Reviewer Comments:
Reviewer 1: Constantinescu et al. present data showing that patients with pheochromocytoma have higher levels of cortisol (and other glucocorticoid metabolites) when compared to patients with PGL, PA, Cushing syndrome, and primary hypertension. They interpret their findings as catecholamines somehow possibly directly increasing cortisol production and that this "elevated" cortisol could potentially contribute to cardiometabolic adverse effects in Pheo patients.
The strengths of the study include relatively large sample sizes, data pre- and post-op, use of centralized steroid measurements. I find several weaknesses that limit the interpretations that are presented. Namely, the measurement of morning steroids (rather than dex suppressed or 24h urinary), the lack of exact and consistent mechanism to differentiate whether this observation represents an increased HPA axis/stress response vs primary adrenal production, and assumptions that the elevated cortisol level is abnormal (vs normal physiologic).

Specific comments

My understanding is that steroid profiling was done from blood samples collected at 8am. These were not done after dex suppression and there was no 24h urinary collection. Thus, these are morning peak cortisol and steroid levels. I am concerned that this is not a reliable measurement of "dysregulated" cortisol production or a reproducible method, see next.

Au: We appreciate the comment. The samples for steroid profiling were indeed obtained in the morning between 8 and 11am (line 125, methods section). In patients with a suspicion of a catecholamine-producing tumor a dexamethasone suppression test is not routinely performed. Exceptions may include patients who present with an adrenal incidentaloma. Even in such cases this may present problems since administration of exogenous steroids, including dexamethasome, can evoke hypertensive crises in some cases leading to death (see PMID:17973541; PMID: 18299478; PMID: 20963573). Similarly, because most of our cases of pheochromocytoma were not tested based on findings of incidentaloma we do not have access to sufficient data for 24 hr urinary free cortisol to address the second part of the referee's comment. The study was retrospective; however, even for a prospective design it would be difficult to ethically defend incorporation of dexamethasone testing within a clinical protocol. Furthermore, we do not indicate in the manuscript that patients with pheochromocytoma have hypercortisolism, as defines patients with subclinical Cushing syndrome; thus, positive results for a dexamethasone test would not be expected. We further clarify that nowhere in the original or revised manuscript do we claim that the reported findings indicate either hypercortisolism or dysregulated cortisol production. This, however, does not mean that the higher plasma concentrations of cortisol and other glucocorticoids observed in patients with pheochromocytoma lack clinical relevance. In the revised manuscript we now acknowledge that use of morning plasma measurements and absence of 24-hour urinary free cortisol measurements are study limitations (lines 312-313).

In a prior paper including several of the current authors, Arlt et al. in JCI Insight 2017 described excess cortisol production is highly prevalent in patients with primary aldosteronism. The results showed that using steroid profiling from an integrated 24h urine collection, that PA patients frequently made excess cortisol, so much so, that they termed this the ConnShing syndrome. In the current paper, Figure 1 first panel on cortisol, patients with PA and controls with hypertension have the same cortisol production.

How could this be? The most likely explanation is that using a single morning blood draw is not an adequate or appropriate method to evaluate cortisol/steroid dysregulation or excess. Using dex suppressed or 24h urinary measures would be more discriminatory. In this regard, I question how useful these control groups are, how strongly one can claim that the higher cortisol in pheo is "abnormal" vs an appropriate physiologic increase in response to a catecholamine producing tumor.

Au: Thank you for the comment. We have largely addressed the issues concerning the dexamethasone suppression test and 24 hr urinary measurements of free cortisol in our response above. We further clarify that although patients with primary aldosteronism may show excess production of glucocorticoids, this does not translate to hypercortisolism as in patients with subclinical Cushing syndrome. Indeed, dexamethasone is often employed in patients with primary aldosteronism to rule out glucocorticoid remediable aldosteronism. Failure to suppress cortisol is not commonly observed.

Regarding Connshing, the manuscript by Arlt et al employed GC-MS for measurements of 24 hr urinary steroids. As reported by Ann McCormack (PMID: 29039221) assessment of hypercortisolism using mass spectrometric measurements of 24-hr urinary free cortisol is less reliable than by immunoassay measurements; this appears related to the fact that the former measurements are less susceptible to interferences from glucocorticoid precursors and metabolites that contribute to the diagnostic signal of immunoassays. The importance of measuring glucocorticoid precursors and metabolites is also indicated by the report of Arlt et al. in which patients with PA showed increased urinary outputs of cortisol precursors and other metabolites, including 11-deoxycorticosterone (tetrahydro-11-deoxycorticosterone) and corticosterone (tetrahydrocortisone). Furthermore, there have been other reports that such mass spectrometric measurements of these urinary metabolites are particularly useful for diagnosis of adrenal cortical tumors, including those involving hypercortisolism (PMID: 27370636; PMID: 28814383; PMID: 28904054).

