Practical Laboratory Medicine 5 (2016) 39–46

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Evaluating the optimum rest period prior to blood collection for fractionated plasma free metanephrines analysis

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ARTICLE INFO

Article history:
Received 9 March 2016
Received in revised form
5 May 2016
Accepted 6 May 2016
Available online 7 May 2016

Keywords:
Plasma metanephrine
Plasma normetanephrine
Phaeochromocytoma
Diagnostic sensitivity
Diagnostic specificity

ABSTRACT

Introduction: The high diagnostic accuracy of plasma metanephrines (PMets) in the diagnosis of Phaeochromocytoma/Paraganglioma (PPGL) is well established. Considerable controversy exists regarding optimum sampling conditions for PMets. The use of reference intervals that do not compromise diagnostic sensitivity is recommended. However, the optimum rest period prior to sampling has yet to be clearly established. The aim of this study was to evaluate PMets concentrations in paired blood samples collected following 30 and 40 min seated-rest prior to sampling, in patients in whom it was clinically reasonable to suspect that PPGL may be present.

Design and Methods: A retrospective cross-sectional study design was used. PMets results from paired blood samples collected after 30 and 40 min seated-rest between January 2009 and June 2015 were recorded. Results were interpreted using reference intervals established in subjects seated and supine.

Results: A total of 410 patient results were eligible for analysis. There was no statistical difference between plasma normetanephrine (NMN) or metanephrine (MN) concentrations in samples collected following 30 and 40 min seated-rest in subjects with PPGL (n=11), post-resection of PPGL (n=20) or in whom PPGL was excluded (n=379). Using reference intervals established in the seated position, diagnostic sensitivity was 100% at 30 min and 90.9% at 40 min. Diagnostic specificity was approximately 95% at both time points. When supine reference intervals were used, diagnostic sensitivity was 100% and diagnostic specificity was reduced by $\approx$22% at both time points.

Conclusion: Based on these data, we recommend at most 30 min continuous rest prior to sampling for PMets measurement.

1. Introduction

Phaeochromocytomas and Paragangliomas (PPGL) are rare neuroendocrine catecholamine-producing tumours that arise from adrenal and extra-adrenal chromaffin tissue, respectively [1,2]. PPGL occur sporadically or as a result of a germ line mutation in susceptible genes [3]. The incidence of PPGL varies between $<$1–8/million per year [4]. The prevalence of PPGL in patients with hypertension attending a general outpatient clinic has been estimated at between 0.2% and 0.6% [5–8]. PPGL can present with a myriad of symptoms including headache, hypertension, hyperhidrosis and palpitations. Undiagnosed

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http://dx.doi.org/10.1016/j.plabm.2016.05.001

PPGL can have deleterious consequences in terms of cardiovascular morbidity and mortality [3,9]. Therefore, a screening test with high negative predictive value and high diagnostic sensitivity is required. The diagnostic accuracy of fractionated plasma free metanephrine (PMets) measurements is well established [10–14].

Considerable controversy exists over sampling conditions for PMets [15,16]. While the most recent Endocrine Society Clinical Practice Guideline [1] recommends sampling patients in the supine position after a period of rest (at least 30 min) and using reference intervals established in the same position, the optimum period of rest has yet to be clearly identified. These recommendations recognise the rapid clearance of metabolites (the estimated half-life of PMets is <4 min, similar to that of the parent catecholamines [17]), the strong influence of sympathetic activation and upright posture to stimulate the release of norepinephrine and its metabolism to normetanephrine (NMN), and the likely absence of a response to these stimuli in patients with PPGL [10,18,19]. Moreover, reference intervals for PMets established using a seated-sampling protocol are higher than those defined using a supine-sampling protocol [1]. Lenders et al. found that, in patients with severe hypertension after 30 min supine rest, plasma NMN concentrations decreased by 30% ($p<0.001$) and plasma metanephrine (MN) concentrations decreased by 27% ($p<0.001$) [15] compared to a baseline value measured in the seated position. Variations in concentrations of PMets in response to changes in posture have been demonstrated in other studies [20,21]. Peaston et al. observed no statistically significant differences in PMets between duplicate blood samples collected by venepuncture at baseline (time 0) and after time 10 min ($p=0.53$) [22]. To date, it has not been established if remaining in a rested unstimulated state for longer than 30 min would improve the diagnostic sensitivity and specificity of PMets.

