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Review

Endocannabinoids as potential biomarkers: It's all about pre-analytics

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ABSTRACT

Introduction: Arachidonoyl ethanolamide (AEA) and 2-arachidonoyl glycerol (2-AG) are central lipid mediators of the endocannabinoid system. They are highly relevant due to their involvement in a wide variety of inflammatory, metabolic or malign diseases. Further elucidation of their modes of action and use as biomarkers in an easily accessible matrix, like blood, is restricted by their susceptibility to deviations during blood sampling and physiological co-dependences, which results in high variability of reported concentrations in low ng/mL ranges.
Objectives: The objective of this review is the identification of critical parameters during the pre-analytical phase and proposal of minimum requirements for reliable determination of endocannabinoids (ECs) in blood samples.
Methods: Reported physiological processes influencing the EC concentrations were put into context with published pre-analytical research and stability data from bioanalytical method validation.
Results: The cause for variability in EC concentrations is versatile. In part, they are caused by inter-individual factors like sex, metabolic status and/or diurnal changes. Nevertheless, enzymatic activity in freshly drawn blood samples is the main reason for changing concentrations of AEA and 2-AG, besides additional non-enzymatic isomerization of the latter.
Conclusion: Blood samples for EC analyses require immediate processing at low temperatures (>0 °C) to maintain sample integrity. Standardization of the respective blood tube or anti-coagulant, sampling time point, applied centrifugal force and complete processing time can further decrease variability caused by sample handling. Nevertheless, extensive characterization of study participants is needed to reduce distortion of clinical data caused by co-variables and facilitate research on the endocannabinoid system.

Introduction

The endocannabinoids (ECs) 2-arachidonoyl glycerol (2-AG) and arachidonoyl ethanolamide (AEA, anandamide) belong to the group of highly potent lipid mediators in mammals [1]. Primarily, they are the endogenous ligands for the cannabinoid receptors 1 and 2 (CBR1/2), which owe their name to their discovery using natural and synthetic cannabinoids [2]. AEA and 2-AG are full agonists on either CBR1 or CBR2 and build the core of the complex endocannabinoid system (ECS), which also includes all relevant receptors and enzymes involved in synthesis and degradation of ECs [3,4]. 2-AG originates from phosphatidylinositol 4,5-bisphosphate-derived (PIP_2) diacylglycerols (DAGs) that are stereoselectively converted by diacylglycerol lipases (DAGL), while AEA can be synthesized via different pathways from *N*-arachidonoyl phosphatidylethanolamines [3,5]. Both compounds can be enzymatically hydrolyzed by fatty acid amide hydrolases (FAAH), and 2-AG additionally by monoacylglycerol lipases (MAGL) [3,5]. Structurally related compounds to arachidonic-acid containing ECs AEA and 2-AG

Abbreviations: ECs, endocannabinoids; ECS, endocannabinoid system; AEA, arachidonoyl ethanolamide; 2-AG, 2-arachidonoyl glycerol; CBR, cannabinoid receptor; EC-like, endocannabinoid-like; 1-AG, 1-arachidonoyl glycerol; PEA, palmitoyl ethanolamide; OEA, oleoyl ethanolamide; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; (U)HPLC, (ultra) high performance liquid chromatography; MS/MS, tandem mass spectrometry; LLE, liquid–liquid extraction; SPE, solid-phase extraction; O-AEA, virodhamine; BMI, body mass index; HDL, high density lipo protein; WB, whole blood; EDTA, ethylenediaminetetraacetic acid; RT, room temperature; FT, freezing temperature; PAF, platelet-activating factor; FTC, freeze–thaw cycles; PMSF, phenylmethylsulfonyl fluoride; KSCN, potassium thiocyanate.

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are called EC-like substances and can be differentiated based on their *N*-acyl group, like palmitoyl ethanolamide (PEA, palmitic acid) and oleoyl ethanolamide (OEA, oleic acid) or are constitutional isomers like 1-arachidonoyl glycerol (1-AG) and virodhamine (O-AEA). They do not, or just marginally, interact with the CBRs and can interact with other receptors or modify the effect of AEA and 2-AG [4,6]. EC-like substances are now examined more frequently to elucidate their function in the ECS and have been found in a wide variety of human matrices [7–10]. In case of 1-AG it's still under debate whether this compound is a biologically inactive artifact resulting from isomerization of 2-AG, or has a relevant function in the mammalian organism [11,12].

