# Validation study to compare effects of processing protocols on measured $N^{\varepsilon}$-(Carboxymethyl)lysine and $N^{\varepsilon}$-(Carboxyethyl)lysine in blood

**George L.J. Hull[1], Jayne V. Woodside[2], Jennifer M. Ames[3,4] and Geraldine J. Cuskelly[1,*]**

[1]Institute for Global Security, School of Biological Sciences, Queen's University Belfast, BT7 5AG, Northern Ireland, UK.

[2]Centre for Public Health, Queen's University Belfast, Belfast, BT12 6BJ, Northern Ireland, UK.

[3]School of Life Sciences, Northumbria University, Newcastle upon Tyne, NE1 8ST, England, UK.

[4]Faculty of Health and Life Sciences, University of the West of England, Bristol, BS16 1FT, England, UK.

***To whom correspondence should be addressed. Correspondence author**:

E-mail: g.cuskelly@qub.ac.uk

**Epidemiological studies show that elevated plasma levels of advanced glycation end products (AGEs) are associated with diabetes, kidney disease, and heart disease. Thus AGEs have been used as disease progression markers. However, the effects of variations in biological sample processing procedures on the level of AGEs in plasma/serum samples have not been investigated. The objective of this investigation was to assess the effect of variations in blood sample collection on measured $N^{\varepsilon}$-(carboxymethyl)lysine (CML), the best characterised AGE, and its homolog, $N^{\varepsilon}$-(carboxyethyl)lysine (CEL). The investigation examined the effect on CML and CEL of different blood collection tubes, inclusion of a stabilising cocktail, effect of freeze thaw cycles, different storage times and temperatures, and effects of delaying centrifugation on a pooled sample from healthy volunteers. CML and CEL were measured in extracted samples by ultra-performance liquid chromatography-tandem mass spectrometry ~~(UPLC–MS/MS)~~. Median CML and CEL ranged from 0.132 to 0.140 mM/M lys and from 0.053 to 0.060 mM/M lys, respectively. No significant difference was shown CML or CEL in plasma/serum samples. Therefore samples collected as part of epidemiological studies that do not undergo specific sample treatment at collection are suitable for measuring CML and CEL.**

***Key Words*: advanced glycation end-products, $N^{\varepsilon}$-(carboxymethyl)lysine, $N^{\varepsilon}$-(carboxyethyl)lysine, epidemiology, blood sampling**

Advanced glycation end products (AGEs) are a heterogeneous group of bioactive compounds, which can be formed from the non-enzymatic glycation of proteins, with reducing sugars, such as glucose.[1] Glycation occurs through the formation of a Schiff base intermediate followed by an Amadori rearrangement to give the ketoamine adduct. When glucose is the reducing sugar, the Amadori rearrangement product (ARP) is known as fructoselysine (FL). Further rearrangement, fragmentation and oxidation reactions of FL lead to the formation of ~~advanced glycation end-products (~~AGEs~~)~~, such as $N^{\epsilon}$-(carboxymethyl)lysine (CML). AGEs can be formed endogenously in the body under normal physiological conditions,[2] and also exogenously during food processing.[3,4] CML is one of the best characterised AGEs, and has been frequently used as a marker of the Maillard reaction in human tissue.[5] CML formation can result from two main pathways involving glucose as a precursor. The first is through glyoxal formed by autoxidation of glucose or the Schiff base.[6,7] The second route is from FL, by oxidative cleavage between C2 and C3 of the sugar residue.[8] In addition, CML may form via lipid autoxidation. $N^{\epsilon}$-(Carboxyethyl)lysine (CEL), the homolog of CML, is formed during the reaction of methylglyoxal, typically formed from arachidonic acid, with the ε-amino group of lysine.[9]