At our institution and during the period of this review, blood samples for PMets analysis were drawn after 30 and 40 min of continuous seated-rest from each individual patient.

The aim of this study was to evaluate PMets concentrations in paired blood samples collected following 30 and 40 min rest prior to blood draw in patients in whom it was clinically reasonable to suspect that PPGL may be present. The results will inform the optimum approach to pre-test patient preparation for PMets analysis.

2. Methods

Ethical approval was granted by the Research Ethics Committee at Galway University Hospitals (GUH). The study was conducted in accordance with the principles of the Helsinki declaration.

2.1. Study design

A retrospective cross-sectional study design was used. All subjects who had plasma NMN and MN sampled in GUH between January 2009 and June 2015 were identified following interrogation of the laboratory information system. Patient demographics including age and gender were recorded. Clinical details were obtained following interrogation of our electronic laboratory and radiology systems. Samples for PMets analysis were collected from subjects with a clinical suspicion of PPGL due to a suggestive clinical history, an adrenal incidentaloma, a significant family history or clinical follow-up of a pathologically proven PPGL. Subjects were divided into three groups: no PPGL, PPGL and post-resection of PPGL (post-PPGL).

The study inclusion criteria were paired samples taken for PMets analysis and clearly identified as having been collected following 30 and 40 min rest. The exclusion criteria were the absence of paired samples either due to an insufficient sample, haemolysis or failure to follow the standard protocol (Fig. 1).

2.2. Plasma NMN and MN sampling

The protocol for patient preparation for PMets sampling at the Centre for Endocrinology, Diabetes and Metabolism (CEDAM) for the period of this study is as follows (Fig. 1): the subject is cannulated and advised to remain seated and at rest in an unstimulated environment until test completion. Following 30 and 40 min rest, whole venous blood (10 mL) is sampled into appropriate specimen tubes (containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant). Specimen tubes on ice are delivered without delay to the laboratory for immediate processing (specimen centrifugation, separation and freezing of plasma at $-20^{\circ}C$) prior to analysis. During the period of this study, PMets were measured using liquid chromatography with tandem mass spectrometry (LC–MS/MS) at the ISO15189:2012 accredited laboratory, Freeman Hospital, Newcastle, United Kingdom. The method has been published in detail with the upper reference limits (URLs) established in subjects ($n=113$) in the seated position in whom the diagnosis of PPGL had been excluded (NMN set at 1180 pmol/L and MN set at 510 pmol/L) [14]. The laboratory is a participant of the Royal College of Pathologists of Australasia (RCPA) Quality Assurance Programme for PMets. Compliance with ISO15189 and Good Laboratory Practice requires the laboratory to carry out verification of assay performance and reference intervals at intervals concomitant with instrument upgrades/method optimisation.

The variables recorded for analysis were age (years), sex, ultimate clinical diagnosis, and biochemical parameters (plasma NMN and MN). Age was defined as the age of subject on the day of sampling.

