technical considerations for these techniques is
192 beyond the scope of this review; the reader is directed to the following for more detail
193 [1,2,16].
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195
196 Much of the progress over the past 5-years has been in how these novel D₂O based
197 techniques can be applied (rather than further development of the isotopic theory of
198 the models *per se*), particularly in terms of health, disease and the rapidly evolving
199 discipline of personalized medicine. For example these techniques have helped to
200 highlight the mechanisms underlying impaired adipose lipid metabolism in insulin
201 resistant humans (e.g. highlighting decreased adipose DNL and TG synthesis: [17]),
202 the mechanisms driving the increase in adiposity associated with chronic insulin
203 treatment (through an increase in triglyceride synthesis or inhibition of lipolysis and
204 the alterations to cholesterol flux due to dyslipidemia and coronary heart disease
205 [18]. However, more recently there has been marked progress in their implementation

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206 alongside high throughput OMIC technologies, in order to gain a more holistic insight
207 into the metabolic regulation of health and disease [16]. This has been in a large part
208 driven by the rapid evolution of new mass spectrometry technologies, in particular
209 the introduction of high resolution mass spectrometers (HRMS) such as Fourier
210 Transform-MS and Orbitrap MS, which can provide isotopic resolution as high as
211 500,000 for some instrumentation. This increase in resolution when combined with
212 liquid chromatography (i.e. LC-HRMS) has provided capabilities for measuring low
213 levels of ^2H enrichment (comparable to that of traditional “gold standard” GC-IRMS
214 techniques) of free fatty acids in a high throughput manner, alongside the
215 measurement of associated whole lipid/lipoprotein species (in the form of
216 lipidomics/proteomics). This has provided a unique analytical platform capable of
217 determining how changes in lipid flux interact to influence the whole
218 lipidome/lipoproteome, hence providing exquisite insight the regulation and control of
219 lipid metabolism and its interaction with other aspects of metabolism under health
220 and disease *in vivo* using D_2O . For example, incorporating traditional lipidomics with
221 D_2O permitted the measurement of dynamic changes in lipid profiles associated with
222 dietary manipulation in animal models. Moreover the simultaneous incorporation of
223 D_2O into high density lipoproteins, alongside cholesterol allows the measurement of
224 the kinetics of HDL *in vivo*, an important technique which could greatly benefit the
225 development of HDL targeted therapies in conditions such as dyslipidemia and
226 atherosclerosis. Indeed, this has been the target in recent years with a number of
227 recent studies utilizing these D_2O techniques to provide a greater insight into the
228 mechanisms and efficiency of a number of LDL-cholesterol lowering therapies in
229 particular. For example, D_2O techniques have help to identify that the cholesterol
230 ester transfer protein (CETP) inhibitor anacetrapib was effective in promoting pre β
231 HDL formation potentially helping to lower LDL-cholesterol levels, acting as a
232 beneficial treatment for coronary heart disease. In addition, the administration of the
233 cholesterol lowering drug ezetimibe was shown to increase the flux of plasma-

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234 derived cholesterol into fecal neutral sterols and hence increased excretion of
235 cholesterol from the body, thereby helping to reduce LDL-cholesterol formation and
236 hence atherosclerosis. These studies highlight the added insight the inclusion of D₂O
237 to lipidomics can provide, and how these techniques will continue to benefit medical
238 and pharmaceutical insight in future when combined alongside standard biochemical
239 techniques and novel high throughput OMICs platforms; this is clearly where the
240 future lies for this niche technique.

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241 **Recent progress in the use of D₂O in the study of nucleotide turnover**

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242 There are many scenarios whereby the ability to quantify DNA and RNA turnover is
243 desirable (e.g. tumourogenesis, skeletal muscle satellite cells, ribosomal biogenesis
244 etc.). Yet to date, advances in the dynamic measurement of nucleotide metabolism
245 have considerably lagged behind that of proteins and lipids due to the lack of suitable
246 precursor compounds. Bromodeoxyuridine and tritiated (radio-active) thymidine have
247 been utilized, although they are incorporated via salvage pathways that are variable
248 and affected by extracellular nucleoside concentrations. Moreover, these analogues
249 are toxic and cannot be used in humans. The potential use of D₂O overcomes many
250 of these restrictions by labeling nucleosides via *de novo* synthesis - a pathway (figure
251 1) that is up regulated during cellular division, is unaffected by extracellular
252 nucleoside concentrations and rarely relies on reutilization. As such methods that are
253 safe for human use and measure cellular division are available [19].
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257 Initial measures of cellular proliferation using D₂O in humans were that of fast
258 turnover blood cells such as PBMC's. Outside of this, these techniques have been
259 used over extended periods (4-6 weeks) to quantify DNA synthesis in skeletal
260 muscle in response to nutritional and exercise interventions [2,20]. This is an area of
261 specific current interest since controversy still exists to the role of skeletal muscle
262 stem cells (satellite cells) e.g. in sarcopenia and exercise adaptation [21,22]. With