For measurements in plasma, it is already well known that morning plasma concentrations of cortisol are inferior to 24 hr urinary free cortisol; this is discussed above and in the manuscript (lines 302-306). However, measurements of plasma cortisol have also been consistently shown to offer an inferior biomarker for hypercortisolism or disordered glucocorticoid production than other steroids, including 11-deoxycortisol, 11-deoxycorticosterone and corticosterone (PMID: 29208661; PMID: 30977834; PMID: 32348959). It is therefore unsurprising that we failed to establish increased plasma concentrations of cortisol in our patients with primary aldosteronism; however, for these patients we did establish increased plasma concentrations of 11-deoxycortisol, corticosterone and 11-deoxycorticosterone. These findings are entirely consistent with those about Connshing in the article by Arlt et al (PMID: 28422753).

We appreciate that clinicians who regularly rely on currently available routine tests and the recommendations concerning use of these tests may not be conversant with advances that have not yet reached the clinical routine mainstream. We have therefore briefly covered these advances in lines 236-239 of the revised discussion. We have also further clarified in the revised manuscript how the presently reported findings are entirely consistent with those of Arlt et al (lines 296-300). Our response to the reviewer will be included with the supplement in order to provide more in depth coverage of the details outlined above.

In this regard, what evidence do the authors provide to claim that the higher peak morning circulating cortisol in pheo patients is "abnormal"? Why is it not a "physiologic or normal" HPA stress response? For

example, high catecholamine levels may increase the stress-response and result in greater cortisol levels, just as pheo patients with high catecholamines have increases in BP, heart rate, brown fat, bone turnover, etc. I would advise not making claims about normal vs abnormal - this observation may represent a physiologic response to high catecholamines.

Au: The manuscript does not claim that morning cortisol concentrations are abnormal. Furthermore, both the original and the revised manuscript do in fact indicate that the likely basis of the increased plasma concentrations of corticosteroids in patients with pheochromocytoma involves a response to catecholamines. Although the data suggest a higher likelihood of an impact of locally released catecholamines on corticosteroid production, both the original and revised manuscript also clarify that other mechanisms of impact cannot be excluded (lines 265-269).

Alternatively, the authors discuss issues with production, but what about increased binding globulin (CBG) or decreased clearance/metabolism? The term "Steroid production" and "steroidogenesis" are used throughout - is this really the correct term or should it be "levels"? Would advise avoiding the assumption that this is entirely a production issue - it could also be a binding and/or clearance issue that also affects the proximal parts of the axis.

Au: The reviewer has raised a valid point that the higher plasma concentrations of cortisol and other glucocorticoids (11-deoxycortisol, corticosterone, 11-deoxycortisosterone) in patients with pheochromocytoma than primary hypertensives might reflect increased binding to transcortin (CBG) and subsequently decreased clearance/metabolism rather than increased adrenal production of these steroids. The referee is particularly correct to indicate that plasma concentrations of any substance reflect not only the rate of entry of that substance into the circulation, but also the clearance of that substance from the circulation. The equation E = CL x C defines this relationship, where E equals the rate of entry of a substance into the circulation (e.g., ng/min), where CL is the circulatory clearance (e.g., L/min) and where C is plasma concentration (e.g., ng/L). Cortisol has a particularly slow circulatory clearance of 0.16 to of 0.13-0.26 L/min reported in one study (PMID: 874909) and 0.10-0.47 L/min in another (PMID: 17370058). The slow clearance is largely due to the substantial binding of circulating cortisol to transcortin, with more binding leading to slower clearance (PMID: 7883829). Thus, steroids that do not show the same high degree of protein binding are characterized by more rapid clearances and lower plasma concentrations. For example, in one study (PMID: 874909) aldosterone had a mean plasma clearance of 1.28 L/min, which was 6.5-fold higher than that of cortisol (0.20 L/min), which contributes to the much lower and more difficult to measure plasma concentrations of aldosterone compared to cortisol.