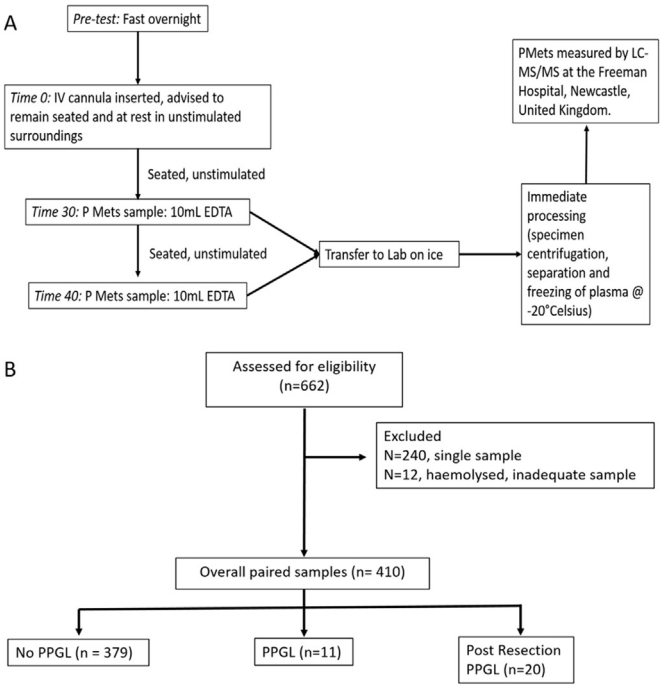

Fig. 1. A. Sampling strategy for plasma free metanephrines & B. Recruitment schematic.

2.3. Outcomes

The primary outcome was a significant difference in NMN/MN in all samples collected at two time points (30 and 40 min). The secondary outcome was a significant difference in NMN/MN between three subcategories of subjects (no PPGL, diagnosis of PPGL, post-resection of PPGL). A further secondary outcome explored the impact of seated-/supine-reference intervals on diagnostic sensitivity and specificity at the two time points studied.

To eliminate the potential bias of a large change in NMN or MN in subjects who were diagnosed with a PPGL, statistical analysis was conducted in subjects with PPGL, subjects post-resection of PPGL and subjects without a diagnosis of PPGL together and independently.

2.4. Interpretation of plasma NMN and MN results

For interpretation of results when a seated sampling protocol is employed for PMets testing, the recently published Endocrine Society Clinical Practice Guideline for PPGL [1] recommends the use of reference intervals that do not compromise diagnostic sensitivity. For this study, we employed supine URLs for NMN and MN of 610 pmol/L and 310 pmol/L respectively, defined using High Performance Liquid Chromatography (HPLC) with electrochemical detection (ECD) in subjects resting supine for at least 20 min [1,10,20]. In this paper, we will present the proportion of false-positive results, diagnostic sensitivity and specificity analysis based on reference intervals established in subjects seated [14] and supine [1,20] and applied to NMN/MN results from samples collected from patients in the seated position.

2.5. Statistical Analysis

Statistical analyses were performed using R (V 3.2.0, R Foundation for Statistical Computing, Vienna, Austria; accessible at www.r-project.org). Summary statistics for normally distributed continuous variables were given by mean (standard deviation) and frequencies (percentages) for categorical variables. Data that were not normally distributed were presented as median (minimum to maximum). When distributions were skewed, a natural log transformation was employed to normalise the data. Comparisons of NMN/MN concentrations between time points (overall, no PPGL, PPGL and post-PPGL) were performed using Mann-Whitney on the normal scale and the paired *t*-test on the transformed data. A p-value < 0.05 was deemed to be statistically significant. Diagnostic sensitivity and specificity was calculated using both seated and supine URLs at 30 and 40 min.

Table 1
Baseline clinical demographics of study subjects.

	Total cohort:	No PPGL	PPGL	Post PPGL	P-value
Number[a]	410	379	11	20	n/a
Age (SD)[*]	51.8 (15.1)	50.78 (15.6)	61.36 (10.6)	57.33 (10.5)	0.005
Male (%)	210 (51.2%)	197 (52.0%)	3 (27.3%)	10 (50%)	0.042

n/a: Not applicable.
[*] Age in years;
[a] participant samples.