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263 many disorders originating from altered cellular proliferation, these techniques have
264 also been used to investigate i) B and T cell kinetics in patients with leukemia or HIV
265 ii) breast epithelial cells in both normal and tumor tissues and, iii) in cellular areas
266 defined as benign or cancerous from prostate tissue - all showing altered rates of
267 proliferation. These methods have again therefore shown great potential for
268 application in a clinical setting. Most recently, D_2O was used to measure B cell
269 proliferation in patients with chronic lymphocytic leukemia (CLL). Deuterium was first
270 incorporated into CLL cells before treatment with the Bruton's tyrosine kinase
271 inhibitor ibrutinib. By monitoring CLL DNA enrichment over the following weeks, it
272 was demonstrated that ibrutinib dramatically decreases CLL cell birth via the lack of
273 deuterium label dilution and hence proliferation of new cells [23].

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274 In addition to circulating cells, DNA synthesis rates have recently been made from
275 tissue biopsies of fat, in attempts to link fat metabolism with obesity and insulin
276 resistance. Storage of excess fat involves adipocyte hypertrophy along with
277 preadipocyte and adipocyte proliferation, with fat distribution and storage related to
278 obesity related diseases. To investigate this, pure adipocytes and preadipocytes
279 were isolated after D_2O labeling, identifying abdominal and femoral fat depots have
280 different proliferation kinetics [24]. Furthermore the rate of adipocyte replacement
281 rates positively correlated with BMI and visceral adiposity but negatively correlated
282 with insulin sensitivity – all signs of impaired metabolic health [25].

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284 Measures of RNA synthesis are also possible with D_2O and have the potential to
285 inform on dynamic ribosomal biogenesis- a primary determinant of protein synthesis
286 rates during growth, cellular proliferation and homeostasis. Deoxynucleotides are
287 reduced from nucleotides and as such opportunities arise for the measurement of
288 RNA synthesis using D_2O ; generally abiding by the same principles as above.
289 Measurements of RNA synthesis in rodent liver have recently been made using D_2O
290 [26]; however currently there is a lack of routine methods in the measurement of
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292 human RNA synthesis, particularly that in slow turnover tissues i.e. muscle. The
293 development of such methods will have considerable impact in the clinical field,
294 especially due to the loss of cell cycle control in many conditions such as cancer [27].

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Conclusion

300 D₂O applications hold considerable promise to generate unheralded insight into
301 dynamic metabolism in 'free living' and clinical environments. With development of
302 high-resolution mass spectrometry enabling "D₂O-MICS" (protein/lipids/metabolites)
303 a single bolus of D₂O coupled to a tissue biopsy can reveal a more holistic picture of
304 *in vivo* metabolic phenotypes and mechanisms of interventions than has ever been
305 possible in a clinical (i.e. studies in humans) context. Crucially, D₂O-MICS can also
306 give rise to 'translationally relevant' predictive, diagnostic and therapeutic biomarkers
307 in humans, reflecting disease progression and responses to therapeutic
308 interventions.

309

Key Points

311

312 - Having methods that reveal the dynamic turnover of metabolic substrates are
313 of great importance in unraveling diseases processes and in the future of
314 personalized medicine.

315

316 - D₂O has shown effectiveness at providing longer-term, integrated,
317 multisubstrate measures (proteins, lipids, nucleic acids) across a range of
318 tissues and populations.

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320 - The ease of application and opportunities created to measure a range of
321 substrates combined with the development of OMIC's methodologies, D₂O

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322 has great potential to provide a more holistic picture of *in vivo* metabolic in
323 clinical populations.

