

Clinical and Genetic Aspects of Prolactin Hypersecretion

Sunita Maria Christina De Sousa

MBBS (Hons) MSc FRACP

for the Degree of Doctor of Philosophy

School of Medicine, University of Adelaide

January 2020

TABLE OF CONTENTS

| | |
|--|-------|
| Abstract..... | vi |
| Declaration..... | ix |
| Acknowledgements..... | x |
| Publications..... | xiii |
| List of Figures | xvii |
| List of Tables..... | xviii |
| Abbreviations | xix |
| Chapter 1: Introduction..... | 1 |
| 1.1 Prolactin physiology | 1 |
| 1.2 Prolactin measurement..... | 6 |
| 1.3 Biological perturbations in prolactin | 9 |
| 1.3.1 Prolactinomas..... | 13 |
| 1.3.2 Hyperprolactinaemia in association with Cushing’s syndrome..... | 19 |
| 1.3.3 Hyperprolactinaemia in association with carotid aneurysms | 21 |
| 1.4 Genetic aspects of hyperprolactinaemia..... | 22 |
| 1.4.1 Germline genetic changes in patients with hyperprolactinaemia..... | 22 |
| 1.4.2 Somatic genetic changes in sporadic prolactinomas..... | 26 |
| 1.4.3 Next generation sequencing in endocrinology..... | 28 |
| 1.5 Thesis aims | 29 |
| Chapter 2: Serum prolactin overestimation and risk of misdiagnosis..... | 31 |

| | | |
|--|-------------------|----|
| 2.1 | Introduction..... | 31 |
| 2.2 | Methods | 31 |
| 2.3 | Results | 32 |
| 2.4 | Discussion | 36 |
| 2.5 | Conclusion | 39 |
| Chapter 3: Dopa-testotoxicosis: disruptive hypersexuality in hypogonadal men with prolactinomas treated with dopamine agonists | | |
| | | 40 |
| 3.1 | Introduction..... | 40 |
| 3.2 | Methods | 40 |
| 3.3 | Results | 41 |
| 3.4 | Discussion | 44 |
| 3.5 | Conclusion | 54 |
| Chapter 4: Impulse control disorders in dopamine agonist-treated hyperprolactinaemia: prevalence and risk factors | | |
| | | 55 |
| 4.1 | Introduction..... | 55 |
| 4.2 | Methods | 58 |
| 4.3 | Results | 62 |
| 4.4 | Discussion | 74 |
| 4.5 | Conclusion | 85 |
| Chapter 5: Prolactin correction for adequacy of petrosal sinus cannulation may diminish diagnostic accuracy in Cushing's disease | | |
| | | 86 |
| 5.1 | Introduction..... | 86 |

| | | |
|--|--------------------|-----|
| 5.2 | Methods | 89 |
| 5.3 | Results | 92 |
| 5.4 | Discussion | 95 |
| 5.5 | Conclusion | 102 |
| Chapter 6: Vasculogenic hyperprolactinaemia: severe prolactin excess in association with internal carotid artery aneurysms..... | | |
| | | 103 |
| 6.1 | Introduction | 103 |
| 6.2 | Methods | 104 |
| 6.3 | Results | 105 |
| 6.4 | Discussion | 113 |
| 6.5 | Conclusion | 116 |
| Chapter 7: The genomic landscape of sporadic prolactinomas | | |
| | | 117 |
| 7.1 | Introduction | 117 |
| 7.2 | Methods | 119 |
| 7.3 | Results | 122 |
| 7.4 | Discussion | 131 |
| 7.5 | Conclusion | 138 |
| Chapter 8: The aryl hydrocarbon receptor (<i>AHR</i>) gene in cyclical Cushing's disease: interaction with the cell cycle and clock genes..... | | |
| | | 139 |
| 8.1 | Introduction | 139 |
| 8.2 | Methods | 140 |
| 8.3 | Results | 144 |

| | | |
|---|-------------------|-----|
| 8.4 | Discussion | 151 |
| 8.5 | Conclusion | 156 |
| Chapter 9: Deep intronic <i>SDHC</i> mutation in familial paraganglioma with prolactinoma: a new mechanism of succinate dehydrogenase-related tumorigenesis..... | | |
| | | 157 |
| 9.1 | Introduction..... | 157 |
| 9.2 | Methods | 162 |
| 9.3 | Results | 169 |
| 9.4 | Discussion | 177 |
| 9.5 | Conclusion | 186 |
| Chapter 10: Conclusions..... | | |
| | | 187 |
| Appendices | | |
| | | 195 |
| Appendix 1: Aggressive pituitary tumours and pituitary carcinomas..... | | |
| | | 195 |
| Appendix 2: Germline variants in familial pituitary tumour syndrome genes are common in young patients and families with additional endocrine tumours..... | | |
| | | 229 |
| Appendix 3: Genetic testing in endocrinology..... | | |
| | | 240 |
| Appendix 4: Case report of whole genome sequencing in the XY female: identification of a novel <i>SRY</i> mutation and revision of a misdiagnosis of androgen insensitivity syndrome..... | | |
| | | 253 |
| Appendix 5: Familial <i>GATA6</i> mutation causing variably expressed diabetes mellitus and cardiac and renal abnormalities | | |
| | | 261 |
| Appendix 6: <i>ARMC5</i> is not implicated in familial hyperaldosteronism type II (FH-II).268 | | |
| | | 268 |
| Bibliography | | |
| | | 272 |

Abstract

Hyperprolactinaemia is the commonest pituitary endocrinopathy. The degree of prolactin elevation is integral to patient assessment, necessitating vigilance in serum prolactin measurement. Treatment with dopamine agonists is usually highly effective; however, some patients experience intolerable side effects or fail to achieve normoprolactinaemia and/or adequate prolactinoma shrinkage. The side effect profile has traditionally focused on gastrointestinal and cardiovascular symptoms, but mounting evidence suggests that hyperprolactinaemic patients are at risk of the same dopamine agonist-induced impulse control disorders frequently observed in the Parkinson's disease and restless legs syndrome populations. Extrapolating from limited data, the overall prevalence of prolactinoma patients with dopamine agonist treatment failure is at least 50 per million population. Additionally, prolactinomas are one of the commonest subtypes of aggressive pituitary tumours and pituitary carcinomas. Prolactinomas also feature prominently in the familial pituitary tumour syndromes, including the recently recognised '3P' association of pituitary adenomas, pheochromocytomas and paragangliomas due to germline mutations in the succinate dehydrogenase genes. In contrast, the somatic mutational events underlying prolactinomas are unknown. By unclear mechanisms, prolactin hypersecretion may also coexist with Cushing's disease and carotid aneurysms, which may cloud patient assessment.