Epidemiological studies have shown that elevated plasma or serum advanced glycation end-products levels are associated with disease conditions such as diabetes, kidney disease, and heart disease.[10-12] There has been some debate and speculation as to the requirement for a stabilisation step at the time of blood collection to prevent artefactual CML and/or CEL formation. All pathways to CML and CEL require an oxidation step and there are various reports that chelation of metal ions, such as copper and iron, by e.g. ethylenediaminetetraacetic acid (EDTA) and diethylenetriamine pentaacetic acid (DTPA) ~~[13]~~ inhibits formation of these AGEs in *in vitro* systems.[13] This has led some investigators to propose including a protocol to prevent artefactual formation of CML and CEL (as well as AGEs requiring oxidation steps for their formation) at the time of blood collection (Professor SR Thorpe, University of South Carolina,

61 USA, personal communication) in a similar way to the stabilisation of ascorbic acid in serum and
62 plasma samples using metaphosphoric acid at time of sample collection. However, it has not
63 been shown whether this additional step in the collection protocol is necessary to prevent
64 artefactual CML and/or CEL formation. Whether additional treatments are required at the time
65 of blood collection is important as including additional steps in handling of blood samples at
66 collection would inevitably increase the costs associated with CML and/ or CEL analysis, and
67 reduce the likelihood of such samples being collected in large epidemiological studies. The need
68 for these additional treatments also inevitably limits the use of blood samples that have already
69 been collected and have not undergone pre-treatment at time of collection; thus excluding many
70 cohorts of blood samples from the opportunity to analyze AGEs.
71 The aim of this validation study was therefore to determine the effect of variations in blood
72 processing conditions, common across epidemiological studies, on the level of CML and CEL in
73 blood samples quantified using a validated ultra-performance liquid chromatography-tandem
74 mass spectrometry (UPLC-MS/MS) method adapted from.[14] The rationale for the various
75 treatments tested in this validation study are summarised in Tables 1 and 2.
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## Materials and Methods

**Materials.** Sodium hydroxide 98%, indomethacin, butylated hydroxytoluene (BHT), DTPA, ethanol 99 %, phosphate buffered saline (PBS), sodium bicarbonate, boric acid 99.5%, sodium borohydride, trifluoroacetic acid (TFA), trichloroacetic acid (TCA), nonafluoropentanoic acid (NFPA) 97%, acetonitrile (HPLC grade), L-Lysine, and $C_{18}$ (Supelclean™ LC-18) SPE tubes were purchased from Sigma (Gillingham, UK). Other chemicals and their suppliers are as follows: EDTA, serum and lithium heparin blood collection tubes (Vacuette Greiner, Frickenhausen, Germany). Methanol (HPLC grade) (VWR International, Lutterworth, UK), hydrochloric acid 37%, (J.T. Baker, Devender, The Netherlands), chloroform (Bios Europe, Skelmersdale, UK), CML, $d_2$-CML, CEL, and $d_4$-CEL (NeoPMS, Strasbourg, France), $d_4$-Lysine Cambridge Isotopes (Andover, MA~~, USA~~).

**Subjects.** The study was conducted according to the guidelines laid down in the declaration of Helsinki and all procedures involving human subjects were approved by School of Medicine Dentistry & Biomedical Sciences Research Ethics Committee in Queen's University Belfast. Each participant signed before donating blood to indicate informed consent. Blood samples (~40 ml) were collected from healthy volunteers ($n$=10) who had been fasting for 12 h.

**Pooling of blood samples.** The serum supernatants were collected and combined to create a pooled sample ($n$=10) for subsequent treatments as described below. A similar method was employed for pooling plasma.

**Preparation of stability cocktail.** Indomethacin (20 mM; prepared in 5% $NaHCO_3$), was diluted to 0.2 mM indomethacin by addition of PBS (1X). BHT (5 mM) was prepared in ethanol. DTPA (10 mM, pH 7.4) was prepared in PBS (1X), and then the pH was adjusted firstly with 6N NaOH for coarse adjustment and then with 1N NaOH for fine adjustment. The stabilisation

cocktail was comprised of the following per 1 ml of blood: 75 µl of indomethacin, 4 µl of BHT and 10 µl of DTPA. It was added to the collection tubes before blood collection. Similar additions were made for serum and plasma where applicable.