As indicated in table 1 of the manuscript, plasma concentrations of cortisol were 38% higher in patients with pheochromocytoma than primary hypertensives compared to respective 79%, 52% and 76% higher plasma concentrations of 11-deoxycortisol, corticosterone and 11-deoxycorticosterone. For these differences to reflect an impact of transcortin, such an impact would need to be proportionally higher and clearances lower for 11-deoxycortisol, corticosterone and 11-deoxycorticosterone than for cortisol. However, as outlined in the study by Peitzsch et al (PMID: 25312486), cortisol has a much slower plasma clearance than other steroids and this contributes to its poorer utility compared to other steroids for indicating selectivity during adrenal venous sampling. Although some corticosteroids can be produced outside the adrenals (PMID: 21540450, PMID: 128232560), the main site by far remains the adrenal. Circulatory clearances can therefore be estimated from the data of Peitzsch et al (PMID: 25312486),

using the increases in plasma concentrations from peripheral to adrenal venous plasma concentrations. From analysis of those data (see table below), the circulatory clearance of cortisol can be estimated at 0.278 L/min, which is within range of the studies reported above (PMID: 874909; PMID: 17370058). In contrast, 11-deoxycortisol, corticosterone and 11-deoxycorticosterone have respective clearances 5.8-, 2.9 and 2.7-fold higher than cortisol. Also in line with previous observations (PMID: 874909), aldosterone has a much higher clearance than cortisol.

Table: Circulatory clearances (CL) of cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone and aldosterone, as derived rates of entry (E) of the steroids into the circulation from the adrenals.

Steroid	PV (ng/mL)	AV (ng/mL)	Δ (ng/mL)	E (ng/min)	CL (L/min)
Cortisol	114	1170	1056	31680	0.278
11-Deoxycortisol	0.33	18.19	17.86	536	1.624
Corticosterone	2.48	69.05	66.57	1997	0.805
11-Deoxycorticosterone	0.04	1.05	1.01	30	0.758
Aldosterone	0.05	4.24	4.19	126	2.514

Data for peripheral venous (PV) and adrenal venous (AV) plasma concentrations of steroids are from table 1 of Peitzsch et al (PMID: 25312486). The rate of entry (E) of steroids from the adrenals into the circulation was estimated from the product of the PV to AV increase in plasma concentrations (Δ) and the blood flow through the adrenals, which was assumed to equal 30 mL/min, as based on previously published data (PMID: 2697487). Circulatory clearance of steroids (CL) were estimated according to the formula E = C x CL, where C the central venous plasma concentration is assumed to be equivalent to PV.

Although transcortin binds mainly cortisol, other steroids such as 11-deoxycorticosterone and corticosterone can also bind to CBG with high affinity (PMID: 618541, PMID: 1149255 PMID: 3665128, PMID: 26522460). CBG is also bound to progesterone with an affinity similar to deoxycorticosterone (PMID: 618541). While the corticosteroids were increased in patients with pheochromocytoma, plasma progesterone concentrations were lower compared to all other groups (table 1). Based on all above presented considerations and data it appears highly unlikely that the variable increases in cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone in patients with pheochromocytoma, but unchanged or reduced concentrations of other steroids, could be due to an effect of binding to CBG or variably reduced circulatory clearances of some steroids and unchanged or even increased clearances of other steroids.

The referee suggests use of the term “levels”. The term “levels” is imprecise since it implies a steady state in plasma concentrations, when in fact these concentrations can be highly dynamic. The term “levels” is therefore not employed in the manuscript. Furthermore, the original hypothesis for this retrospective study, as stated in the abstract and introduction, was that secretion of catecholamines from PPGLs may result in altered steroid production or steroidogenesis. This hypothesis still remains, although we now clarify in the discussion that differences or changes in plasma concentrations of any substance can reflect either increased entry of such substances into the circulatory compartment or decreased clearance from that compartment (lines 283-290). Thereby the increased plasma concentrations of glucocorticoids, as well as unchanged concentrations of most steroids, but decreased concentrations of other steroids, such as DHEAS, rather than reflecting changes in steroidogenesis could remotely reflect divergent changes in clearance of these steroids. This is now acknowledged in the

revised discussion with a reference to the supplement, where the above arguments are presented. On that basis we leave it up to readers to determine whether the differences in plasma concentrations likely reflect differences in clearance of steroids or differences in entry of those steroids into the circulation. Although the differences in plasma concentrations support the original hypothesis no claim is made in the discussion that the findings actually prove a direct influence on steroid production or steroidogenesis.

Did the authors measure ACTH? It would be helpful to know if ACTH were elevated as a sign of an increased central stress response, or if they were suppressed as a sign of increased adrenal steroidogenesis. Conversely, what do the authors make of the lower DHEAS levels in pheo? This implies that steroidogenesis was independent of ACTH? How likely is that?