AEA and 2-AG have been investigated in the context of several diseases, illustrating the important role of these compounds, as for example in multiple sclerosis [13], Alzheimer's and Parkinson's disease [3], autism spectrum disorder [14], major depression [15], inflammatory bowel disease and colorectal cancer [16], complex regional pain syndrome [17] or sepsis and septic shock [18]. Furthermore, the ECS and its complexity have been described in several reviews in detail as for example by Lu and Mackie [19] or Hillard [4], including information on the potential of the ECS as a therapeutic target [20,21]. As the ECs might be powerful diagnostic or prognostic biomarkers relevant in a broad field of diseases, the reliable quantification in easily accessible matrices is of crucial importance. Determination of EC concentrations in human samples was performed among others in CSF [22,23], salvia and breast milk [8,24], and urine [10,25–28]. However, blood is by far the most widely employed and characterized matrix for the determination of ECs in humans (Table 1) and was, therefore, chosen for this review.

Exact concentrations of the ECs are needed to prove their relevance as biomarkers in clinical studies and to make them later applicable as diagnostic tools for the clinical routine investigation of single patients. Consequently, standardized analytical methods, as well as protocols for blood sample collection (whole blood, plasma or serum) during the pre-analytical phase, are necessary to guarantee comparable results when analyzing samples from individual patients. Comparing the measured concentrations of AEA and 2-AG in healthy controls reported in different studies indicate clearly that this standardization is not achieved yet (Table 1). Parameters and therefore possible influences of (pre-) analytical workflows (e.g., blood processing or missing chromatographic separation) are manifold, additionally often underreported and, in consequence, not fully comparable by themselves. Adequate sample handling is of absolute importance for correct quantification of ECs and EC-like substances [29–32], which is why this review is focused on the pre-analytical phase spanning from the selection of suitable participants to blood processing. Furthermore, analytical challenges associated with EC quantification are shortly summarized to depict all critical parameters in the overall process of research on ECs. We aim to draw attention to potential pitfalls and provide an overview of relevant issues to be considered when planning a study including the analyses of AEA and 2-AG.

Analytical challenges of AEA and 2-AG determination

The basis for robust quantification of AEA and 2-AG is the use of validated bioanalytical methods for reliable determination in biological matrices even at low concentrations. Most of the hitherto published methods have used (ultra) high performance liquid chromatography in combination with tandem mass spectrometry [(U)HPLC-MS/MS] after applying liquid–liquid extraction (LLE) or solid-phase extraction (SPE) [1,6,7,28–30,51–53]. Furthermore, GC–MS/MS methods are used for quantification e.g. of AEA in biological matrices like plasma [9,54]. The occurring analytical challenges have been intensively studied and are generally considered when analyzing AEA and 2-AG.

In the case of 2-AG, isomerization to 1-AG during sample preparation is the major challenge in the analysis and has been intensively investigated [28,30,55,56]. The process of isomerization by acyl migration (Fig. 1) depends primarily on the solvents used and can be prevented by using non-protic solvents like toluene or ethyl acetate and avoiding protic solvents like water, methanol or ethanol, especially when the evaporation of extracted samples is necessary [28,30]. Additionally, acyl migration is enhanced by elevated temperatures [57] and is base catalysed (pH 8, $t_{½} = 8.5$ min) [58]. Higher concentrations of proteins like albumin also increase the rate of acyl migration, but the mechanism is not yet elucidated [58]. In parallel with isomerization, degradation of 2-AG and 1-AG can take place during sample preparation [30]. Fortunately, degradation of analyte can be minimized by using non-protic solvents and adapted for by the addition of stable isotopically labelled internal standards as early as possible in the sample preparation process. Hereby, the analytical challenges of isomerization, degradation and also adsorption on glass and plastic surfaces [58] of 2-AG and 1-AG can be solved satisfactorily. However, the distinct quantification of 2-AG and 1-AG is necessary due to the lack of isomerically pure standards and the unsolved debate over the physiological importance of 1-AG [56]. Differentiation can be achieved by an appropriate (U)HPLC method providing baseline separation of both analytes, as distinguishing the compounds by tandem mass spectrometry is not possible. Quantification of AEA is less demanding and reliably possible using an appropriate (U)HPLC method. The occurrence of virodhamine (O-AEA, structural isomer to AEA) [30,59] is well known and has generally been considered in the process of method development [8,29,30]. The rearrangement of these isomers is strongly pH-dependent [60] and is, overall, not addressed as a major issue during analytical workflows, probably due to low conversion rates and the rarity of quantification of O-AEA. O-AEA rearranges to AEA under basic conditions (approx. 0.5% conversion at pH 9.2 after 30 min) while the conversion of AEA to O-AEA needs more drastic acidic conditions, like a strong acid in combination with heat or sonication and does, therefore, not proceed spontaneously [60]. Chromatographic separation of these respective isobars (AEA/O-AEA) is a necessity due to their inherent differences in physiological function and their varying ratio in different tissues. O-AEA has only a partial-agonistic activity on CBR1 and might be able to antagonize the effect of other ECs while also surpassing the concentration of AEA itself in peripheral tissues [59].