**Stabilization cocktail treatment.** Samples treated with stabilisation cocktail were kept at 4°C for 30 min and then centrifuged (2400 x g) for 20 min at 4°C. Unless otherwise stated, untreated EDTA and lithium heparin samples were centrifuged immediately after blood collection. Untreated serum samples were allowed to clot before centrifugation. In all experiments, unless otherwise stated, the stability cocktail was prepared on the day of experiment and stored at 4°C.

**Experimental design.** The series of 5 experiments are described briefly below and summarised in Table 1. Unless otherwise stated in an experimental manipulation, samples were stored at -80°C following treatment and measurement of CML and CEL were conducted immediately after blood collection. Samples were prepared in triplicate in all experiments.

*Experiment 1: To test the combined effects of blood collection tube type and inclusion of stabilization cocktail*. Blood was collected in 3 different blood tube types (EDTA, serum and lithium heparin) either with or without stabilisation cocktail (6 treatments in total). Corresponding serum and plasma sample aliquots were collected, extracted, and analyzed for CML and CEL. immediately.

*Experiment 2: To test the effect of using fresh versus pre-prepared stabilisation cocktail.* Experiment 1 showed that there was no difference between blood collection tube type, so all subsequent experiments were conducted on EDTA tubes only. The stability cocktail was prepared on the day, the day before, and 1 week before blood collection, and stored in the cold room at 4°C prior to the addition in the appropriate collection tubes. Stabilisation cocktail (that had been (i) prepared fresh or (ii) prepared 24 h in advance or (iii) prepared 7 days, in advance)

was added to EDTA blood collection tubes. Samples were then kept at 4°C for 30 min, and centrifuged (2400 x g) for 20 min at 4°C.

*Experiment 3: To test the effect of multiple freeze-thaw cycles.* Blood samples were collected in EDTA collection tubes and then centrifuged (2400 x g) for 20 min at 4°C. From the pooled sample 3 x 1 ml aliquots were transferred to different tubes. One sample was analyzed immediately for AGE levels, and the remaining 2 treatment samples were put through the freeze thaw cycle (-80°C) either 1 or 2 times, before CML and CEL analyzes.

*Experiment 4: To test the effect of frozen storage temperatures and immediate versus delayed measurement of AGE.* Blood samples were collected in EDTA collection tubes and then centrifuged (2400 x g) for 20 min at 4°C. The samples were then stored at either -20°C or -80°C for varying storage periods (1, 2, 4, and >6 months).

*Experiment 5: To test the effects of immediate versus delayed centrifugation and/or addition of stabilising cocktail solution.*

*Experiment 5.1: Effect of immediate versus delayed centrifugation.* Blood samples were collected in EDTA tubes (with or without stabilisation cocktail) and stored at either 4°C or 21°C for varying periods of time (0, 1, 2, 4, 8, and 24 h). Samples were removed from storage at the corresponding time, kept at 4°C for 30 min (stabilisation cocktail-treated samples only) and all samples were then centrifuged (2400 x g) for 20 min at 4°C.

*Experiment 5.2: To test the effect of delay in stabilisation.* Blood samples were collected in EDTA tubes and kept at 4°C for 30 min followed by centrifugation (2400 x g) for 20 min at 4°C. Samples were then stored at either 4°C or 21°C for varying periods of storage time (0, 1, 2, 4, 8, and 24 h), following which stability cocktail was added to samples at the corresponding delay time.

153 *Experiment 5.3: Effect of conducting immediate versus delaying both stabilisation and*
154 *centrifugation.* Blood samples were collected in EDTA tubes and stored at either 4°C or 21°C
155 for varying periods of time (0, 1, 2, 4, 8, and 24 h). Samples were removed from storage at each
156 time point and the stability cocktail was added. Samples were kept at 4°C for 30 min followed by
157 centrifugation (2400 x g) for 20 min at 4°C.

