Energy-Resolved Fragmentation Aiding the Structure Elucidation of Steroid Biomarkers.

Christopher C. J. Fitzgerald [a], Christopher Bowen [b], Madysen Elbourne [c], Adam Cawley [d], and Malcolm D. McLeod [a*]

[a] Research School of Chemistry, Australian National University, Canberra, Australian Capital Territory, 2601, Australia.

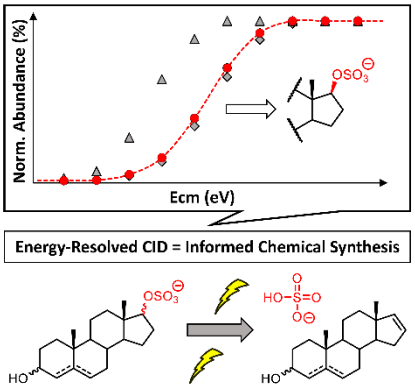
[b] Mass Spectrometry Business Unit, Shimadzu Scientific Instruments (Australasia), Rydalmere, New South Wales, 2116, Australia

[c] Centre for Forensic Science, University of Technology Sydney, Broadway, New South Wales, 2007, Australia

[d] Australian Racing Forensic Laboratory, Racing NSW, Sydney, New South Wales, 2000, Australia.

*Corresponding Author

ABSTRACT: The identification and confirmation of steroid sulfate metabolites in biological samples is essential to various fields, including, anti-doping analysis and clinical sciences. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) is the leading method for the detection of intact steroid conjugates in biofluids, but due to the inherent complexity of biological samples and low concentration of many targets of interest, metabolite identification based solely on mass spectrometry remains a major challenge. The confirmation of new metabolites typically depends on the comparison to synthetically derived reference materials that encompass a range of possible conjugation sites and stereochemistries. Herein, energy-resolved collision induced dissociation (CID) is used as part of UHPLC-HRMS/MS analysis to distinguish between regio- and stereo-isomeric steroid sulfate compounds. This wholly MS-based approach was employed to guide the synthesis of reference materials to unambiguously confirm the identity of an equine steroid sulfate biomarker of testosterone propionate administration.



INTRODUCTION

Anabolic androgenic steroids (AAS) are among the most commonly detected group of compounds in world sport.[1] The use of AAS is proscribed by sporting bodies and included in the World Anti-Doping agency's (WADA) Prohibited List and the International Federation of Horseracing Authorities (IFHA) International Agreement on Breeding, Racing and Wagering.[2,3] The development of new methods to detect these steroids and their metabolites form an essential and ongoing part of anti-doping efforts to maintain fair competition and protect the welfare of participants.

Endogenous and exogenous AAS are mainly excreted in urine as their phase II metabolites.[4] In phase II metabolism, steroids undergo enzymatically driven conjugation which is dominated by two major classes; sulfates and glucuronides. These conjugates account for up to 97% of excreted steroid metabolites.[5–7] In equine AAS metabolism a larger proportion of sulfated conjugates has been reported relative to that observed in humans.[8] Intact phase II metabolites play an important role in anti-doping analysis as long-term biomarkers. Several studies have demonstrated longer urinary excretion times for sulfate metabolites.[9–15] Common to these studies is the confirmation of new biomarkers using chromatographic retention time and tandem mass spectrometry (MS/MS) experiments. In the case of AAS sulfate conjugates the spectra are usually dominated by the precursor ion and transitions so sulfate-derived fragments, including hydrogen sulfate, *m/z* 97, HSO_4^-.[9,16–19] This fragmentation behavior has been a key identifier in various anti-doping and clinical studies characterizing steroid monosulfates.[17,20–24]

One limiting factor in identifying new steroid sulfate biomarkers is determining the position and stereochemistry of the sulfate ester. Typically, such studies rely solely on mass spectrometric methods, as insufficient material is available to perform more comprehensive nuclear magnetic resonance (NMR) spectroscopic analysis or X-ray crystallography. Confirmation by MS/MS thus requires access to a wide range wide of synthetically derived regio- and stereoisomeric reference materials that are then compared to the

new biomarker using chromatographic and MS/MS analysis. This approach is dependent on highly specialized synthetic skills, is labor intensive and expensive.[18] Alternatively, hydrolysis followed by gas chromatography-mass spectrometry (GC-MS) analysis can be used to identify the unconjugated metabolite by comparison to the wide range of commercially available unconjugated reference materials, but depending on the structure, the original site of conjugation may not be readily ascertained.[16,18,25,26]