This thesis evaluates pitfalls in the assessment and management of patients with prolactin excess due to prolactinomas or related disorders and the pathogenesis of prolactin excess. The key finding of the clinical section is that treating prolactinomas with dopamine agonists poses a high, previously underestimated risk of impulse control disorders. We documented this in a case series of eight men with prolactinomas and dopamine agonist-induced hypersexuality, which we referred to as 'dopa-testotoxicosis' to highlight the apparent

additive effects of dopamine receptor stimulation and testosterone normalisation. We subsequently undertook the largest reported cross-sectional analysis of the risk of impulse control disorders in 113 hyperprolactinaemic patients vs. 99 controls. Our findings highlight the need for screening for dopamine agonist side effects, particularly those that may drive behavioural disturbances, as they may not be appreciated in routine practice. The other clinical studies of this thesis were also practice-changing. In a study of 58 cases, we showed that serum prolactin is overestimated by the Roche platform, with the potential for patient mismanagement as physiological and statistical prolactin variations may be misclassified as tumoural hyperprolactinaemia. A retrospective cohort study of 13 patients who underwent inferior petrosal sinus sampling in the evaluation of Cushing's syndrome revealed consistent co-lateralisation of prolactin and adrenocorticotrophic hormone and demonstrated how prolactin-corrected adrenocorticotrophic hormone concentrations may threaten test accuracy, arguing against routine prolactin measurement in petrosal sinus samples.

The major molecular study of this thesis involved next generation sequencing (NGS) of paired germline and tumour DNA from 12 patients with sporadic prolactinomas. This was the first pangenomic study of a pure prolactinoma cohort investigating both point mutations and copy number variants. We found a high burden of copy number variation and a paucity of point mutations. In another NGS study of two families with familial paragangliomas and other tumours including a prolactinoma, we demonstrated a novel *SDHC* deep intronic mutation which is the first reported deep intronic mutation amongst the succinate dehydrogenase genes. We also employed NGS in the first molecular study of cyclical Cushing's disease, finding a putative novel role for the aryl hydrocarbon receptor (*AHR*) gene as a link between pituitary tumorigenesis and clock genes. Targeted RNA sequencing was employed in a study of two patients with the rare association of marked hyperprolactinaemia and carotid aneurysms. We introduced the term 'vasculogenic

hyperprolactinaemia' to describe this association and performed the first molecular study of the disorder; however, our investigation of candidate prolactin secretagogues was negative.

Taken together, these studies have produced new knowledge in the important clinical field of prolactin hypersecretion; each of the studies either impacts upon diagnosis and/or therapy in this field, or points towards new strategies for further scientific study.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holders of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

.....

Sunita Maria Christina De Sousa

January 2020

Acknowledgements

I am most indebted to my principal supervisor, Professor David Torpy, who inspired me firstly to train in endocrinology and secondly to embark on research; two separate but readily intertwined paths as shown by his unrivalled example. Thank you for creating research opportunities that have been compelling and challenging, providing freedom for me to pursue niche interests, guiding me through all roadblocks, and generally being a friend and trusted mentor. Learning from you has been instrumental to my career, and I look forward to continuing to do so as I begin working alongside you later this year.

I would also like to thank my co-supervisors, Professor Hamish Scott and Dr Lucia Gagliardi. In warmly welcoming me into the Genetics and Molecular Pathology Laboratory of SA Pathology, Hamish introduced me to the rich yield offered by next generation sequencing and the importance of leaving no question – or case – unanswered. His unrelenting care for patients and nurturing of all staff members, including clinicians who may have otherwise felt out of place, make him the lynchpin of our laboratory.

Given the breadth of the clinical and scientific topics in this thesis, I have relied upon the generous support of many other colleagues over the last 3.5 years. The staff of the Genetics and Molecular Pathology Laboratory – most notably: Dr Paul Wang, Dr Jinghua (Frank) Feng, Dr Andreas Schreiber, Dr Chris Hahn, Ms Milena Babic, Mr Hung (Andy) Nguyen, Dr Wendy Parker and Ms Rosalie Kenyon – has been instrumental in patiently teaching and assisting me in laboratory experiments and data analysis. Special mention must also be made of:

- Dr John Baranoff and A/Prof Louise Rushworth, who provided invaluable psychological and statistical expertise, respectively, in the impulse control disorder cross-sectional study;

- A/Prof Ann McCormack, A/Prof Warrick Inder, Prof Ian Chapman, A/Prof Christopher Yates, Dr Shaun McGrath, A/Prof Morton Burt, Dr Carmela Caputo and A/Prof Henrik Falhammar who contributed cases from across Australia to the hyperprolactinaemia and Cushing's disease studies;
- A/Prof Roderick Clifton-Bligh and Dr Nicola Poplawski, who assisted in the clinical and genetic characterisation of the families in the *SDHC* study;
- Mr Stephen Santoreneos, who welcomed me into his theatre and kindly provided operative specimens for the pituitary studies; and
- The Australian Cancer Research Facility, which performed the whole exome sequencing described in Chapters 7-9 and the RNA-Seq in Chapter 9.

I would also like to thank all study participants and acknowledge the specific contributions of the following people and their staff:

- Chapter 2: Dr Mohamed Saleem, who performed prolactin measurements in the split serum samples and reviewed the original Roche reference interval data.
- Chapter 6: Dr Emily Meyer and Dr Wayne Rankin, who measured serum prolactin-releasing hormone by ELISA, and Mr Peter Brautigan, who assisted in the RT-PCR experiments of prolactin secretagogues.
- Chapter 7: Dr Angeline Shen (collation of clinical data and operative specimens from interstate participants), Dr Barbara Koszyca and Dr Samuel Roberts-Thomson (histological review), Dr Leila Eshraghi (assistance in point mutation analysis) and Dr Paul Wang (design of the integrated somatic variant calling pipeline and assistance in copy number variation analysis).

- Chapter 8: Dr Jinghua (Frank) Feng (assistance in point mutation analysis), Dr Paul Wang (assistance in copy number variation analysis), Dr Sarah Moore (SNP array of tumour DNA) and Mr Jim Manavis (tumour immunohistochemistry).
- Chapter 9: Dr Jinghua (Frank) Feng (assistance in point mutation analysis), Dr Paul Wang (assistance in copy number variation analysis), Ms Rachel Hall (Sanger sequencing), Mr John Toubia (transcriptome analysis), Dr Tristan Hardy (haplotype analysis), A/Prof Roderick Clifton-Bligh (metabolomic profiles) and A/Prof Michael Buckland (*SDHC* promoter hypermethylation studies).