Au: As was recognized by referee 2 (see point 2), the original manuscript clarified that ACTH was not measured. Since the study was retrospective and not prospective, appropriate samples were not collected for ACTH (see response above). Therefore, the study lacks data on ACTH and it cannot definitively determined that the increased glucocorticoids reflect either altered HPA function or a more direct paracrine action of catecholamines. Indeed, as the referee points out the lower plasma concentrations of DHEAS in patients with pheochromocytoma do not support an influence of ACTH. Sustained suppression of ACTH is established to result in a reduction of DHEAS (PMID: 9536209). Similarly reduced plasma concentrations of DHEAS are well established to occur in patients with subclinical Cushing syndrome and other clinical conditions associated with increased adrenal production of glucocorticoids, such as adrenal Cushing syndrome and PBMAH (PMID: 25146553; PMID: 28490185; PMID: 27797672; PMID: 29208661; PMID: 30977834; PMID: 32348959). Since DHEAS is responsive to ACTH, the lowered plasma concentrations of DHEAS might reflect lower plasma concentrations of ACTH, which may result from feedback inhibition of steroids on the hypothalamo-pituitary adrenal axis.

We therefore thank the referee for making this point, which we have built on further in the revised manuscript to offer support for a paracrine rather than stress effect (lines 272-282). Nevertheless, without measurements of ACTH, this remains unclear. Thus, although the presented data support a paracrine effect more than a stress response, both the original and revised manuscript do not offer any firm conclusion concerning this point (see lines 270-271).

There are many comparisons between the pheo and PGL group, but there is no table showing the relative functional status of each. Comparing the degree of metanephrine/catecholamine production between the Pheo and PGL groups is worthwhile so readers can sense whether it is the anatomical location or the catecholamine production. Grouping them together, as in table 2, does not help resolve this. Having a large Table 1 with all features would be really helpful in the main text.

Au: A table with all steroid concentrations in all patient groups is now provided as table 1. Due to the amount of information, placing all data into a single large table was not practical. Instead, the revised manuscript now includes data on urinary catecholamines as well as plasma and urinary free metanephrines in patients with paraganglioma and pheochromocytoma (table 3). Moreover, we have revised table 4, which now presents data for relationships, excluding patients with paraganglioma. It is additionally clarified in the text that while there were significant positive relationships between

corticosteroids and catecholamines or metanephrines for patients with pheochromocytoma, relationships were not significant for patients with paragangliomas. The referee is also referred to our response to first point of referee 2.

The methods seem to indicate that blood sampling for PPGL was done 12-36 months AFTER the tumors were removed - is that correct? The text seems to suggest sampling was done with the tumor in place.

Au: In a subset of 100 patients with PPGLs a second blood sample was taken after surgical resection of tumors. This time interval varied between 12 to 36 months. The relevant section of the methods has been rewritten for clarity (lines 134-138).

Reviewer 2:

This is a very well written manuscript, which describes a retrospective study from 12 European referral centers with 182 patients with pheochromocytoma, 36 with paraganglioma and 270 primary hypertensives. In addition, patients with primary aldosteronism (n=461) and Cushing syndrome (n=124) were included. The investigators looked at differences in mass spectrometry-based profiles of 15 adrenal steroids between groups and after surgical resection of PPGLs. Positive relationships were observed between several glucocorticoids and plasma and urinary markers of catecholamine excess. The authors speculate that the data raises the possibility that
elevated levels of glucocorticoids due to the paracrine effect of adrenal medullary catechols might contribute to the cardiovascular and metabolic complications caused by pheochromocytomas.

Major Comments:

1. The authors suggest that because patients with pheochromocytoma and not paraganglioma showed increased circulating concentrations of glucocorticoids, that it is the locally produced and not the circulating catecholamines that are responsible for effects on enhanced steroidogenesis (Figure 2). The authors should clarify that all of the paragangliomas were catecholamine-secreting and that the blood and urine concentrations of norepinephrine and normetanephrine were not different between the pheochromocytoma and paraganglioma patients.

Au: We appreciate the comment. As outlined in our response to referee 1, we have included an additional table to clarify this issue and we also refer referee 2 to our response to that comment.

All paragangliomas were functional and were characterized by increased urinary outputs of norepinephrine. Lack of production epinephrine and metanephrine in paragangliomas compared to pheochromocytomas is well established and is secondary to reduced expression of phenylethanolamine-N-methyltransferase (PNMT) in extra-adrenal compared to adrenal tumors; this in turn is believed to be secondary to proximity to adrenal corticosteroids, which induce expression of PNMT to facilitated conversion of norepinephrine to epinephrine.