3. Results

In total, 662 patient samples were assessed for inclusion in our study. 252 patients were excluded. 240 patients had only a single blood sample collected. A further 12 patients' samples were either insufficient or haemolysed and unsuitable for laboratory analysis at one or more time points. In total 410 paired patients' samples were used in this study. PPGL was excluded based on a combination of clinical, biochemical and imaging findings. In the post-PPGL group, not all of the patients were diagnosed in GUH. Some patients initially had urinary metanephrines and not PMets analysed to establish the diagnosis. This fact, combined with several patients having multiple samples taken post-resection of PPGL as part of their follow-up, accounts for the larger number of patients in the post-PPGL group compared to the PPGL group. The mean age and gender of subjects studied at time of sampling are shown in Table 1. A significant difference in age was noted between the three categories studied; the PPGL group were on average 10 years older than the no PPGL group. There was also a significant difference in gender between the groups with 52% men and 50% men in the no PPGL and post-PPGL groups, respectively compared to only 27.3% men in the PPGL group.

Table 2 and Fig. 2 outlines the median plasma NMN and MN concentrations at each time point. Fig. 2 illustrates the natural log of the mean concentration for NMN and MN at each time point. There was no statistically significant difference in NMN or MN between paired blood samples collected at either 30 or 40 min post rest in either the total study group, subjects without PPGL, with PPGL or post-PPGL.

Table 3 illustrates the diagnostic sensitivity and specificity of plasma NMN and MN using seated and supine decision thresholds for samples collected from subjects in the seated position after 30 and 40 min. Of concern is the finding that biochemical evidence of an incomplete resection could have been missed if only seated sampling URLs were applied to PMets measured in the sample collected after 40 min of seated-rest. This sample was drawn from a 75-year old male patient who had a PPGL resected 20 years previously. Following recurrence, further surgery with curative intent was performed with samples for PMets taken 1-month post resection. The NMN concentration was 1314 pmol/L in the sample collected at 30 min (a positive biochemical screen: $>$URL set at 1180 pmol/L) but decreased to 911 pmol/L in the sample collected at 40 min (a negative biochemical screen). Of note, at the time of biochemical testing, the patient reported episodes of

Table 2
Plasma NMN and MN results collected after 30 and 40 min seated rest.

Time (min)		30	40	P-value
Plasma Normetanephrine NMN (pmol/L)				
Overall	Median (range)	439 (40 – 14,488)	413 (40 – 12,068)	0.142
	Log mean (SD)	6.12 (0.65)	6.06 (0.68)	0.208
No PPGL	Median (range)	429 (40–2161)	401 (40 – 1938)	0.122
	Log mean (SD)	6.05 (0.53)	5.99 (0.57)	0.115
PPGL	Median (range)	3365 (1314 – 14,488)	4136 (911 – 12,068)	0.847
	Log mean (SD)	8.31 (0.76)	8.31 (0.74)	0.994
Post-PPGL	Median (range)	524 (161 – 1297)	530 (169 – 1289)	0.978
	Log mean (SD)	6.30 (0.53)	6.29 (0.56)	0.970
Plasma Metanephrine MN (pmol/L)				
Overall	Median (range)	170 (40–25000)	168 (40–24,641)	0.999
	Log mean (SD)	5.12 (0.63)	5.11 (0.66)	0.954
No PPGL	Median (range)	171 (40–471)	170 (40–1110)	0.912
	Log mean (SD)	5.10 (0.48)	5.10 (0.51)	0.941
PPGL	Median (range)	465 (40–25000)	428 (40 – 24,641)	0.922
	Log mean (SD)	6.67 (2.00)	6.63 (2.03)	0.972
Post-PPGL	Median (range)	104 (40–316)	94.5 (43 – 239)	0.507
	Log mean (SD)	4.64 (0.49)	4.55 (0.46)	0.571

*Upper Reference Limit; NMN: 1180 pmol/L; MN: 510 pmol/L.