In summary, the analytical challenges for the determination of AEA and 2-AG are well studied and can be solved using non-protic solvents during sample preparation, as well as stable isotopically labelled internal standards in combination with an appropriate (U)HPLC method (Fig. 1). These measures are incremental for the reproducible determination of EC concentrations in any biological matrix and should be considered when planning EC analyses.

Pre-analytical challenges of AEA and 2-AG

Pitfalls during the pre-analytical phase of EC analyses have been identified to be caused either by human variability, the choice of the examined blood component or inadequate handling of blood samples. The following describes these pre-analytical challenges in detail and discusses potentials solutions.

Human variability

Investigation of ECs in clinical trials or under pathophysiological conditions are in need of an adequate control group for comparison. This is routinely done in an age- and sex-dependent manner and is discussed to be highly relevant for 2-AG and AEA. While Fanelli et al. did report significantly different levels of 2-AG, but not AEA between sexes [30], exactly the opposite was the case for a study by Blüher et al. [39]. This was attributed to different triglyceride levels between participants of the two studies. Accordingly, 2-AG is reported to positively correlate with a wide range of metabolic (surrogate) parameters like triglyceride levels [30,61], body mass index (BMI) [61,62], percentage of body fat [39] and fasting insulin levels [39,61] while high-density lipoprotein (HDL) cholesterol and adiponectin are negatively correlated [61]. AEA on the

Table 1
Selected mean values of AEA and 2-AG concentrations (in ng/mL) in human blood samples from healthy volunteers or control cohorts reported by independent research groups. Sorted from lowest to highest reported 2-AG concentration. Data as mean ± SD if not stated otherwise. ns: not specified, na: not applicable, sum: summarized reporting of 2-AG and 1-AG concentrations, separate: specified separate reporting of 2-AG concentrations without 1-AG, # concentration extracted from graphical data, † median is reported, ‡ information extracted from cited publications.