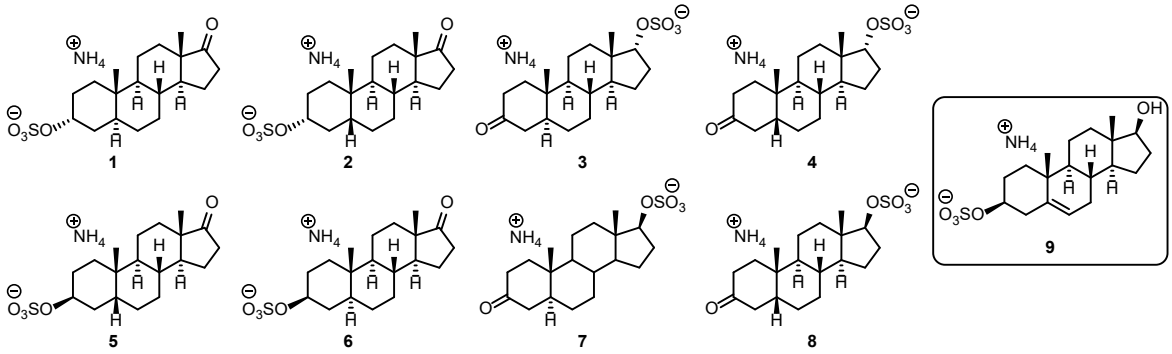


Figure 1. Synthesized library of reference materials (**1-9**). Including hydroxyandrostanone monosulfates (**1-8**) and an androstenediol monosulfate (**9**).

Recently developed MS-based methodologies such as energy-resolved collision induced dissociation (CID) and ion mobility spectrometry (IMS) experiments can provide additional information to aid the characterization of steroid biomarkers. In IMS the use of collision cross section (CCS) values has been demonstrated to be an effective additional molecular descriptor allowing for increased selectivity and improvements in the detection of phase I and intact phase II sulfate and glucuronide conjugates of AAS at biologically relevant concentrations in urine, although the resolution of isomers remains challenging.[27–29] The CCS has also been shown to be reproducible across different instrumental platforms, allowing for a consolidation of steroidal CCS data in databases.[29]

Energy-resolved CID experiments, where the fragmentation of precursor ions is studied across a wide range of applied collision energies has been shown to strengthen structural interpretation in distinguishing isomeric small molecules and metal oxide clusters alike, based on experimentally derived appearance energies (AE).[30–34] The use of estimated threshold energies ($E_{5\%}$) have also been applied to explore competing modes of bond homolysis in alkoxyamines.[35] Energy-resolved CID has also been used to study the dissociation pathways of the Beta-2 agonist class of performance enhancing of drugs,[36] and to aid the structural characterization of N-glycans from their fragmentation patterns.[37] Recently, these approaches have been extended to reveal characteristic differences between sets of related steroid monosulfates based on estimated threshold energies ($E_{5\%}$).[38] A common drawback in this energy-resolved CID analysis is the sensitivity of the measured energies to small changes in experimental and instrumental parameters.[39] This variability suggested the need for a reference set of sulfated molecules to improve the reliability of experimentally measured threshold energies when used on different systems.[39]

This work describes the use of energy-resolved CID methods on an ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) instrument equipped with a high-speed quadrupole-tine of flight (Q-ToF) mass analyzer to differentiate the sulfate ester conjugation site and stereochemistry for a set of isomeric steroid sulfate compounds. The threshold energies ($E_{5\%}$) derived from single injections are used to aid in the structure elucidation of an unknown equine urinary metabolite found at elevated levels after testosterone propionate administration to a thoroughbred horse. The information derived from this approach helped guide the synthesis of candidate reference materials, thereby leading to the rapid identification and confirmation of a novel metabolite.

METHODS

Experimental. Materials and instruments associated with chemical synthesis and characterization are reported in detail in the supplementary material (S2).