158 **CML and CEL extraction.** The samples were extracted based on the method previously
159 reported.[11] Briefly, a 10 µl portion of the supernatant was reduced overnight at 4°C in sodium
160 borate buffer (0.5 M, pH 9.2, 400 µl) and 2 M sodium borohydride in 0.1 M NaOH. Proteins
161 were then precipitated by addition of 200 µl of 60% TCA and pelleted by centrifugation (2000 x
162 g for 10 min). The supernatant was carefully removed by aspiration with a Pasteur pipette. The
163 protein pellet was washed once by resuspension in 1 ml of 20% TCA and centrifuged (2000 x g
164 for 10 min). The internal standards (CML, $d_2$-CML, CEL, and $d_4$-CEL) were added and samples
165 were hydrolyzed in 6 N HCl (1 ml) at 110°C for 24 h. The acid was removed under vacuum
166 (Genevac evaporator (EZ-2), Genevac, Ipswich, UK), and the dried hydrolysates were
167 reconstituted in 5 mM NFPA, and solid phase extracted using a $C_{18}$ cartridge.

168 **UPLC-MS/MS analysis.** Lysine, CML, and CEL concentrations of hydrolysates were
169 determined by a Waters Acquity Ultra Performance LC system coupled to a Waters Micromass
170 Quattro Premier XE tandem quadruple mass spectrometer (Manchester, UK). Briefly, protein
171 hydrolysates (0.25 µl plasma/serum, 5 µl injection) were injected onto a Waters BEH $C_{18}$ UPLC
172 column, 1.7 µm, 2.1mm × 50 mm, housed in a column oven at 50°C. The flow rate was 0.3
173 ml/min. Solvent A was aqueous nonafluoropentanoic acid (NFPA, 5 mM) and solvent B was
174 acetonitrile. Gradient elution was employed starting at 10% solvent B which was maintained for
175 0.4 min, followed by a linear gradient from 10% to 80% solvent B in 3.8 min, with a hold at 80%
176 solvent B for 1.5 min, and then returned to 10% solvent B for 2 min. The run time was 7.5 min.
177 The analysis was performed using a Waters Acquity UPLC (Manchester, UK) coupled to a triple

quadruple MS operating in multiple reaction monitoring (MRM) mode. The MS was operated in electrospray ionization (ESI) positive mode using MRM. Lysine, CML, and CEL were quantified with the use of isotopically labelled internal standards and by reference to an external standard calibration curve. Data were reported as median ± interquartile range of triplicates.

**Statistical analysis.** Statistical analyzes were performed with SPSS 12.0 for Windows. Significance of differences between 2 and 3 groups was tested by the nonparametric Mann–Whitney *U* test and Kruskall Wallis tests, respectively, for independent samples. A two-tailed probability $<0.05$ was considered significant.

186 **Results**

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188 Samples were extracted in triplicate and inter-sample variability was <7%. The inter-day and
189 intra-day variability of the UPLC system was calculated by analysing a pooled plasma sample
190 (*n*=10), over five consecutive days (inter-day), and four times on the same day (intra-day). The
191 coefficients of variation obtained for the reproducibility tests described above, were <10%. The
192 LOD (0.01 μM/L) and LOQ (0.02 μM/L) were calculated at a signal-to-noise ratio of 3 and 10,
193 respectively, for both CML and CEL.
194 Calibration curves, obtained by linear regression of a plot of the analyte/internal standard
195 peak-area ratio versus analyte concentration, were linear over the range of concentrations tested
196 with correlation coefficients >0.98. Selected results of experiments 1, 2, 3, 4, 5.1, 5.2 and 5.3 are
197 shown in Tables 2 - 4. There was no statistically significant effect of either blood tube type or
198 inclusion of stabilisation cocktail on CML or CEL levels ($p > 0.48$ in all cases; Table 2). When
199 the cocktail was prepared in advance, there was similarly no effect on CML or CEL levels (data
200 not shown). Varying frozen storage temperature (Table 3), delay after blood collection until
201 AGE analysis (Table 3), and combined delay in both stabilisation and centrifugation (Table 4) all
202 similarly had no effect on measured AGE levels. Repeat (up to 2) freeze-thaw cycles, delaying of
203 centrifugation only, delayed addition of stabilisation cocktail only (data not shown) all similarly
204 had no effect on measured AGE levels.
205