Ref.	Research focus	*N* (m/f)	Age	Population (characterization)	Matrix	AEA	2-AG	Instrumentation (LLOQ, IS)
Matias et al. (2006) [33]	(Dys-)regulation and function of ECs in obesity and hyperglycemia	8 (5/3)	62.3 ± 7.6	Healthy volunteers (BMI, glucose, triglycerides, cholesterol)	Serum	0.61#	0.28# (ns)	HPLC-APCI-MS (AEA: ns, AEA-d8‡; 2-AG: ns, 2-AG-d8‡)
Hahnefeld et al. (2020) [34]	Fluoride/citrate as stabilizing agent for clinical routine	10 (4/6)	34.6 ± 9.5	Healthy volunteers (fasted)	Plasma (NaF/citrate)	0.13	0.30 (separate)	UHPLC-ESI-MS/MS (AEA: 0.0125 ng/mL, AEA-d8; 2-AG: 0.0313 ng/mL, 2-AG-d5)
					Plasma (EDTA)	0.19	0.35 (separate)	
Monteleone et al. (2005) [35]	Blood EC levels in anorexia nervosa, binge-eating disorder and bulimia nervosa	15 (0/15)	22.9 ± 3.8	Healthy controls (SCID-I, family history, menstruation cycle, medication, BMI, body fat and lean mass, plasma leptin)	Plasma (EDTA)	0.88#	0.39# (ns)	HPLC-APCI-MS (AEA: ns, AEA-d8‡; 2-AG: ns, 2-AG-d8‡)
Fanelli et al. (2012) [30]	Estimation of reference intervals for circulating ECs in humans	76 (0/76)	44.5†	Healthy subjects (BMI, waist circumference, blood pressure, triglycerides, cholesterol, glucose, insulin, uric acid, disease status, medication)	Plasma (EDTA)	0.34†	0.54† (separate)	2D-HPLC-APCI-MS/MS (AEA: 0.007 ng/mL, AEA-d8; 2-AG: 0.030 ng/mL, 2-AG-d5)
		45 (45/0)	43.0†			0.34†	0.65† (separate)	
Caraceni et al. (2010) [36]	Circulating and hepatic ECs in cirrhosis patients	14 (11/3)	49.5 ± 11.4	Healthy controls (cardiac index, stroke index, heart rate, plasmatic renin activity, mean arterial pressure)	Plasma (ns)	1.77	0.62# (ns)	HPLC-APCI-MS (AEA: ns, AEA-d8; 2-AG: ns, 2-AG-d5)
Gatta-Cherifi et al. (2012) [37]	Postprandial deregulation of ECs	12 (2/10)	39.1 ± 3.7	Normal-weight subjects (BMI, waist circumference, blood pressure, glucose, insulin, cholesterol, triglycerides, ASAT, ALAT, γ-GT)	Plasma (heparin)	0.16#	1.06# (ns)	HPLC-APCI-MS/MS (AEA: 0.010 ng/mL, AEA-d8; 2-AG: 0.114 ng/mL, 2-AG-d5)
Di Marzo et al. (2009) [38]	Insulin as regulator of plasma ECs in obese subjects	10 (6/4)	44 ± 2	Normoweight volunteers (BMI, waist circumference, triglycerides, fasting glucose and insulin, visceral and subcutaneous fat)	Plasma (heparin)	1.56#	1.26# (sum)	HPLC-APCI-MS (AEA: ns, AEA-d8; 2-AG: ns, 2-AG-d5)
Blüher et al. (2006) [39]	Dysregulation of peripheral and adipose tissue ECS in obesity	10 (10/0)	48 ± 13	Lean subjects (medication, weight fluctuations, BMI, waist circumference, HbA_{1c}, fasting glucose and insulin, subcutaneous and visceral fat, FFAs, cholesterol, triglycerides, adiponectin, Leptin, IL-6)	Plasma (ns)	0.73#	1.94# (ns)	HPLC-APCI-MS (AEA: ns, AEA-d4‡; 2-AG: ns, ns)
		10 (0/10)	43 ± 14			0.94#		
Sipe et al. (2010) [40]	Biomarkers of ECS activation in obesity	48 (17/31)	66.4 ± 6.7	Normal-weight subjects (BMI, waist circumference, FAAH 385 genotype)	Plasma (EDTA)	4.45	2.12 (ns)	HPLC-ESI-MS/MS (AEA: 0.05 ng/mL‡, AEA-d8; 2-AG: ns, 2-AG-d5)
Schroeder et al. (2009) [41]	Circulating ECs during orthostatic stress	23 (12/11)	29 ± 9.6	Healthy volunteers in supine position (BMI)	Plasma (ns)	0.68#	2.97# (sum)	HPLC-ESI-MS/MS (AEA: ns, AEA-d4‡; 2-AG: ns, ns)
Bilgin et al. (2015) [42]	Profiling of ECs in lipoproteins	5 (ns)	ns	Anonymous volunteers (fasting state)	Plasma (EDTA)	5.07#	4.47# (separate)	μLC-ESI-MS/MS (AEA: ns, AEA-d8; 2-AG: ns, 2-AG-d8)
Schaefer et al. (2014) [43]	ECs in borderline personality and complex PTSD	30 (3/27)	31.5 ± 10.6	Healthy volunteers (BMI, nicotine and cannabis consumption)	Serum	0.74	4.74 (ns)	HPLC-ESI-MS/MS (AEA: 0.070 ng/mL‡, AEA-d4‡; 2-AG: ns, ns)
Engeli et al. (2005) [44]	Activation of peripheral ECS in obesity	20 (0/20)	57 ± 4.5	Lean women (BMI, waist circumference, blood pressure, heart rate, cholesterol, triglycerides, glucose, insulin, disease status, weight constancy, absence of hormonal replacement therapy)	Blood (ns)	0.73#	6.07# (sum)	HPLC-ESI-MS/MS (AEA: ns, AEA-d4‡; 2-AG: ns, ns)
Gachet et al. (2015) [45]	Method development for arachidonic acid, prostanoids, steroids and ECs in human plasma	32 (32/0)	ns	Healthy volunteers (ns)	Plasma (EDTA)	0.59	6.25 (sum)	HPLC-ESI-MS/MS (AEA: 0.04 ng/mL, AEA-d4; 2-AG: 0.08 ng/mL, 2-AG-d5)
Balvers et al. (2009) [46]	Method development of ECs and related compounds in human plasma	23 (0/23)	ns	Healthy volunteers (postmenopausal)	Plasma (EDTA)	0.24	7.09 (sum)	HPLC-ESI-MS/MS (AEA: 1.28 ng/mL (plasma), AEA-d8; 2-AG: 17.5 ng/mL (plasma), 2-AG-d8)
Hill et al. (2008) [47]	ECs in females with depressive disorders	11 (0/11)	30.2 ± 6.9	Matched control subjects to minor depression cohort (BMI, regular menses, no regular medication regimen, cigarette and alcohol consumption, education, disease status)	Serum	0.21	6.88 (sum)	HPLC-APCI-MS (AEA: ns, AEA-d8; 2-AG: ns, 2-AG-d8)
		16 (0/16)	27.9 ± 9.2	Matched control subjects to major depression cohort (see above)		0.25	7.42 (sum)	
Hill et al. (2009) [15]			25.9 ± 6	Matched control subjects (BMI, no regular medication regimen, cigarette	Serum	0.92#	149.24# (sum)	HPLC-APCI-MS (AEA: ns, AEA-d8; 2-AG: ns, 2-AG-d8)