UHPLC-HRMS/MS detection of AAS intact phase II metabolites. Energy-resolved CID experiments employed a 9030 Q-ToF mass spectrometer equipped with an ESI source interfaced to a Nexera LC-40D X3 pump system for chromatographic separation (all from Shimadzu, Rydalmere, Australia). The column used was a Shim-pack Velox C18 column (1.8 μm, 2.1 x 100 mm, Shimadzu). The UHPLC separation was performed at flow rate 0.4 mL/min, using gradient mixing of two phases: Solution A; 20 mM ammonium formate in 100% water; and solution B; 20 mM ammonium formate in 100% methanol. The gradient was 0-0.5 min (10% B), 0.5-20.5 min (10-100% B), 20.5-23.0 min (100% B), 23.0-23.01 min (100-10% B), 23.01-27.5 min (10% B). The injection volume was 5 µL and column oven temperature was 40 ^{0}C.

Parameters for energy resolved CID MS/MS experiments. Steroid sulfate compounds readily ionized as their monoanions ($[M\text{-}NH_4]^-$) under negative mode ESI. Scan MS (*m/z* 100-1000) were collected between 0.5 and 20.5 minutes. For the duration of this acquisition energy-resolved CID MS/MS acquisition (*m/z* 50-1000) was performed for each reference material targeting the precursor ion *m/z* 369.1740 with a Q1 width of *m/z* 1. Energy-resolved CID MS/MS spectra were acquired over a CE ramp from 10-65 eV in increments of 5 eV and immediately after higher energies were sampled at 75 and 80 eV. This gave a loop time (duty cycle) of 0.80 s comprising of one scan MS event (0.1 s) and 14 MS/MS events (0.05 s), giving a total scan speed of approximately 19 Hz. Resolution was approx-

imately 30,000 (FWHM) for scan MS and MS/MS experiments. The peak areas for precursor (*m/z* 369.1740) and sulfate derived product ions (*m/z* 96.9596 & 79.9568) were calculated from extracted ion chromatograms with a tolerance of ± 10 ppm using LabSolutions Insight software (Shimadzu).

Calculation of estimated threshold energy ($E_{5\%}$). Breakdown curves were obtained by plotting the normalized abundance (peak area) of the product ion(s) of interest, against the energy in the center-of-mass frame (E_{cm}), followed by least-squares fitting to the sigmoidal function of the type:

$$I_i(E_{cm}) = \frac{BR_i}{1+e^{(E_{1/2}-E_{cm})b_i}} \qquad \text{(Eq. 1)}$$

Where; I_i is the normalized abundance of the product ion of interest, BR_i is the branching ratio of the product ion, b_i describes the rise of the sigmodal curve, and $E_{1/2}$ is the energy at which the function has reached half of its maximum value.[33–35,40] Curve fitting was performed using KaleidaGraph® 4.5 by Synergy Software. Where possible, the ions derived from secondary fragmentation were summed back to their primary product ion. The estimated threshold energies ($E_{5\%}$) were derived from the energy (E_{cm}) when I_i = 5, i.e. the calculated energy (E_{cm}) required to achieve 5% fragmentation, and were calculated using as previously described.[35,38]

RESULTS AND DISCUSSION

A novel metabolite of testosterone propionate in equine. In a recent study aimed at the untargeted profiling of equine urinary sulfate metabolites after testosterone propionate administration,[41] two putative steroid sulfate metabolites with the same exact mass (*m/z* 369.1739) were discovered. The identity of one of these metabolites was confirmed as epiandrosterone sulfate (**6**) after matching it to synthetically derived reference materials (**1**-**8**, Figure 1). The reference materials possessed all possible regio- and stereo-isomeric combinations at the C3, C5 and C17 positions along a general C_{19} hydroxyandrostanone backbone. However, no match was found for the second metabolite, which showed a significant elevation (500-fold change) 12 h after drug administration. The new biomarker was observed to have an earlier retention time (7.69 min vs 10.16 min for epiandrosterone sulfate) in reverse phase chromatography, leading to the inference that the remaining metabolite possessed higher polarity than epiandrosterone sulfate. Two assumptions were made about the structure of the unknown metabolite: 1) an increase polarity resulted from an additional hydroxyl group on the steroid backbone, generally at the C3 or C17 positions;[8] 2) the addition of a C=C double bond was required to maintain the correct accurate mass, with this type of functionally commonly occurring at the C4 or C5 positions amongst androstenones, *c.f.*, testosterone and dehydroepiandrosterone (DHEA).[8] Based on these assumptions, sixteen possible C_{19} androstenediol monosulfate isomers, stemming from four general core structures, can be considered as potential candidates for the unknown metabolite (Figure 2 and Figure S1).