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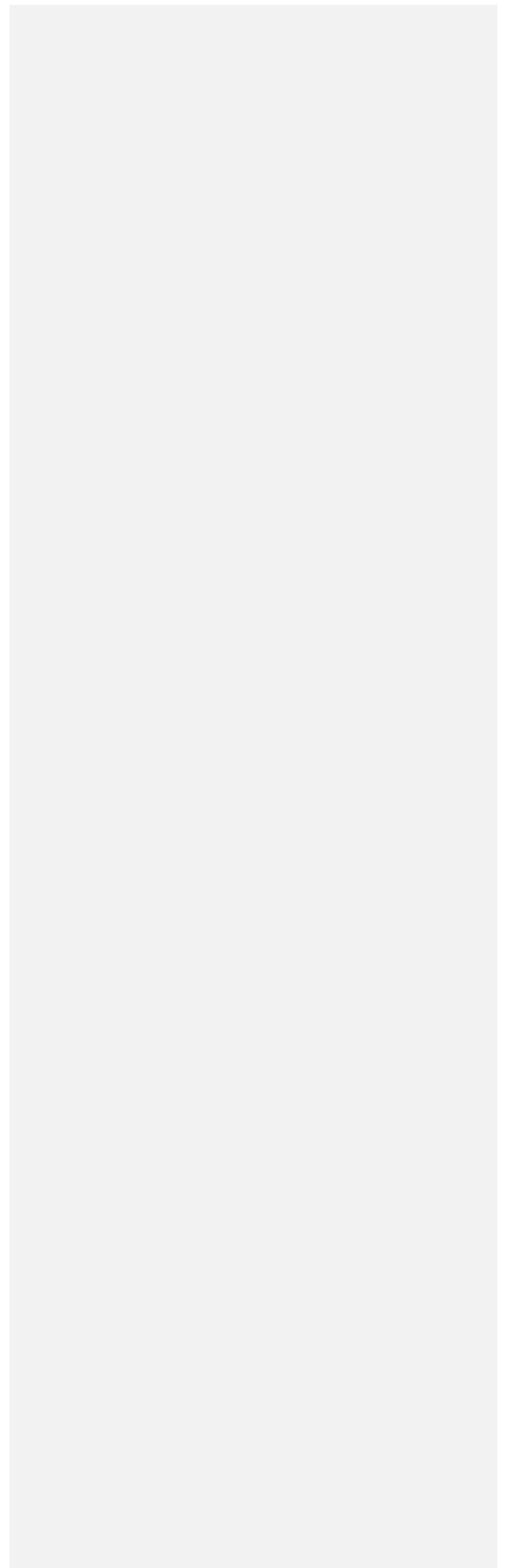
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350 **Figure Legend**

351 **Figure 1**

352 **D₂O – A multivalent tracer.** D₂O can be simply administered by oral consumption
353 and becomes rapidly equilibrated within body water. Subsequently, deuterium
354 becomes predictably incorporated into many precursors in which their metabolic fate
355 can be followed.

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357 **Table Legend**

358 **Table 1 –D₂O loading regimes.** Table 1 shows the dose of D₂O and the analytical
359 machinery required to ensure detection of desired substrates. The doses are taken
360 from published examples or experimental calculations and can inform on the
361 necessary D₂O administration depending on the mass spectrometry instrumentation
362 available. (*) Turnover rates of individual proteins and lipids vary and so earlier
363 sampling is preferable to capture maximum number of analytes. **AP**, atom percent.

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364 **LOD**, limit of detection. **GC-MS**, gas chromatography mass spectrometry. **GC-pyr-**
365 **IRMS**, gas chromatography pyrolysis isotope ratio mass spectrometry. **LC-HRMS**,
366 **liquid chromatography high resolution mass spectrometry.**

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387 [All authors contributed equally to this manuscript](#)

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392 **Conflicts of interest**

393 There are no conflicts of interest

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411 *Macdonald et al 2015- Used D₂O in the measurement of muscle protein synthesis in
412 patients with upper GI cancer, demonstrating altered muscle protein synthesis rates
413 and the application of the D₂O approach within a clinical setting.

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414 * Brook et al 2016 Application of D₂O techniques to measure long term muscle
415 protein synthesis in young and old individuals, demonstrating impaired anabolic
416 response to resistance exercise with age.