(*continued on next page*)

Table 1 (*continued*)

Ref.	Research focus	*N* (m/f)	Age	Population (characterization)	Matrix	AEA	2-AG	Instrumentation (LLOQ, IS)
	Circulating ECs in major depression and after exposure to social stress	14 (0/14)		and alcohol consumption, disease status, blood glucose, leptin and insulin)				
Lam et al. (2010) [8]	Method development for NAEs in human bio-matrices	9 (ns) 7 (ns)	ns	Volunteers (ns)	Plasma (EDTA) Serum	0.25 0.35	na	UHPLC-ESI-MS/MS (AEA: 0.070 ng/mL, AEA-d8; 2-AG: na)
Ottria et al. (2014) [26]	Method development for NAEs in human bio-matrices	10 (ns)	ns	Healthy volunteers (ns)	Plasma (EDTA)	0.23	na	UHPLC-ESI-QTOF-MS (AEA: 0.125 ng/mL, SEA- or LNEA-d4; 2-AG: na)
Schreiber et al. (2007) [48]	Method development for NAEs in human serum	8 (4/4)	ns	Healthy volunteers (medication, no alcohol consumption for 1 week, no diagnosis according to DSM-IV)	Serum	0.32	na	HPLC-ESI-MS/MS (AEA: 0.070 ng/mL, AEA-d4; 2-AG: na)
Balvers et al. (2013) [49]	Method development for free and esterified NAEs in human plasma and blood cells	10 (ns)	ns	Healthy volunteers (fasted)	Plasma (ns)	0.37	ns	UHPLC-ESI-MS/MS (AEA: 0.056 ng/mL, AEA-d8; 2-AG: 0.771 ng/mL, 2-AG-d8)
Zoerner et al. (2009) [9]	Method development for FAEAs in human plasma	16 (7/9)	39 ± 8.2	Healthy individuals (BMI)	Plasma (EDTA)	0.47	na	GC–MS/MS (AEA: < 0.087 ng/mL , AEA-d4; 2-AG: na)
Lin et al. (2012) [50]	Method development for free and esterified NAEs in human plasma and blood cells	5 (ns)	ns	Humans (fasted)	Plasma (heparin)	0.90	na	UHPLC-ESI-MS/MS (AEA: 0.2 ng/mL, AEA-d8; 2-AG: na)