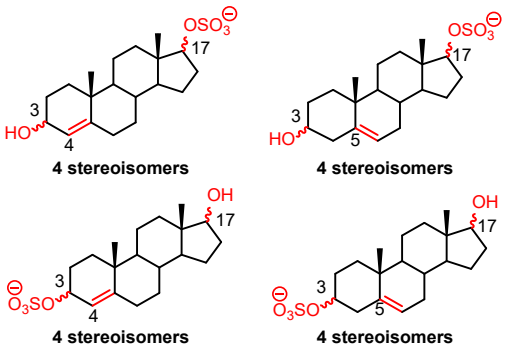


Figure 2. Possible isomers for the unknown metabolite (*m/z* 369.1741). There are 16 possible isomers for the androstenediol monosulfate class of molecules when the conjugation site is varied between the C3 and C17 positions and the double bond is either in the C4 or C5 positions. All 16 isomers are shown in the supplementary information, Figure S1.

Stereochemical determination of sulfate conjugation site using mass spectrometry. The general workflow used to acquire an energy-resolved breakdown curve of a sulfate ion is now described. After UHPLC separation and negative mode ESI, the compound undergoes scan MS followed by MS/MS. In MS/MS the collision energy is applied in a stepped fashion over 12 scans. The high scan speeds available for the Q-TOF mass analyzer allowed for the sampling of product ion data from low to high energies in small energy increments (5 eV, 10-65 eV) and at high resolution (30,000 FWHM). The energy profiles derived from the chromatographic peaks from single injections were then plotted, and the threshold energy calculated after least-squares fitting of the data to equation 1. To minimize the effects of inter-run variability on the measured energy profiles,[39] all reference materials and samples were run in triplicate in the same injection sequence.

The energy-resolved CID profile of the unknown metabolite was compared against the library of synthesized isomeric reference materials (**1**-**8**, Figure 1). These were chosen as they were readily accessible from their free steroid using previously established synthetic methodology.[42] It was hypothesized that the profiles of the 8 hydroxy-androstane sulfate isomers could be used as proxies to identify the stereochemistry at the conjugation site of the new biomarker, ultimately informing synthesis of the relevant reference material. The energy-resolved CID profile of androst-5-ene-3β,17β-diol 3-sulfate (**9**, boxed) was also obtained, as it was readily available from a previous synthesis.[18]

The results of the energy profiling workflow are displayed in Figure 3A and show the estimated threshold energies ($E_{5\%}$) of the nine synthesized isomers (**1-9**) and unknown metabolite (Metabolite).