My research would not have been possible without the following financial support:

- NHMRC Medical Postgraduate Scholarship (Jul 2015 - Dec 2015)
- Royal Adelaide Hospital A. R. Clarkson Scholarship (Jan 2016 - Jun 2019)
- RACP Servier Staff 'Barry Young' Research Establishment Fellowship (Jul - Dec 2019)
- Health Services Charitable Gifts Board grants (2016 - 2020)
- Royal Adelaide Hospital Clinical Project Grant (2019 - 2020)

Finally, I am forever grateful for my parents, Agnelo and Matilda, who have supported and believed in me at every stage, and taught me that your education is one thing that can never be taken away from you. Also infinitely supportive of my career from medical school to now is my husband, Tristan. An ever-loyal audience to my work, you make all of this possible, worthwhile and fun. Last but not least, my beautiful baby girl, Clementine – you may have added a pregnant pause to this work over the final months, but you are by far my proudest achievement and the greatest lesson I have received in the wonders of prolactin and genetics.

Tristan and Clementine, I dedicate this thesis to you both.

Publications

Publications related to work presented in this thesis:

1. **De Sousa SMC**, Manavis J, Feng J, Wang P, Schreiber AW, Scott HS & Torpy DJ. A putative role for the aryl hydrocarbon receptor (*AHR*) gene in a patient with cyclical Cushing's disease. *BMC Endocr Disord.* 2020;20:18.
2. **De Sousa SMC**, Baranoff J, Rushworth RL, Butler J, Sorbello J, Vorster J, Thompson T, McCormack AI, Inder WJ & Torpy DJ. Impulse control disorders in dopamine agonist-treated hyperprolactinaemia: prevalence and risk factors. *J Clin Endocrinol Metab.* 2020;105:e108-e118.
3. **De Sousa SMC**, Wang PPS, Santoreneos S, Shen A, Yates CJ, Babic M, Eshraghi L, Feng J, Koszyca B, Roberts-Thomson S, Schreiber AW, Torpy DJ & Scott HS. The Genomic Landscape of Sporadic Prolactinomas. *Endocr Pathol.* 2019;30:318-328.
4. **De Sousa SMC**, Toubia J, Feng J, Wang P, Hardy TSE, Babic M, Geoghegan J, Hall R, Scott G, Rawlings L, Schreiber A, Luxford C, Clifton-Bligh R, Poplawski N, Scott H & Torpy D. OR34-6 A Novel Mechanism of SDH-Deficient Tumorigenesis and Implications for Genetic Testing in Patients with Pheochromocytoma-Paraganglioma. *J Endocr Soc.* 2019;3(Suppl 1).
5. **De Sousa SMC**, Saleem M, Rankin W & Torpy DJ. Serum prolactin overestimation and risk of misdiagnosis. *Endocrinol Diabetes Metab.* 2019;2:e00065.
6. **De Sousa SMC**, Meyer EJ, Rankin W, Brautigan PJ, Burt MG & Torpy DJ. Vasculogenic hyperprolactinaemia: severe prolactin excess in association with internal carotid artery aneurysms. *Pituitary.* 2017;20:676-682.

7. **De Sousa SMC**, McCormack AI, McGrath S & Torpy DJ. Prolactin correction for adequacy of petrosal sinus cannulation may diminish diagnostic accuracy in Cushing's disease. *Clin Endocrinol (Oxf)*. 2017;87:515-522.
8. **De Sousa SM**, Chapman IM, Falhammar H & Torpy DJ. Dopa-testotoxicosis: disruptive hypersexuality in hypogonadal men with prolactinomas treated with dopamine agonists. *Endocrine*. 2017;55:618-624.

Other publications produced during candidature:

1. McCabe MJ, Gauthier MA, Chan C, Thompson TJ, **De Sousa SMC**, Puttick C, Grady JP, Gayevskiy V, Tao J, Ying K, Cipponi A, Deng N, Swarbrick A, Thomas ML, kConFab10, Lord RV, Johns AL, Kohonen-Corish M, O'Toole S, Clark J, Mueller SA, Gupta R, McCormack AI, Dinger ME & Cowley MJ. Development and validation of a targeted gene sequencing panel for application to disparate cancers. *Sci Rep*. 2019;9:17052.
2. **De Sousa SMC** & McCormack AI. Aggressive Pituitary Tumors and Pituitary Carcinomas. In KR Feingold, B Anawalt, A Boyce, G Chrousos, K Dungan, A Grossman, JM Hershman, G Kaltsas, C Koch, P Kopp, M Korbonits, R McLachlan, JE Morley, M New, L Perreault, J Purnell, R Rebar, F Singer, DL Trence, A Vinik & DP Wilson (Eds.), *Endotext*. South Dartmouth, MA: MDText.com Inc. 2019.
3. Du YT, Moore L, Poplawski NK & **De Sousa SMC**. Familial *GATA6* mutation causing variably expressed diabetes mellitus and cardiac and renal abnormalities. *Endocrinol Diabetes Metab Case Rep*. 2019;19-0022.
4. **De Sousa SMC** & Jesudason D. Rebound vertebral and non-vertebral fractures during denosumab interruption in a postmenopausal woman. *Clin Endocrinol (Oxf)*. 2019;90:250-252.

5. **De Sousa SM**, Hardy TS, Scott HS & Torpy DJ. Genetic Testing in Endocrinology. *Clin Biochem Rev.* 2018;39:17-28.
6. **De Sousa SMC**, Sheriff N, Tran CH, Menzies AM, Tsang VHM, Long GV & Tonks KTT. Fall in thyroid stimulating hormone (TSH) may be an early marker of ipilimumab-induced hypophysitis. *Pituitary.* 2018;21:274-282.
7. Hardy TSE, **De Sousa SMC** & Norman RJ. Obesity and Reproduction. In MK Skinner (Ed.), *Encyclopedia for Reproduction* (2nd edn). Waltham, MA: Elsevier. 2018.
8. **De Sousa SMC**, Stowasser M, Feng J, Schreiber AW, Wang P, Hahn CN, Gordon RD, Torpy DJ, Scott HS & Gagliardi L. *ARMC5* is not implicated in familial hyperaldosteronism type II (FH-II). *J Hum Hypertens.* 2017;31:857-859.
9. **De Sousa SM**, McCabe MJ, Wu K, Roscioli T, Gayevskiy V, Brook K, Rawlings L, Scott HS, Thompson TJ, Earls P, Gill AJ, Cowley MJ, Dinger ME & McCormack AI. Germline variants in familial pituitary tumour syndrome genes are common in young patients and families with additional endocrine tumours. *Eur J Endocrinol.* 2017;176:635-644.
10. **De Sousa SM**, Kassahn KS, McIntyre LC, Chong CE, Scott HS & Torpy DJ. Case report of whole genome sequencing in the XY female: identification of a novel *SRY* mutation and revision of a misdiagnosis of androgen insensitivity syndrome. *BMC Endocr Disord.* 2016;16:58.
11. **De Sousa SM** & Norman RJ. Metabolic syndrome, diet and exercise. *Best Pract Res Clin Obstet Gynaecol.* 2016;37:140-151.