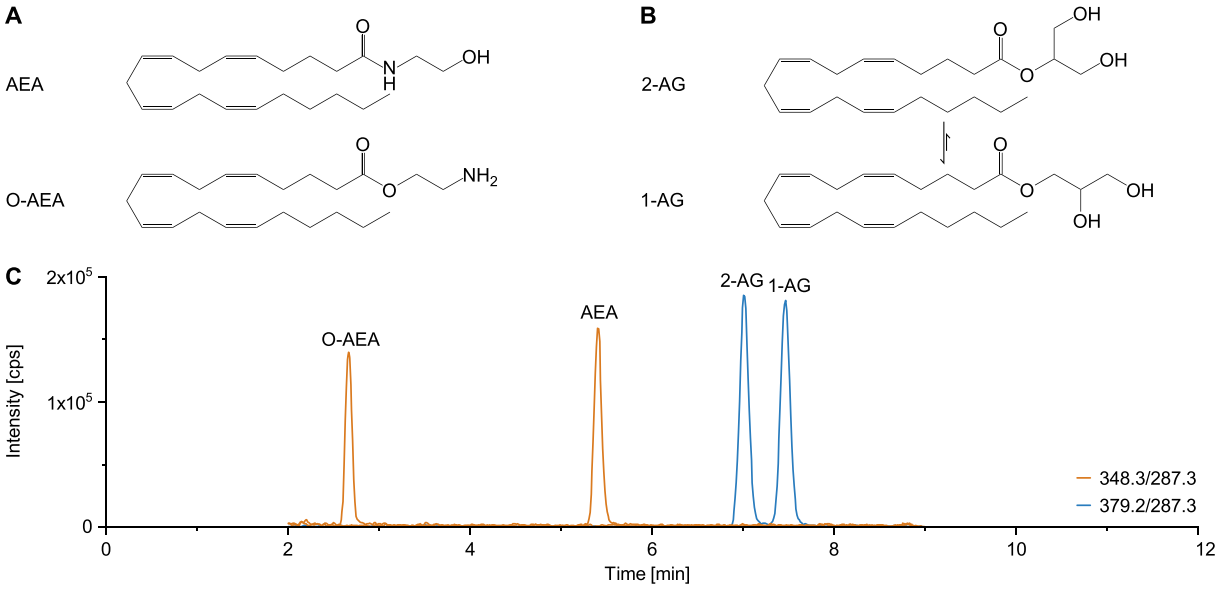

Fig. 1. Chemical structures and chromatographic separation of AEA/O-AEA and 2-AG/1-AG. Chromatographic separation between isomers is achieved employing a previously published RP-UHPLC-MS/MS method [29]. Detailed fragment ion spectra of AEA and 2-AG are available in a publication by Zoerner et al. [6].

other hand is only reported to be negatively correlated with intra-abdominal adiposity [61].

Additionally, inter- and intra-subject variability is reported to be increased by diurnal changes of physiological EC levels in humans [63]. Vaughn et al. reported changing AEA levels at different times of the day and the impact of sleep deprivation [64]. And while this was not the case for 2-AG, Hanlon et al. found peak physiological levels of 2-AG around noon [65]. This time of the day was not included in the previous study and might explain this discrepancy. The influence of fed status on 2-AG and AEA levels is still under debate [63,66] and while being plausible due to the extensive connection between metabolic parameters and EC levels, it is not completely proven.

The high number of influential factors due to the broad involvement of the ECS in physiological processes and diseases has been extensively addressed [4] and might explain a greater proportion of the differences in EC levels determined in control groups in different clinical studies. This huge variety of influential factors makes completely matching control and study groups nearly impossible, but additional standardization of sampling time and fasting status might already decrease variability in clinical data. Furthermore, adjustment of observed EC concentrations for proven influences might be suitable to reduce bias, but needs extensive characterization of every participant [62].

Blood components: whole blood, plasma, or serum?

Plasma and serum are the most used whole blood-based (WB) components in clinical analyses of ECs, and are both depleted of blood cells using centrifugation. WB itself is seldom used even though this would allow for short sample processing time [51], because it puts the sample at great risk for hemolysis [67] and distortion of physiological concentrations [68]. Detailed pre-analytical stability studies of ECs are only available for plasma or anti-coagulated WB, but not for serum. And

while plasma and serum are described to be comparable in relation to overall metabolomic features they still can be differentiated on the basis of their sample processing, specifically due to mandatory coagulation of serum samples [69,70]. Briefly, the formation of serum is started by initiating the coagulation cascade, for example, through clot-activators like silica, which leads to the activation of platelets and formation of fibrin clots [71,72]. Activated platelets among other things secrete mediators, like thromboxane A_2, that can modulate the coagulation cascade [73], and the involvement of ECs, especially 2-AG, is discussed intensively since 2-AG has been shown to activate platelets [74–76]. Furthermore, Berdyshev et al. have reported that 2-AG release is initiated by platelet activation after interaction of the platelet-activating factor (PAF) with its respective receptor [77]. It can, therefore, be assumed that activation of the coagulation cascade alters observed 2-AG concentrations in serum. This is supported by recent findings of Pedersen et al. who reported increased 2-AG concentrations attributed to platelet activation in serum while AEA concentrations remained stable [66]. Another explanation for differing EC concentrations in serum compared to plasma can presumably be found in the so-called volume displacement effect [70]. This effect describes the change of the forming supernatant volume in relation to the volume of removed protein fractions [78,79]. Fittingly, serum is reported to contain less protein and yield a smaller supernatant volume than plasma per initial volume of whole blood [69]. The amount of soluble analytes in serum is consequently distributed in a reduced supernatant volume compared to plasma and yields higher concentrations. This might also apply for ECs as they are not associated with lipoproteins and the majority is said to be not protein bound [4,42]. Overall, these reports indicate that obtained values of 2-AG and AEA in serum and plasma samples are not comparable, but both might still be suitable for use in studies when employing an appropriate control group.