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417 * Wilkinson et al 2015. First demonstration of D₂O in the acute measurement of
418 human muscle protein synthesis (≤3h), providing a less invasive and cost effective
419 method. Additionally demonstrated synthesis rates determined by D₂O to be
420 equivalent to those using traditional amino acid tracer approaches.

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421 * Burger et al 2017. Provided the first in vivo demonstration in the effectiveness of drug
422 treatment ibrutinib on cellular proliferation in CLL patients by monitoring deuterium
423 incorporation into DNA

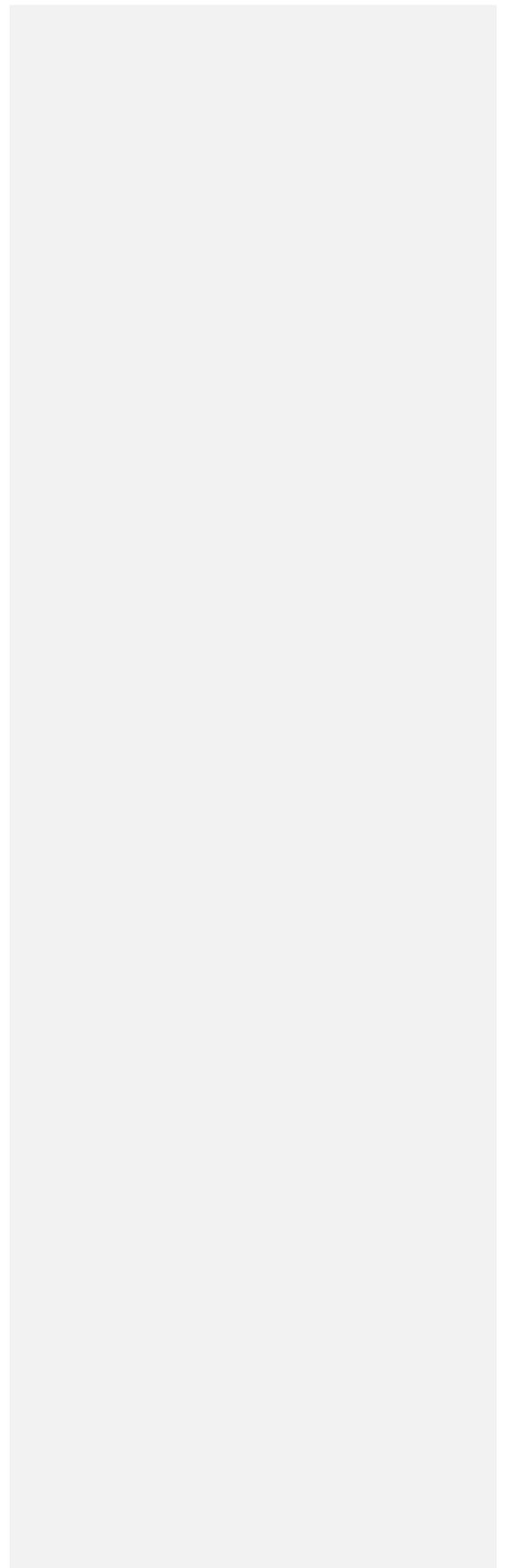
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Application	Dose of D2O (70AP)	Measurement Duration	Minimal Analytical Requirement	LOD
Protein turnover				
Acute	400ml	3hrs	GC-pyr-IRMS	0.0005%
Chronic	150ml + 50ml/wk 150ml + 100-150ml/d	2d – 6wk 4wk– 6wk	GC-pyr-IRMS GC-MS	0.0005% 0.5%
Individual*	150-400ml + 80-100ml/d	1d-4wk	LC-HRMS	0.0005%
Lipid turnover				
Acute	150ml + 50ml/wk	2d – 6wk	LC-HRMS	0.0005%
Chronic	150-400ml + 100-150ml/d	4wk-10wk	GC-MS	0.5%
Individual*	~300ml	1d- 6wk	LC-HRMS	0.0005%
Nucleic acid turnover				
DNA/RNA fast (>5%.d)	150 ml + 50ml/wk 150ml + 100-150ml/d	1d – 6wk 2d – 6wk	GC-pyr-IRMS GC-MS	0.0005% 0.5%
DNA/RNA Slow (<5%.d)	150 ml + 50ml/wk 150ml + 100-150ml/d	1d – 6wk 4wk – 6wk	GC-pyr-IRMS GC-MS	0.0005% 0.5%

Deleted: %