Manuscripts submitted:

1. **De Sousa SMC**, Toubia J, Hardy TSE, Feng J, Wang P, Schreiber AW, Geoghegan J, Hall R, Rawlings L, Buckland M, Luxford C, Novos T, Clifton-Bligh RJ, Poplawski NK,

Scott HS & Torpy DJ. Deep intronic *SDHC* mutation in familial paraganglioma: a new mechanism of succinate dehydrogenase-related tumorigenesis. *J Endocr Soc*.

2. O'Reilly MW, **De Sousa SMC** & Torpy DJ. Adrenal disease in pregnancy. In J Wass, W Arlt & R Semple (Eds.), *Oxford Textbook of Endocrinology and Diabetes* (3rd edn). Oxford, UK: Oxford University Press.

List of Figures

| | |
|---|-----|
| Figure 2.1. Comparative performance of prolactin by Roche Cobas vs. Siemens Centaur | 36 |
| Figure 4.1. ICD frequency and mean HBCS and DASS21 scores in patients vs. controls | 67 |
| Figure 5.1. Mean ACTH and prolactin values on ACTH dominant and non-dominant sides ... | 92 |
| Figure 6.1 T2 MRI images of the ICA aneurysms | 107 |
| Figure 6.2 Hormonal profiles in response to therapeutic interventions | 108 |
| Figure 6.3 Overlapping <i>HDC</i> circulating RNA levels between patients and controls..... | 109 |
| Figure 7.1. Hematoxylin & eosin appearance of prolactinomas at medium power | 124 |
| Figure 7.2. Examples of somatic point variants as shown in Integrated Genomics Viewer .. | 128 |
| Figure 7.3. Examples of tumour DNA calls of CNVs and copy-neutral LOH..... | 130 |
| Figure 8.1. Radiological and biochemical features of the patient..... | 141 |
| Figure 8.2 Position of <i>AHR</i> variant..... | 147 |
| Figure 8.3 <i>AHR</i> and <i>AIP</i> tumour immunohistochemistry (100x magnification)..... | 148 |
| Figure 8.4 Structure of human wild-type and mutant <i>RXRG</i> protein | 149 |
| Figure 8.5. Tumour ploidy count | 150 |
| Figure 8.6. Mutational signature of tumour DNA..... | 151 |
| Figure 9.1. Pedigrees showing succinate dehydrogenase-related and other tumours | 162 |
| Figure 9.2. DNA and RNA representations of the <i>SDHC</i> variant, c.20+74A>G..... | 172 |
| Figure 9.3. Chr 1 and 11 loss in the paraganglioma of II.4 from Family 1 | 174 |
| Figure 9.4. Haplotype analysis using exome data from the 22 autosomes | 176 |

List of Tables

| | |
|--|-----|
| Table 2.1. Serum prolactin interassay discordance in clinical practice | 33 |
| Table 2.2. Potential implications of serum prolactin overestimation | 37 |
| Table 3.1. Local data of eight men with dopa-testotoxicosis..... | 42 |
| Table 3.2. Published reports of 12 men likely affected by dopa-testotoxicosis..... | 49 |
| Table 4.1. Demographic and clinical data of study participants | 63 |
| Table 4.2. Differences in patients screening positive vs. negative for ICDs | 70 |
| Table 4.3. Questionnaire excerpts of DA side effects in hyperprolactinaemic patients | 73 |
| Table 4.4. ICD prevalence in studies of DA-treated hyperprolactinaemic patients | 76 |
| Table 5.1. Published equations employing prolactin measurement in IPSS interpretation.... | 88 |
| Table 5.2. Characteristics of patients with Cushing’s syndrome who underwent IPSS..... | 91 |
| Table 6.1. Published cases with apparent vasculogenic hyperprolactinaemia | 111 |
| Table 7.1. Clinical characteristics of the patient cohort..... | 123 |
| Table 7.2. Pathological characteristics of the patient cohort..... | 124 |
| Table 7.3. Somatic sequence variants of interest | 127 |
| Table 7.4. Somatic copy number variant analysis results | 129 |
| Table 7.5. Paired tumour-germline pangenomic studies in prolactinoma patients..... | 132 |
| Table 8.1. Germline variants in genes of interest | 145 |
| Table 9.1. Clinical characteristics of affected relatives in Family 1 and 2 | 163 |

Abbreviations

| | |
|--------|---|
| 1KGP | 1000 Genomes Project |
| 3PAs | 3P association |
| 5-HT1 | 5-hydroxytryptamine type 1 |
| 5-HT2B | 5-hydroxytryptamine type 2B |
| 95% CI | 95% confidence intervals |
| ACMG | American College of Medical Genetics and Genomics |
| ACRF | Australian Cancer Research Facility |
| ACTH | adrenocorticotrophic hormone |
| AHR | aryl hydrocarbon receptor |
| AIP | aryl hydrocarbon receptor-interacting protein |
| APT | aggressive pituitary tumour |
| ARNT | Ah receptor nuclear translocator |
| BMAL1 | brain-muscle-aryl hydrocarbon nuclear translocator-like protein 1 |
| c/p | central-to-peripheral |
| CADD | combined annotation dependent depletion |
| CD | Cushing's disease |
| CLOCK | circadian locomotor output cycle kaput |
| CNV | copy number variant |
| COSMIC | Catalogue of Somatic Mutations in Cancer |
| CRH | corticotrophin-releasing hormone |
| CS | Cushing's syndrome |
| D1 | dopamine type 1 |
| D2 | dopamine type 2 |

| | |
|------------------|--|
| D3 | dopamine type 3 |
| DA | dopamine agonist |
| DASS21 | Depression Anxiety Stress Scale 21-item |
| dbSNP | Single Nucleotide Polymorphism Database |
| DOG1 | Discovered on GIST-1 |
| <i>DSM-V</i> | <i>Diagnostic and Statistical Manual of Mental Disorders (5th edn)</i> |
| E-box | enhancer-box |
| EAS | ectopic ACTH syndrome |
| EGFR | epidermal growth factor receptor |
| ELISA | enzyme linked immunosorbent assay |
| ESP | Exome Sequencing Project |
| ExAC | Exome Aggregation Consortium |
| FDA | Food and Drug Authority |
| FFPE | formalin-fixed, paraffin-embedded |
| FH2 | familial hyperaldosteronism type 2 |
| FPTS | familial pituitary tumour syndromes |
| GATK | Genome Analysis Toolkit |
| GERP | genomic evolutionary rate profiling |
| GH | growth hormone |
| GIST | gastrointestinal stromal tumours |
| gnomAD | Genome Aggregation Database |
| GQ | genotype quality |
| GTE _x | Genotype-Tissue Expression |
| HBCS | Hypersexual Behaviour Consequences Scale |