Influence of blood sample handling

Clinical blood sample handling has the function of acquiring and preserving systemic information from singular patients in the form of blood samples and is, therefore, of major importance. It can be divided into blood drawing, blood processing and, finally, storage and transport for laboratory analysis. The pre-processing of WB into plasma or serum for analysis starts after the blood draw and ends with the separation from cellular components after centrifugation. WB for serum preparation needs to be stored at room temperature (RT) for 20–60 min before centrifugation in order for clotting to take place. In comparison, serum is lacking all clotting factors (e.g. fibrinogen), which isn't true for plasma, as clotting is inhibited in plasma by the use of anti-coagulants, like K_3EDTA, lithium-heparin or sodium fluoride-citrate. WB for plasma preparation can either be directly centrifuged or stored at RT or under chilled conditions (>0 °C, FT), respectively. These parameters need to be carefully selected and controlled as they can critically influence observed EC concentrations. Jian et al. reported a rapid *ex vivo* increase of AEA in freshly drawn WB samples that were stored at RT or FT for up to 2 h before centrifugation and final analysis of AEA in plasma [63]. This process was slowed down by storing WB at reduced temperatures. This *ex vivo* increase of AEA in anti-coagulated WB cannot be completely prevented and was, since then, confirmed multiple times by independent research groups [7,29–31]. Furthermore, stability assessment of AEA in WB also proved enzymatic degradation by FAAH [63]. 2-AG on the other hand is reduced by prolonged storage of anti-coagulated WB at RT and FT [29,30], which might be caused by uptake and degradation in platelets by FAAH [75] or MAGL [80] and apparent stable concentrations might be actually an incidental equilibrium between the synthesis and degradation of 2-AG [7]. Furthermore, isomerization of 2-AG to 1-AG takes place and is, thus, changing the ratio and concentrations of 2-AG/1-AG in a time-dependent manner [56]. The amount of isomerization (during sample pre-processing, especially inter-individual) cannot be foreseen because of its dependency e.g. on protein concentration and pH [58] and is still relevant at very low temperatures [57]. This can potentially disguise further underlying stability issues as the rate of increase of 1-AG does not match the rate of increase or decrease of 2-AG in the aforementioned stability studies and also highlights the need for separate quantification. Beyond that, 2-AG levels, and as a consequence of that also 1-AG levels, are reported to increase with rising centrifugal force which is not the case for AEA [29,77], making the pre-analytical process even more complex.

After centrifugation, the resulting supernatant needs to be carefully transferred into a new sample tube without disturbing the lower layer and is thereby separated from cellular components. The high stability of AEA at RT and FT in resulting plasma samples [7,29,30] suggests the crucial involvement of protein bound enzymes, [63] or the release of AEA by blood cells [31,81], and separation of cellular components enables stabilization of AEA levels. 2-AG is reported to rise at RT in plasma samples and while Gurke et al. [29] and Hahnefeld et al. [34] have observed a stabilization of measured 2-AG concentrations at FT, this is not always the case [7,30,82]. Röhrig et al. proposed a residual enzymatic activity of lipases in plasma that gradually releases 2-AG from its precursors [7].

For subsequent analysis it might be beneficial to split the transferred supernatant into suitable aliquots to avoid unnecessary freeze–thaw cycles (FTCs). For plasma samples, AEA is reported to endure up to two [29] or four [30] FTCs, while stability of 2-AG during FTCs is under debate, as just one [29] or up to four [30] FTCs seem to be possible without altering 2-AG levels. The same applies for 2-AG in serum samples, which is again described to be susceptible to FTCs and further delays before final freezing, which was not the case for AEA [82]. On the other hand, Schreiber et al. report decreasing levels of AEA due to one FTC in combination with two months of storage at −80 °C [48].

The underlying influencing factors during blood processing are reported to be predominantly of enzymatic nature and cannot be attributed to changes due to the coagulation process, as all of these stability studies were conducted using an anti-coagulant. For AEA, the influence of the anti-coagulation mechanism on rising levels in WB can be excluded, as heparinized WB has shown similar results to WB stabilized with EDTA [31]. Different approaches have been proposed to further enhance stability of ECs during pre-analytical workflows. These span from acidification of blood samples [7] to the addition of specific inhibitors, like orlistat [57] or phenylmethylsulfonyl fluoride (PMSF) [63]. Also more unspecific procedures, like the addition of KSCN [7] or the anti-coagulant sodium fluoride-citrate [34], have been reported to be beneficial. Nevertheless, the complex biological activity in blood and, therefore, alterations of physiological EC levels can only be addressed adequately by (I) lowering the processing temperature, (II) rapid removal of cellular components and (III) standardization of processing times to reduce the impact of time-dependent processes like isomerization or residual enzymatic activity.