As is recognized by the reviewer, it is however blood and urine concentrations of norepinephrine and normetanephrine that are important to consider. Among these two amines, the metabolite has no established physiological action and serves simply as a marker of the size of vesicular norepinephrine

stores. Although, paragangliomas were characterized by lower plasma concentrations and urinary outputs of normetanephrine than pheochromocytomas, this is expected since the former tumors are characterized by lower vesicular stores of catecholamines than pheochromocytomas (PMID: 25946206); consequently these tumors do not produce as much metabolites within tumor cells from the catecholamines leaking for vesicular stores (PMID: 14586065). What is important is the observation that urinary outputs of norepinephrine did not differ significantly between patients with pheochromocytima and paraganglioma. This reflects established observations that noradrenergic PPGLs have much higher rate constants for catecholamine secretion than adrenergic tumors, this despite the lower tumor tissue concentrations of catecholamines of the former than the latter tumors (PMID: 21051559).

2. If indeed there is increased primary adrenal production of glucocorticoids in patients with pheochromocytoma (and thus suppressing ACTH secretion), why was DHEA-S not significantly lower in these patients prior to surgery compared to postop data (Figure 3)? It is unfortunate that serum ACTH concentration measurement was not part of the protocol.

Thank you for the comment. Indeed the referee is correct to point out that the post-operative measurements (between 12 and 36 months) showed no increase in plasma concentrations of adrenal androgens compared to decreases in corticosteroids and aldosterone. As we indicate in the discussion this might reflect differences in post-operative compensation of the different adrenal zones of steroid production (lines 255-257). As was outlined in the original manuscript as well as the revised manuscript, it also remains possible that the post-operative changes in steroids might also reflect affects of adrenalectomy with accompanying reduced adrenal cortical reserve and steroid production, which may also vary among the three zones. We agree that having ACTH levels would have been very helpful (lines 270-271, 310-312). Again, as in the original version of the manuscript, the revised version also clarifies that the data cannot fully exclude a central nervous system effect on ACTH

3. The authors suggest that elevated levels of glucocorticoids might also contribute to the cardiovascular and metabolic complications in patients with pheochromocytoma. They indicate that the effect would be similar to that reported for patients with primary aldosteronism. However, the authors need to address the data shown in Figure 1 where the serum cortisol concentration in patients with primary aldosteronism and primary hypertension appear identical. Do the data from this study support or refute the concept of increased cortisol levels in patients with primary aldosteronism?

Au: Thank you for the comment. We refer the reviewer to our response to the first comment from referee 1.

4. One could argue that, although important to include, patients with primary hypertension may not be the best control group. Do the authors suggest that the degree of stable blood pressure control and stress are similar in patients with pheochromocytoma compared to those with primary hypertension? Thus, the drop in cortisol postoperatively (shown in Figure 3) would not be surprising in any stressed patient group. How can the authors assure the reader that this effect is specific to adrenal medullary catechol secretion?

Au: With referee's point in mind, patients tested for pheochromocytoma in whom tumors have been subsequently excluded could represent a better-suited control group than the primary hypertensives of

the present study. However, our own previous work has shown that markers of adrenal medullary function are similar in such patients compared to reference populations of normotensives and hypertensives (PMID: 30097498). Similar results have also been published by us for plasma concentrations of steroids in patients tested for hypercortisolism, in whom disease was excluded, compared to age and sex matched hypertensives (PMID: 30977834). Furthermore, with the exception of 17-hydroxyprogesterone and 21-deoxycortisol, we have observed no differences in steroid concentrations between hypertensive and normotensive populations (PMID: 28479316). On the basis of these observations, differences in steroids between patients tested for pheochromocytoma in whom tumors are excluded compared to either primary hypertensive or normotensive groups seem unlikely. Patients with primary hypertension therefore provide a reasonable control group.

Minor points:

5. Figure 1 - shades of gray are not visually different enough. Suggest open (white) column, filled (black) column, hatched column, etc. Similarly, for Figure 3, use filled (black) and open (white) columns
6. Page 8, line 159: "androstendione"
7. Page 11, line 234: "patents"
8. Page 11, line 236: "metanrephrines"
9. Page 12, line 246: "glucorticoids"

All minor points have been addressed.

Finally, thank you very much for the careful review of the manuscript and comments for improvement.