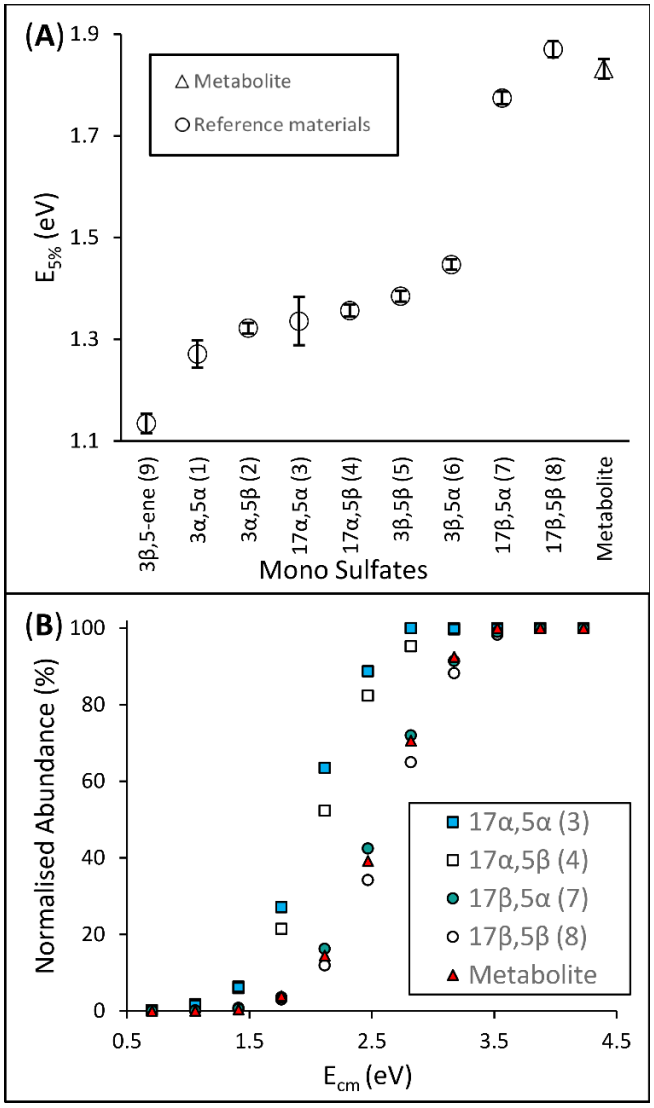


Figure 3: *Evaluation of synthesized reference materials and the new biomarker*. (**A**) Mean threshold energies ($E_{5\%}$ ± SD, n = 3) of the saturated steroid mono-sulfate reference materials (**1**-**9**) and the new biomarker (Metabolite), (**B**) Breakdown curves for the four 17-conjugated reference materials (**3**, **4**, **7** & **8**) and the new biomarker (Metabolite). Each reference material is identified by the stereochemistry at the conjugation site followed by the stereochemistry of the 5-position (i.e., 17β-hydroxy-5α-androstan-3-one, 17-sulfate (**7**) becomes 17β,5α (**7**))

Table 1: ***Confirmation using UHPLC retention time and MS/MS analysis.***

Reference material					Metabolite			
Compound name	RT (min) (%RSD, *n* =12) [theoretical *m/z*]	Product ion (*m/z*)	RA (%)	Threshold energy ($E_{5\%}$, eV) (SD, *n*=3)	RT (min) (%RSD, *n* =3) [tolerance]	RA (%)	AORC[43] [%Tolerance]	Threshold energy ($E_{5\%}$, eV) (SD, *n*=3)
androst-4-ene-3β,17β-diol, 17-sulfate (**10a**)	9.49 (0.02) [369.1740]	369.1721	38	1.82 (0.01)	9.49 (0.13) [9.45-9.55]	45	[27-50]	1.83 (0.02)
		351.1598	2			2	[0-12]	
		337.1466	39			31	[27-51]	
		96.9588	100			100	[70-100]	
		79.9567	1			2	[0-11]	

The new biomarker (metabolite) was detected the sulfate fraction of equine urine after administration of testosterone propionate. It was matched against the synthesized reference material (**10a**) according to Association of Official Racing Chemists (AORC) retention time and MS/MS criteria.[43] All CID product ion spectra used for confirmation were acquired using a collision energy (CE) of 40 eV derived from the energy-resolved CID experiments. The relative abundance of product ions was derived from the peak area of extracted ion chromatograms with a mass tolerance of ± 10 ppm. The threshold energy ($E_{5\%}$) for both compounds was obtained according to experimental section and matched within one standard deviation (*n*= 3).

All reference materials were chromatographically resolved (Table S1). Moreover, good separation was observed between biologically relevant epimers, such as androsterone sulfate (**1**, RT 11.86 min), epiandrosterone sulfate (**6**, RT 11.00 min) and etiocholanolone sulfate (**2**, 11.72