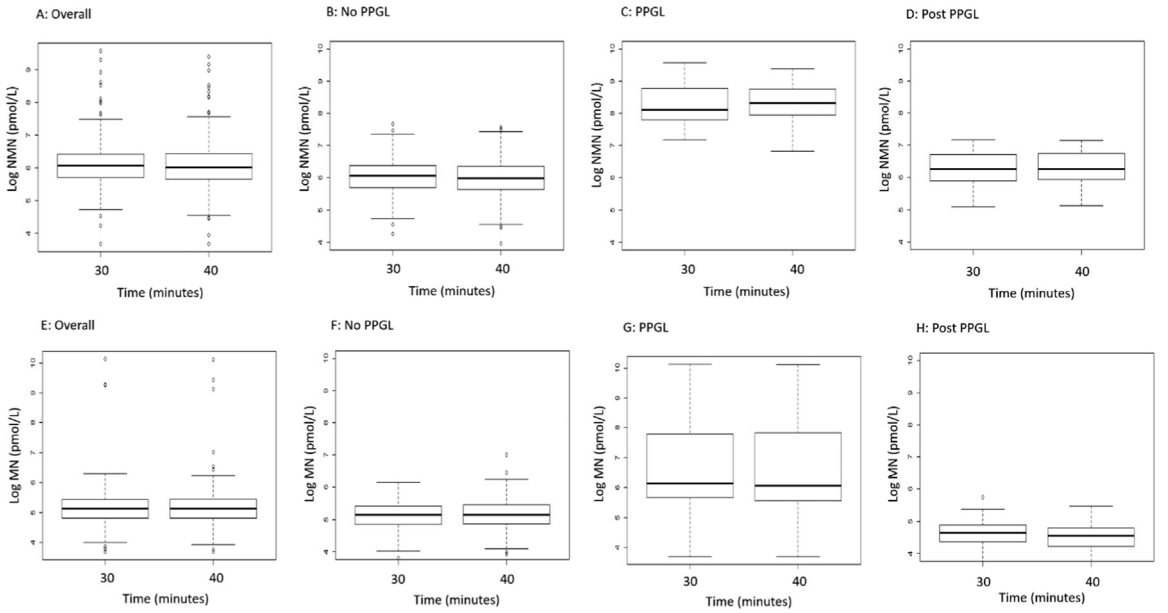

Fig. 2. Schematic of Natural log of plasma metanephrine (MN) and normetanephrine (NMN) for total study group, no PPGL (phaeochromocytoma or paraganglioma), PPGL and post-PPGL groups.

Table 3
Diagnostic sensitivity and specificity using seated and supine URLs at Time 30 and 40 min.

	Sensitivity		**Specificity**	
Time (min)	**30**	**40**	**30**	**40**
Seated URLs				
NMN	100%	90.9%	94.8%	95.6%
MN	36.4%	36.4%	97.9%	97.7%
NMN and/or MN	100%	90.9%	94.6%	94.8%
Supine URLs				
NMN	100%	100%	75.9%	76.2%
MN	72.7%	54.5%	90.9%	90.9%
NMN and/or MN	100%	100%	72.3%	71.5%

URL: Upper Reference Limit, NMN: Normetanephrine, MN: Metanephrine.

hyperhidrosis. As this was the main symptom at index presentation, clinical suspicion for an incomplete surgical resection was high and a metaiodobenzylguanidine (mIBG) scan was ordered. Follow-up confirmed incomplete resection of PPGL.

Diagnostic sensitivity was 100% for PMets using seated and supine URLs after 30 min seated-rest and 100% using supine URLs after 40 min seated-rest. Diagnostic specificity after 30 and 40 min was almost identical, 94.6% v 94.8% when seated URLs were applied for MN and NMN. There was a marked decrease in diagnostic specificity (approximately 22%) when supine URLs were applied. This was similar at both time points (72.3% v 71.5%).

Table 4 details the number of false-positive results using seated and supine URLs at 30 and 40 min. A similar proportion of false positives were identified at both time points.

Table 4
False-positive test results for PMets using seated and supine URLs at time 30 and 40 min.