| | |
|-------|--|
| HBI | Hypersexuality Behaviour Inventory |
| HCC | hepatocellular carcinoma |
| HNPGL | head and neck paraganglioma |
| HPA | hypothalamic-pituitary-adrenal |
| ICA | internal carotid aneurysms |
| ICD | impulse control disorder |
| IGF1 | insulin-like growth factor 1 |
| IHC | immunohistochemistry |
| IPS | inferior petrosal sinus |
| IPSS | inferior petrosal sinus sampling |
| LOH | loss of heterozygosity |
| MEN1 | multiple endocrine neoplasia type 1 |
| MEN4 | multiple endocrine neoplasia type 4 |
| MeSH | Medical Subject Headings |
| MINAS | multiple inherited neoplasia allele syndrome |
| MLPA | multiplex ligation-dependent probe amplification |
| MRI | magnetic resonance imaging |
| NFPA | non-functioning pituitary adenoma |
| NGS | next generation sequencing |
| OR | odds ratio |
| PA | pituitary adenoma |
| PARP | poly(ADP)-ribose polymerase |
| PAS | PER-ARNT-SIM |
| PBS | phosphate-buffered saline |

| | |
|---------|--|
| PC | pituitary carcinoma |
| PEG | polyethylene glycol |
| PGL | paraganglioma |
| PGL1 | hereditary PGL syndrome type 1 |
| PGL3 | hereditary PGL syndrome type 3 |
| PGL4 | hereditary PGL syndrome type 4 |
| POMC | proopiomelanocortin |
| PPGL | phaeochromocytoma/paraganglioma |
| PPNAD | primary pigmented nodular adrenocortical disease |
| PRLH | prolactin-releasing hormone |
| PrRF | prolactin-releasing factor |
| PrRP-31 | prolactin-releasing peptide-31 |
| PTCOE | Pituitary Tumor Centers of Excellence |
| QUIP | Questionnaire for Impulsive-Compulsive Disorders in Parkinson's Disease |
| QUIP-S | Questionnaire for Impulsive-Compulsive Disorders in Parkinson's disease – Shortened version |
| RCC | renal cell carcinomas |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| RTK | receptor tyrosine kinase |
| RXRG | retinoid X receptor gamma |
| SCN | suprachiasmatic nucleus |
| SD | standard deviation |
| SDH | succinate dehydrogenase |
| SDHA | succinate dehydrogenase subunit A |

| | |
|-------------|---|
| SDHB | succinate dehydrogenase subunit B |
| SDHC | succinate dehydrogenase subunit B |
| SDHD | succinate dehydrogenase subunit D |
| SDHAF2 | succinate dehydrogenase assembly factor 2 |
| <i>SDHx</i> | <i>SDHA, SDHB, SDHC</i> and <i>SDHD</i> genes +/- <i>SDHAF2</i> |
| SDRS5 | Social Desirability Response Set Scale |
| SNP | single nucleotide polymorphism |
| SNV | single nucleotide variant |
| TCDD | 2,3,7,8 tetrachlorodibenzo-p-dioxin |
| TRH | thyrotrophin-releasing hormone |
| TSH | thyroid-stimulating hormone |
| UCSC | University of California, Santa Cruz |
| UK10K | United Kingdom 10,000 Genomes Project |
| VAF | variant allele frequency |
| WES | whole exome sequencing |
| WGS | whole genome sequencing |
| WT | wild-type |
| X-LAG | X-linked acrogigantism |

Chapter 1: Introduction

1.1 Prolactin physiology

Human prolactin is a 199-amino acid single-chain polypeptide hormone (Cooke *et al.*, 1981) that is encoded by *PRL*, a 5-exon gene spanning 10 kb on Chr 6 (Owerbach *et al.*, 1981; Truong *et al.*, 1984). Prolactin, growth hormone (GH) and placental lactogen are thought to have derived from a common ancestral gene via gene duplication approximately 400 million years ago (Niall *et al.*, 1971; Cooke *et al.*, 1981), with the amino acid sequences of human prolactin and the principal 191-amino acid human GH exhibiting 42% sequence homology (Smith & Norman, 1990). Prolactin was the last of the human anterior pituitary hormones to be discovered owing to this structural similarity, as well as the significant lactogenic activity of human GH that is exceptional amongst animal species (Bartke & Kopchick, 2015) and the marked abundance of GH relative to prolactin in humans (Friesen *et al.*, 1970). Despite prolactin being identified in sheep, cows, birds and other animals in the early 1930s, human prolactin was not isolated until 1970, when it became apparent that previous notions of a bifunctional GH with added lactogenic capacity were incorrect as GH was not elevated in the serum of lactating women and lactation was possible in women with congenital GH deficiency (Friesen, 1995). Ultimately, using transsphenoidally-retrieved prolactinoma tissue provided by the neurosurgeon Jules Hardy, immunoprecipitation experiments performed in Montreal by Henry G. Friesen and Harvey J. Guyda revealed a distinct hormone peak non-reactive to GH antibodies; further isolation and characterisation led to the chemical description of prolactin (Friesen *et al.*, 1970; Friesen, 1995).

Over 80% of circulating prolactin exists as a 23-kDa monomer that was originally termed 'little' prolactin (Suh & Frantz, 1974; Sinha, 1995). Smaller prolactin molecules are created by alternative splicing and post-translational modifications, such as protein cleavage (Freeman

et al., 2000). Larger molecular forms include a covalently bound dimer known as 'big' prolactin (45-60 kDa), prolactin bound to IgG which is termed 'big, big' prolactin (150-170 kDa) and polymers comprised of monomeric prolactin joined by covalent or non-covalent bonds (up to 500 kDa) (Suh & Frantz, 1974; Sinha, 1995; Freeman *et al.*, 2000; Gibney *et al.*, 2005; Melmed *et al.*, 2011). These larger molecules are collectively referred to as macroprolactin, which mostly consists of big, big prolactin (Melmed *et al.*, 2011).

Almost all circulating prolactin is produced by lactotrophs, which are adenohipophyseal cells derived from the *POU1F1* (formerly, *PIT1*) lineage that also gives rise to somatotrophs and thyrotrophs (Mohammad *et al.*, 2003). Lactotrophs are distributed evenly throughout the anterior pituitary, comprising 17% of adenohipophyseal mass in men and nulliparous females and up to 70% in pregnant women at term (Asa *et al.*, 1982; Horvath *et al.*, 1999). Mammosomatotrophs are an intermediary adenohipophyseal cell type with the capacity to produce both prolactin and GH (Freeman *et al.*, 2000). The impressive plasticity of the pituitary in general and lactotrophs in particular permits the wide variation in prolactin levels observed across life stages (Guyda & Friesen, 1973; Horvath *et al.*, 1999). mRNA expression and other studies have demonstrated extra-pituitary production of prolactin in the central nervous system, mammary glands, uterus, decidual tissue and immunological and endothelial cells that is mediated by a promoter region of the *PRL* gene that is upstream of the usual promoter region responsible for *PRL* expression in the pituitary (Freeman *et al.*, 2000).