min).[8,13,19,44–47] The new biomarker has a high threshold energy, comparable to the 17β-oriented sulfates. The retention time of unsaturated androstenediol mono-sulfate (**9**) was observed to be closer to the unknown metabolite than the eight hydroxyandrostanone mono-sulfate compounds (**1**-**8**), due to the increased polarity resulting from the free hydroxy group. The diol monosulfate compound (**9**) was also found to have the lowest estimated threshold energy ($E_{5\%}$), due to a relatively low energy fragmentation leading to a conjugated diene product. The pattern in the $E_{5\%}$ for compounds (**1**-**8**) indicated that sulfate ester stability is largely governed by the stereochemistry at the conjugation site, while the configuration at a greater distance seems to have little effect (Figure 3A). This pattern is clearly demonstrated in the four reference materials that possess 17-conjugation (**3**,**4**,**7**, & **8**, Figure 3B). Here, despite there being large differences in the configuration of the A-ring, the 17β-oriented sulfates (**7**) and (**8**) show only a small difference in threshold energies. This was also observed for the 17α-oriented sulfates (**3** & **4**). Importantly, a large difference is observed between the 17α and 17β pairs in both their estimated threshold energies (Figure 3A) and energy-resolved CID profiles (Figure 3B). From this analysis we concluded that these functionally simpler hydroxyandrostanone sulfate molecules could be used as proxies for related species so long as the functionality and stereochemistry at the conjugation site is conserved. After evaluation of the new biomarker, the $E_{5\%}$ was found to aligned closely with the two 17β-sulfated reference materials (**7** & **8**, Figure 3). Assuming that the conjugation site and stereochemistry is the main contributor to the observed $E_{5\%}$ energy, we could therefore putatively assign 17β stereochemistry to the sulfate conjugation site of the new biomarker.

Confirmation of a novel metabolite. Using the $E_{5\%}$ energies from the energy-resolved CID as a guide, narrowed the identity of the new biomarker from sixteen to the four isomers, specifically those that contain a 17β-oriented sulfate ester (Figure 2 & Figure S1). Following the synthesis of the four isomers (Scheme S1), the new biomarker was matched to androst-4-ene-3β,17β-diol, 17-sulfate (**10a**, Figure 4). All four isomers were chromatographically resolved (Table S3). The confirmation was performed according to Association of Official Racing Chemists (AORC) retention time and MS/MS criteria, and is summarized in Table 1.[43] Further confirmation was also achieved through standard addition (spiking) of the synthesized reference materials into the urine samples due to the close elution of the **10a** and **11** isomers (Figures S2 & S3). Moreover, compounds **10a** and **11** were also analyzed using the energy-resolved CID. The profile and $E_{5\%}$ of compound **10a** (1.82 eV ± 0.01) also closely match the urinary metabolite (1.83 eV ± 0.02) within one standard deviation. Feasibly, this could be used as a secondary MS based method in the future for matching this type of steroidal metabolite (Table 1, Table S2, Figure S4 & S5). The energy profile of **10b** was not obtained due to low precursor abundance.

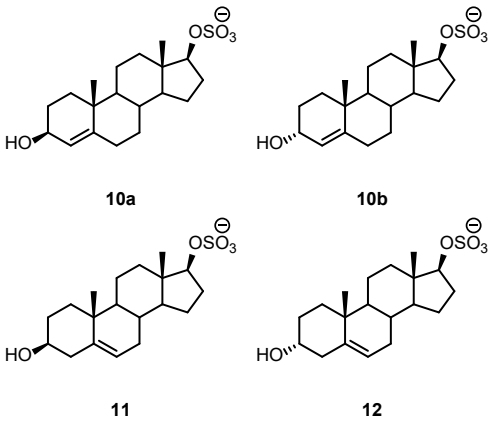


Figure 4. The four synthetically derived 17β-oriented sulfate conjugates.

CONCLUSION

We report a novel application of energy-resolved CID experiments for the structure elucidation of sulfate ester conjugate site and stereochemistry in steroid sulfate metabolites. The energy profiles could be derived from chromatographic peaks observed in single injections and were found to depend on the location and stereochemistry at the conjugation site, with more distant changes to the steroid skeleton having little effect on estimated threshold energies. The energy-resolved CID was used to guide the synthesis of reference materials for an unknown AAS sulfate urinary biomarker detected following testosterone propionate administration to a thoroughbred horse. This study shows that a library of structurally simple reference materials can be used to aid the identification of sulfate ester conjugate site and stereochemistry for a new steroid sulfate biomarker. This energy-resolved CID MS/MS method has wider potential to aid in the structure elucidation of new biomarkers for other metabolite classes where access to suitably diverse collections of reference materials is accessible.

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AUTHOR INFORMATION

Corresponding Author

*Malcolm D. McLeod, **malcolm.mcleod@anu.edu.au**

Author Contributions

CF: Conceptualization, Methodology, Script, Validation, and Writing—Original Draft. CB: Methodology. ME: Methodology: AC: Methodology and Conceptualization. MM: Conceptualization, Methodology, Funding, and Writing—Supervision, review and editing. All authors have given approval to the final version of the manuscript.

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