Upper reference limits	**Seated-sampling**		**Supine-sampling**	
Time (min)	30	40	30	40
False-Positives No. (%)				
NMN	14 (3.5%)	12 (3.0%)	95 (23.8%)	93 (23.3%)
MN	0 (0%)	3 (0.8%)	27 (6.8%)	28 (7.0%)
NMN and/or MN	21 (5.4%)	20 (5.2%)	107 (27.7%)	110 (28.5%)

MN: Metanephrine, NMN: Normetanephrine.

4. Discussion

These data provide useful clinical information. There was no statistically significant difference observed in plasma MN or NMN concentrations between paired blood samples collected after either 30 or 40 min. This was true for all three categories evaluated (diagnosis of PPGL, post-resection of PPGL and patients in whom PPGL was excluded). There was no difference in diagnostic specificity or false-positive rates using seated-sampling URLs after 30 or 40 min seated-rest. Notably, no difference in diagnostic sensitivity and specificity or false-positive rates was determined using supine-sampling URLs at either time point. Of import, one case of incomplete resection of PPGL would not have been diagnosed if seated-sampling URLs were applied solely to the patient's sample collected after 40 min seated-rest. Remarkably, this did not occur in the sample collected at 30 min. No case of PPGL was missed when supine-sampling URLs were applied to either the sample collected after 30 or 40 min seated-rest. Eisenhofer et al. recommend the use of age-adjusted cut offs for plasma NMN in subjects >60 years of age (NMN >1047 pmol/L) to minimize the numbers of false-positive results. Notably, adoption of such cut offs to our study cohort would not have altered the false-negative rate at 40 min [23]. The false-positive result rate of study subjects increased on average by $\approx 22\%$ when supine-sampling URLs were applied. This is not surprising as such findings have been widely reported [1,20,24]. However, the impact is significant, as all patients with elevated PMets require follow-up. The recommended follow-up includes repeat biochemical testing using a fasted/supine-sampling PMets protocol [1,25].

The importance of rest prior to PMets sampling is clearly delineated in multiple studies. Deutschbein et al. demonstrated that 15 min of intense exercise prior to sampling resulted in an 82% increase in MN and an 85% increase in NMN that began to fall (although not back to baseline) after 15 min rest [21]. Raber et al. highlighted that cardiovascular exercise (cycling) resulted in significant elevation in NMN but not MN concentrations in hypertensive patients with type 2 diabetes. The authors also showed a non-significant increase in normotensive patients with type 2 diabetes and hypertensive patients without type 2 diabetes [26]. Bracken et al. found a significant increase in both MN and NMN concentrations above baseline in a cohort of healthy men who completed ergometric sprints [27]. The proportion of false positives decreases following rest post-exercise. The optimum duration of rest has yet to be defined. Studies advocating seated or supine rest prior to sampling typically do not specify the duration of seated/supine rest. We recommend that the duration of rest prior to sampling is disclosed in any future published work involving reference intervals for PMets to permit the development of more prescriptive sampling guidelines. Our data suggests that more than 30 min rest is not required prior to sampling.

In our cohort, only 2.7% of subjects had PPGL and 4.9% were post-resection of PPGL, which confirms that the condition is "frequently sought but rarely found" [28–30]. A patient screened for PPGL at our centre is 36 times more likely to have a negative than a positive screening test. Although it is reported that PPGL occurs equally in both men and women [31–33], only 27.3% of subjects with a new diagnosis of PPGL were male and 73.7% were female; although men represented 51.2% of the overall PPGL cohort (PPGL and post-resection for PPGL). It is possible that this is an artefact of our small sample size. Subjects with a diagnosis of PPGL (mean age 61.36 years) were on average 10 years older than subjects who did not have a diagnosis of PPGL (mean age 51.78 years). Martucci et al. reported that the mean age at diagnosis of PPGL is 43 years [34] while Guerrero et al. reported the mean age to be 47.1 years [33], although PPGL can occur at any age.

This study demonstrates the non-inferiority of a seated sampling protocol with 30 min of prior unstimulated rest when screening for PPGL using PMets. Further, these data confirm that prolonged seated-rest (>30 min) prior to blood draw not only confers no additional diagnostic benefit but also has the potential to miss a diagnosis of PPGL when reference intervals established in the same position are used.