The regulation of prolactin production in the pituitary is unique amongst the adenohipophyseal hormones. Firstly, lactotrophs are constitutively active, with the hypothalamus exerting an inhibitory rather than a stimulatory effect on prolactin production (Talwalker *et al.*, 1963). Secondly, this tonic inhibition is mediated by dopamine, which is a

catecholamine neurotransmitter, as opposed to the hypothalamic peptide hormones that regulate the production of other pituitary hormones (Ben-Jonathan *et al.*, 1980; Grattan, 2015). Thirdly, prolactin lacks a classical endocrine end-organ. Rat models instead demonstrate a short-loop negative feedback circuit whereby prolactin increases tyrosine hydroxylase activity and therefore dopamine production in the dopaminergic neurons of the hypothalamic arcuate nucleus. Dopamine then binds dopamine type 2 (D2) receptors on lactotrophs, in turn inhibiting prolactin production by suppressing adenylate cyclase and decreasing *PRL* gene expression (Grattan, 2015). Reduced sensitivity of this short loop may explain the physiological lactotroph proliferation and hyperprolactinaemia of pregnancy and lactation (Grattan & Averill, 1995).

There are several known and theorised prolactin-releasing factors (PrRF) which may act either directly on lactotrophs or indirectly by reducing dopamine. Oestrogen is one of the most potent stimuli and likely accounts for the larger median pituitary height in women *vs.* men (Tsunoda *et al.*, 1997), the female predominance of prolactinomas (Fernandez *et al.*, 2010), the peaking of prolactinoma incidence during reproductive years (Fernandez *et al.*, 2010), the propensity for prolactinomas to grow during pregnancy (Molitch, 2015) and involute following menopause (Karunakaran *et al.*, 2001), and the lactotroph hyperplasia seen in female but not male D2 receptor-deficient mice (Asa *et al.*, 1999). However, exogenous oestrogen exposure is not typically associated with prolactinoma progression in humans (Fahy *et al.*, 1992; Corenblum & Donovan, 1993). Glycosylated and phosphorylated prolactin molecules, which are generally considered to be less biologically active forms of prolactin, may possess regulatory functions to suppress prolactin release (Freeman *et al.*, 2000).

Prolactin was named for its role in promoting lactation to feed mammalian offspring in response to nipple suckling. Mammary gland growth, milk synthesis and regulation of milk composition remain the most readily apparent functions of prolactin (Freeman *et al.*, 2000). Through alterations in prolactin receptor density, prolactin also facilitates the phenomenon of asynchronous concurrent lactation in marsupials whereby different mammary glands within an individual produce milk of varying composition due to intermammary differences in prolactin binding that allow tandem feeding of young joeys and older offspring (Bird *et al.*, 1994). However, hypoprolactinaemic women can still breastfeed (De Coopman, 1993), and prolactin levels do not correlate with breastmilk yield (Howie *et al.*, 1980). Interestingly, women in the Chinese fishing village of Tanka are able to exclusively breastfeed infants from the right breast with negligible breastmilk production from the left breast (Ing *et al.*, 1977). This argues against a purely endocrine mechanism of lactation, which would be expected to maintain symmetrical breastmilk production, and instead suggests autocrine or paracrine contributors. In addition, most men and even some reproductive age women with marked hyperprolactinaemia never develop galactorrhoea. Thus, prolactin is neither necessary nor sufficient for breastmilk production, and the functions of prolactin in humans likely extend beyond lactation.

In fish, prolactin is critical in ion transport (Breves *et al.*, 2013). In rats, prolactin modulates sexual receptivity and parental behaviour and enhances immune function (Grattan, 2015). Decidual prolactin production has been demonstrated in both humans and rats, putatively contributing to amniotic fluid osmoregulation and growth and immune system development in the embryo/fetus (Freeman *et al.*, 2000). Different animal models have shown interactions between prolactin and receptors in the brain, which abbreviate the cortisol stress response (Grattan, 2015). The prolactin receptor is also expressed in bone, gonads, adrenal cortex, kidneys, prostate, gastrointestinal tract, immune system, adipose tissue and

skin (Grattan, 2015; Melmed *et al.*, 2015). Additional effects of prolactin include increased insulin production, insulin resistance, food intake, adipogenesis and calcium absorption from the gut (Grattan, 2015). Prolactin may also influence personality as hyperprolactinaemic individuals have been shown to be less extroverted with reduced novelty-seeking behaviour and greater social conformity (Athanasoulia *et al.*, 2012a). These personality characteristics interestingly appear the obverse of the hypersexuality and the other impulse control disorders (ICDs) observed in some prolactinoma patients during dopamine agonist (DA) therapy as discussed in Chapters 3 and 4.

The role of prolactin may depend on overall context, with the production and functions of prolactin frequently being dichotomous. Hypothalamic neurons can switch during pregnancy and lactation from being dopaminergic and therefore inhibitory of prolactin production, to enkephalinergic and therefore stimulatory (Merchenthaler, 1994). Progesterone stimulates prolactin production in the decidua (Maslar *et al.*, 1986), but inhibits prolactin production in the myometrium (Gellersen *et al.*, 1991). Prolactin molecules have both pro- and anti-angiogenic effects (Clapp *et al.*, 1998), and trophic and lytic effects on the corpus luteum (Freeman *et al.*, 2000).

Extrapolating from these data, prolactin has been hypothesised to be a far more pleiotropic hormone in humans than its name suggests. In a model proposed by David R. Grattan (2015), prolactin may be considered an overarching child-rearing hormone that reduces stress responses in pregnant and lactating women, enhances nutritional status in offspring, and fosters maternal and even paternal nurturing behaviour. Lactational infertility, most likely due to inhibitory effects of prolactin on gonadotrophin-releasing hormone and kisspeptin, is likely the key role of prolactin from a teleological perspective as it allows for resources to be focused on recent offspring.

1.2 Prolactin measurement

Contemporary prolactin radioimmunoassays are highly specific and easily distinguish prolactin from GH (Melmed *et al.*, 2015). A single measurement is usually sufficient. Serial testing at 15-min intervals may be performed if results are equivocal as prolactin production is pulsatile; however, dynamic testing of prolactin secretion, possible with dopamine antagonist agents such as metoclopramide, is not indicated in clinical practice (Casanueva *et al.*, 2006; Melmed *et al.*, 2011). Prolactin levels follow a circadian rhythm, peaking during sleep and reaching a nadir between 10AM and noon, with larger and more frequent pulses in women compared to men and in younger compared to older individuals (Freeman *et al.*, 2000; Melmed *et al.*, 2015). Prolactin production also varies across the menstrual cycle, peaking at the time of ovulation (Ehara *et al.*, 1973). Such variation is usually accounted for in laboratory reference intervals and prolactin can be measured at any time in clinical practice (Melmed *et al.*, 2011). The gender discrepancy related to the stimulatory effect of oestrogen is typically managed by the use of different reference intervals for men and women. Due to interassay variability, reference intervals should be derived using local normative data and given in mIU/L or mcg/L, where 1 mcg/L is equivalent to 21.2 mIU/L by World Health Organization Standard 84/500 (Casanueva *et al.*, 2006). As highlighted in Chapter 2, prolactin values should be interpreted in relation to the upper limit of normal for a given assay rather than as an absolute value.