Conclusion

Clinical research in the field of the ECS, including analysis of AEA and 2-AG is prone to many pitfalls that potentially reduce inter-study comparability and introduce bias (Fig. 2). Extensive research and validation of bioanalytical methods enable reliable quantification of ECs in blood-based samples and secure sample integrity during analytical workflows. Therefore, data quality is now mainly determined by the pre-analytical phase in which complex biological activity in blood samples encounters challenging logistics involved in clinical practice.

Extensive metabolic characterization of participants is needed to detect underlying co-correlations, and especially longitudinal studies could benefit from standardization of sampling time to reduce intra-individual variability due to diurnal changes. The use of plasma for determination of ECs seems to be the most suitable, as this allows for immediate and cooled blood processing and, thereby, decreases enzymatic artifacts and isomerization, respectively. As processing

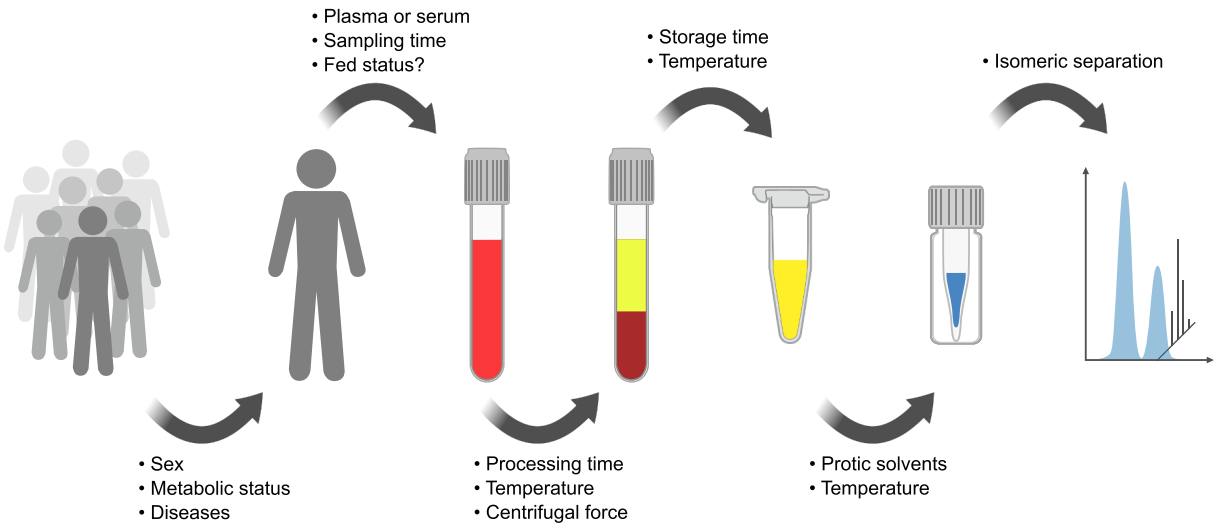

Fig. 2. Critical pre-analytical and analytical parameters during clinical EC analyses

parameters are largely dependent on instrumental availability and number of clinical personnel, the use of serum might be more feasible at some locations and could still be sufficient if coagulation time is strictly standardized. Nevertheless, serum is expected to yield higher AEA concentrations due to the prolonged storage with cellular components at RT and increased 2-AG levels because of the activation of platelets. Unfortunately, systematic comparisons between processing parameters in hitherto published studies are hindered by underreporting of either chromatographic (e.g. separation of 2-AG/1-AG) or process-inherent conditions and the complex multifactorial context. Reported EC levels certainly reflect pre-analytical process parameters and physiological concentrations. The addition of stabilizing agents can reduce enzymatic artifacts at the cost of higher clinical complexity and possibly increased frequency of errors and should, therefore, be carefully evaluated. Best practice should at least include immediate processing of blood samples at the lowest possible temperature (>0 °C) and subsequent (short-term) storage until analysis at −80 °C. Distribution of sample volumes into suitable aliquots for analysis can further increase sample integrity by avoiding unnecessary freeze–thaw cycles. Besides respective blood tubes or anti-coagulant used, sampling time point, processing temperatures, applied centrifugal force and complete processing time, including intermediate storage should be standardized to reduce inter-day variability, especially if clinical personnel are changing or the study is multicentered.

These pre-analytical measures have the potential to greatly reduce the influence of sample handling and harmonize reported EC levels while also lowering the variability of clinical data. This could enable detection of even minor impacts on the ECS by pathophysiological changes. Metastudies of reliable quantitative data could further facilitate elucidation of physiological functions of AEA and 2-AG in the human body, which are currently impossible. While ECs have already proven their relevance, an even deeper understanding of underlying mechanisms is needed to truly establish them as diagnostic or predictive biomarkers outside of academia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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