The collection of paired blood samples for PMets in this study stems from the period when the referral site employed enzyme immunoassay (EIA: LDN 2-Met ELISA (Fast Track) Kit; LDN, Nordhorn, Germany) for measurement. At that time, the recommended pre-analytical sampling protocol for PMets advised collecting blood from patients fasting (overnight) and cannulated for 30 min of continuous supine rest into heparin specimen tubes at two time points, 30 min (baseline) and at 40 min. It is our understanding that the rationale for sampling at two time points was based on evidence from inter-laboratory proficiency testing (RCPA Quality Assurance Programme) for free metanephrines, suggesting that immunoassays demonstrated poorer analytical precision than either LC-ECD or LC–MS/MS [35,36]. When the referral laboratory changed from using EIA to LC–MS/MS for PMets measurement, the sampling protocol changed to a seated sampling protocol, with blood collected (venepuncture) into EDTA specimen tubes at baseline (time 0 min) and after a further 10 min. However, at our institution the phlebotomy practice (indwelling catheter) and sampling at baseline (after 30 min) and again at 40 min was embedded locally and continued.

The half-lives of PMets together with the difference in sampling protocols between the referral site and ourselves (specifically, the cannulated 30 min of seated rest prior to blood draw), most likely explains why Peaston et al. did not observe a difference between plasma NMN and MN in duplicate samples collected by venepuncture ten minutes apart [22]. Moreover, it is reasonable to assume that our phlebotomy practice lessens the effect of sympathoadrenal activation associated with venepuncture/upright posture on PMets that may obscure tumour–related increases in catecholamines/PMets. This, combined with the use of seated reference intervals, increases the potential for a missed diagnosis of PPGL

As a consequence of the current study findings and the recent Endocrine Society Clinical Practice Guideline [1], our pre-analytical practice for PMets has changed. We now use a fasted/supine sampling protocol together with appropriately established reference intervals, with a single EDTA blood sample collected after 30 min of supine rest. A limitation of the current study is the tumour size in the PPGL cohort. PPGL tumour diameter ranged from 2.5 cm to 10 cm. It has been demonstrated that tumour diameter influences PMets secretion, suggesting that up to 50% of PPGL <2 cm would be missed

using seated-sampling URLs for NMN and MN [24,37].

Finally, further work looking at shorter time periods in the supine position (for example 20 min versus 30 min) is warranted. Reducing the length of time that a patient remains supine and unstimulated reduces the potential for non-compliance. A reduction in time necessary for appropriate patient preparation results in health economic benefits (decrease in bed occupancy and healthcare professional time). Should our findings be replicated in patients in the supine position, we would advocate that the Endocrine Society Clinical Practice Guidelines should include the duration of a period of rest prior to blood sampling in subsequent revisions.

5. Conclusion

There is no statistically significant difference between PMets results in paired blood samples collected following 30 and 40 min rest in the study categories evaluated. However, when reference intervals established in the seated position were utilized, the diagnostic sensitivity was 100% in plasma collected after 30 min of seated-rest, but dropped to 90.9% in samples collected after 40 min. Diagnostic specificity was 94.6% and 94.8% respectively. Notably, when supine-reference intervals were employed, diagnostic sensitivity was 100% and diagnostic specificity reduced by ~22% at both time points. Based on these data, we recommend at most 30 min rest prior to blood collection for PMets measurement. We advocate further study to determine if our findings hold true in subjects following 30 v 40 min supine rest.

Acknowledgements

We wish to express our gratitude to all the participants who made this study possible. Special thanks to the scientific, nursing, and medical staff at the Centre for Endocrinology, Diabetes and Metabolism, the Department of Clinical Biochemistry at Galway University Hospitals and Saolta Hospital Group. We wish to express our gratitude to Dr Chris Boot and the scientific staff of Freeman Hospital, Newcastle, UK.

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