The key biochemical cause of a false positive result for hyperprolactinaemia is macroprolactinaemia, which may lead to unnecessary pituitary investigations and DA therapy for an apparent prolactinoma if missed. The molecular size of macroprolactin confines it within the vascular space, which not only limits its biological activity but also reduces its renal clearance and raises the serum concentration of prolactin by most

automated immunoassays (Gibney *et al.*, 2005). Individuals may thus falsely appear to be hyperprolactinaemic despite the absence of prolactin over-production and clinical features of hyperprolactinaemia. Macroprolactinaemia is found in 10-35% of referred individuals, with higher prevalence amongst women, those with milder degrees of hyperprolactinaemia and in referral laboratories receiving samples with equivocal prolactin measurements from elsewhere (Gibney *et al.*, 2005; Casanueva *et al.*, 2006). Prevalence is lowered by the use of newer prolactin assays, which exhibit less cross-reactivity with macroprolactin (Vilar *et al.*, 2014). Gel filtration chromatography accurately distinguishes different prolactin molecules, but macroprolactin can be more cost-effectively excluded by precipitation of serum samples with polyethylene glycol (PEG) and quantification of the remaining monomeric prolactin in the supernatant (Gibney *et al.*, 2005; Vilar *et al.*, 2014). Given PEG precipitation can be performed in large scale, it should be routinely performed in all patients with raised prolactin levels as clinical features cannot reliably differentiate between true hyperprolactinaemia and macroprolactinaemia. Some symptoms, such as amenorrhoea and galactorrhoea, are expectedly more frequent in the context of true hyperprolactinaemia compared to macroprolactinaemia, but none are sufficiently specific. Similarly, luteinising hormone and oestradiol are lower in patients with true hyperprolactinaemia because of the greater ability of monomeric prolactin to suppress the hypothalamic-pituitary-gonadal axis, but values overlap with patients with macroprolactinaemia (Gibney *et al.*, 2005). Following PEG precipitation, true hyperprolactinaemia is best defined by an absolute threshold based on normative data of PEG-treated sera rather than the percentage of recovered prolactin (e.g., >60%). This addresses the 2% risk of a false negative result that arises when a patient has true hyperprolactinaemia in absolute values due to a defined cause in addition to a significant proportion of macroprolactin (Gibney *et al.*, 2005; Vilar *et al.*, 2014). PEG precipitation is only an additional fraction of the cost of prolactin measurement and its

overall cost effectiveness has been demonstrated through cost savings in minimising radiological investigations and DA prescriptions (Gibney *et al.*, 2005). If not done upfront, PEG precipitation should be at least performed in hyperprolactinaemic patients with few or no clinical features of hyperprolactinaemia and/or no apparent cause of hyperprolactinaemia (Gibney *et al.*, 2005; Casanueva *et al.*, 2006; Melmed *et al.*, 2011).

The most important cause of a false negative prolactin result is the 'hook effect', whereby the presence of extreme hyperprolactinaemia saturates both the solid phase (capture) and radiolabelled (signal) antibodies in the prolactin immunoassay, preventing the antibody-antigen-antibody 'sandwich' required for detection. This may occur in up to 14% of patients with pituitary macroadenomas and may lead to unnecessary pituitary surgery for a presumed non-functioning pituitary adenoma (NFPA), as opposed to medical therapy with DAs (Petakov *et al.*, 1998). The hook effect occurs more frequently in men, younger patients and those with giant prolactinomas, all of which are associated with greater degrees of hyperprolactinaemia (Petakov *et al.*, 1998; Casanueva *et al.*, 2006). False negative results due to the hook effect may be determined by an additional washout step to remove unbound prolactin prior to addition of the radiolabelled/signal antibody or by 1:10 or 1:100 dilution (Casanueva *et al.*, 2006; Melmed *et al.*, 2011). These strategies can be employed either routinely or in clinically suspicious cases, such as patients with pituitary macroadenomas, galactorrhoea and a normal or minimally elevated undiluted prolactin level. By contrast, patients with microprolactinomas and patients with a sellar mass but intact hypothalamic-pituitary-gonadal function are unlikely to exhibit the hook effect (Petakov *et al.*, 1998).

1.3 Biological perturbations in prolactin

Hyperprolactinaemia is the commonest endocrinopathy of the hypothalamic-pituitary system (Vilar *et al.*, 2014). Severe hyperprolactinaemia with serum prolactin levels >10-fold normal is almost always due to pregnancy/lactation, where prolactin can reach up to 15-fold normal (Hu *et al.*, 2018), or macroprolactinomas, defined as prolactin-secreting pituitary adenomas (PA) with maximal tumour diameter >10 mm (Casanueva *et al.*, 2006). Serum prolactin usually parallels tumour diameter in prolactinomas, but exceptions include pregnancy, interference by macroprolactinaemia, the aforementioned hook effect, and cystic prolactinomas which contain fewer lactotrophs than the more typical solid prolactinoma (Melmed *et al.*, 2011; Vilar *et al.*, 2014). Very rare causes of severe hyperprolactinaemia include malignant prolactinoma defined by the presence of metastasis (Kars *et al.*, 2006), and ectopic prolactin production in acute leukaemia and rare solid tumours such as perivascular epithelioid cell tumours (Korytnaya *et al.*, 2014). Another rare cause, investigated in Chapter 6, is vasculogenic hyperprolactinaemia, where prolactin can reach up to 190-fold normal in the setting of cavernous carotid aneurysms (De Sousa *et al.*, 2017d).

Microprolactinomas, defined by tumour diameter <10 mm, often produce mild hyperprolactinaemia with serum prolactin levels four- to six-fold normal (Casanueva *et al.*, 2006; Vilar *et al.*, 2008). Other PA subtypes may also result in mild hyperprolactinaemia, usually due to the stalk effect discussed below. Alternatively, these other PA subtypes may produce prolactin *in situ*. This is observed in up to 50% of patients with acromegaly where somatotrophinoma cells may co-secrete prolactin in addition to GH (Melmed *et al.*, 2011). As illustrated in Chapter 5, patients with the pituitary form of Cushing's syndrome (CS) – i.e., Cushing's disease (CD) – may also exhibit mild hyperprolactinaemia due to an apparent

paracrine stimulatory effect on lactotrophs exerted by corticotrophinoma cells (Loli *et al.*, 1998; De Sousa *et al.*, 2017c). Hyperprolactinaemia also frequently accompanies the GH excess of X-linked acrogigantism (X-LAG) due to microduplications involving *GPR101* (Trivellin *et al.*, 2014), whilst pure prolactinomas are amongst the commonest PA subtypes seen in the familial pituitary tumour syndromes (FPTS) associated with *MEN1*, *AIP* and *PRKAR1A* mutations (Elston *et al.*, 2009; Beckers *et al.*, 2013). The genetic aspects of prolactinomas and related tumorigenesis are further explored below and in Chapters 7-9.