206 **Discussion**

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208 The study was designed to mimic the commonly used procedures and storage conditions that
209 occur in an epidemiological study. The results obtained showed no significant difference
210 between any of the blood processing treatments or storage conditions investigated, on either the
211 level of CML or CEL in plasma/serum samples. The study showed that use of the stability
212 cocktail had no effect on the level of CML and CEL, and thus is an unnecessary step in their
213 analysis. The results from the current investigation demonstrate that the levels of CML and CEL
214 in plasma/serum samples are not significantly affected by the normal procedures and conditions
215 employed in an epidemiological study, and thus are suitable for measurement in large scale
216 epidemiological studies. Samples collected across different population studies use collection
217 procedures which can vary by many factors including: (i) delays to centrifugation, (ii) blood
218 collection tube, (iii) blood collection temperature pre-centrifugation, (iv) frozen storage
219 temperature, (v) delay between collection and analysis and (vi) exposure to multiple freeze-thaw
220 cycles. In the case of AGE analysis specifically, variations which may occur include: (i)
221 inclusion of stabilisation cocktail, (ii) advance or fresh preparation of stabilisation cocktail, or
222 (iii) immediate and delayed addition of stabilisation cocktail.

223 An exponential increase in the number of publications relating to AGEs has occurred in the
224 last 30 years. A PubMed search shows that in the 1980s, 1990s, and 2000s, the number of
225 articles on AGEs was 21, 978, and >3700, respectively. Epidemiological studies have suggested
226 that individuals with higher elevated circulating CML, are at greater risk of arterial stiffness,[15]
227 chronic kidney disease,[16] anemia,[17] cardiovascular and all-cause mortality.[18] The data
228 provided here support the use of stored samples, regardless of collection and storage method,
229 from other epidemiological studies for the analysis of CML and CEL to further examine the
230 association between AGEs and disease risk. These results are based on analysis of samples

collected from apparently healthy volunteers whose AGE blood levels are typically low. A comparison of the effect of pre-treatment of samples would need to be conducted on blood samples with higher AGE levels (such as amongst diabetics) to demonstrate if the non-effect of pre-treatment is maintained.

Furthermore, it supports the conclusion that future studies planning to measure CML and CEL do not need to employ additional processing steps at blood collection.

In conclusion additional processing of blood at collection does not elevate measured levels of CML and CEL. This indicates that specific collection protocols are not required for measuring CML and CEL, thus indicating that many samples currently stored for epidemiological studies could be used for CML and CEL analyzes.

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**Abbreviations**

| | |
|---|---|
| AGE | advanced glycation end products |
| BHT | butylated hydroxytoluene |
| CEL | $N^{\epsilon}$-(Carboxyethyl)lysine |
| CML | $N^{\epsilon}$-(Carboxymethyl)lysine |
| DTPA | diethylenetriamine pentaacetic acid |
| EDTA | ethylenediamine tetraacetic acid |
| ESI | electrospray ionization |
| FL | fructoselysine |

| | | |
|---|---|---|
| 256 | LOD | limit of detection |
| 257 | LOQ | limit of quantitation |
| 258 | MRM | multiple reaction monitoring |
| 259 | MS | mass spectrometry |
| 260 | $NaHCO^3$ | sodium bicarbonate |
| 261 | NaOH | sodium hydroxide |
| 262 | NFPA | nonafluoropentanoic acid |
| 263 | PBS | phosphate buffered saline |
| 264 | SPSS | statistical package for the social sciences |
| 265 | TCA | trichloroacetic acid |
| 266 | TFA | trifluoroacetic acid |
| 267 | UPLC | ultra high performance chromatography |

268

269 **Conflict of Interest**

270 None of the authors declared any personal or financial conflict of interest.

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