The differential diagnosis of mild hyperprolactinaemia includes anything that interferes with dopamine secretion, transport or action in the hypothalamic-pituitary circulation. The 'stalk effect' refers to interruption of dopamine transport from the hypothalamus to the pituitary along the portal vessels within the intervening infundibulum or 'stalk'. It may be caused by infundibular compression by sellar or suprasellar masses (e.g., PA, craniopharyngioma, Rathke's cleft cyst, meningioma, metastasis) or infiltration (e.g., lymphocytic hypophysitis, Langerhans cell histiocytosis), or by infundibular damage or transection (e.g., by surgery, irradiation, head trauma). Alternatively, these aetiologies may produce hyperprolactinaemia by direct impairment of hypothalamic dopamine production (Casanueva *et al.*, 2006).

The inhibitory effect of dopamine may also be lost due to medications, which are the commonest cause of non-tumoural hyperprolactinaemia (Melmed *et al.*, 2011). Causative medications include agents which antagonise the dopamine receptor (e.g., risperidone, haloperidol, metoclopramide, domperidone), deplete dopamine stores (e.g., reserpine) or inhibit hypothalamic dopamine production (e.g., methyldopa, verapamil, morphine, heroin) (Molitch, 2005a). The mechanism of hyperprolactinaemia is less clear in other causative drugs, including tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, histamine 2 receptor blockers, anaesthetic agents, sibutramine, cocaine,

amphetamine and marijuana (Molitch, 2005a; Vilar *et al.*, 2014). Antipsychotics and antidepressants are the most frequent interfering medications (Vilar *et al.*, 2008). Typical antipsychotics such as haloperidol and chlorpromazine are especially potent in inducing hyperprolactinaemia, whereas atypical antipsychotics are less potent in this regard, likely due to weaker affinity for the D2 receptor and the combination of antagonist and agonist effects at the D2 receptor (Molitch, 2005a). Aripiprazole is an atypical antipsychotic with hypoprolactinaemic effects which may in fact be used to treat psychiatric disease and concomitant hyperprolactinaemia (Vilar *et al.*, 2014). However, the atypical antipsychotic, risperidone, produces some of the highest degrees of drug-induced hyperprolactinaemia (Molitch, 2005a; Vilar *et al.*, 2008). Variants in the *DRD2* gene encoding the D2 receptor may mediate the degree of dopamine antagonist-induced hyperprolactinaemia (Calarge *et al.*, 2009).

Other non-adenomatous causes of hyperprolactinaemia include primary hypothyroidism (likely due to lactotroph stimulation by thyrotrophin-releasing hormone (TRH)), exogenous oestrogens (e.g., the combined oral contraceptive pill or hormone replacement therapy), chronic kidney disease (due to reduced renal clearance of prolactin and dysregulation of prolactin production), and nipple stimulation or any source of chest wall injury (due to afferent neural pathways decreasing dopamine production) (Casanueva *et al.*, 2006; Melmed *et al.*, 2011; Vilar *et al.*, 2014). Though the mechanisms are unknown, hyperprolactinaemia may occur in cirrhosis (Vilar *et al.*, 2014), and in 12% of women meeting the Rotterdam criteria for polycystic ovary syndrome (Delcour *et al.*, 2019). Prolactin may also rise transiently as a result of psychological or physical stress (as may occur during venepuncture), seizures, or physiological conditions such as coitus, orgasm, exercise, sleep or eating. Hyperprolactinaemia is said to be idiopathic when pituitary imaging is normal and other known causes of prolactin excess are absent. Idiopathic

hyperprolactinaemia is assumed to be due to either occult tumours below the 2-3 mm resolution of pituitary magnetic resonance imaging (MRI) or anti-pituitary antibodies which may be found in >25% of affected patients (De Bellis *et al.*, 2007).

To help discern the myriad causes of hyperprolactinaemia, moderately elevated serum prolactin levels (e.g., up to five-fold elevated) should prompt review of confounding drugs and disorders, and blood collection with as little venepuncture stress as possible for repeat prolactin measurement in addition to β -human chorionic gonadotropin, thyroid-stimulating hormone (TSH), creatinine, liver enzymes and insulin-like growth factor 1 (IGF1) (Casanueva *et al.*, 2006). As MRI demonstrates incidental pituitary lesions in up to 10% of the general population and small adenomas may be missed on MRI, the biochemical diagnosis of hyperprolactinaemia should be secured prior to pituitary imaging (Hall *et al.*, 1994; Casanueva *et al.*, 2006). DA-induced normalisation of hyperprolactinaemia is not a specific feature of prolactinomas as DAs are highly effective in normalising serum prolactin levels from non-tumoural causes such as drugs, the stalk effect, acromegaly and idiopathic hyperprolactinaemia (Gibney *et al.*, 2005). Clinical features may also overlap between hyperprolactinaemia due to prolactinomas and that due to other causes (Melmed *et al.*, 2011). However, significant tumour shrinkage during DA therapy is specific to prolactinomas (Casanueva *et al.*, 2006).

Prolactin is under tonic inhibition by the hypothalamus, hence hypothalamic-pituitary disorders typically increase rather than decrease prolactin. Nonetheless, prolactin deficiency is found in 6-27% of patients with hypothalamic-pituitary disease, presumably due to loss of functioning lactotrophs (Toledano *et al.*, 2007). Congenital prolactin deficiency may occur in the setting of combined pituitary hormone deficiency due to mutations in genes including *POU1F1* and *PROP1* (McLennan *et al.*, 2003). Acquired prolactin deficiency classically occurs

following rapid and severe lactotroph destruction, as may occur in pituitary apoplexy, Sheehan's syndrome, drug-induced hypophysitis (e.g., due to ipilimumab) and surgical damage; it may also rarely develop due to craniopharyngiomas and lymphocytic hypophysitis (Toledano *et al.*, 2007; Vilar *et al.*, 2014). Hypoprolactinaemia is almost always accompanied by other pituitary hormone deficiencies. Prolactin deficiency is not associated with its own clinical syndrome and is instead best regarded as a marker of severe, and likely irreversible, pituitary damage, although the reduced sensitivity of most prolactin assays in the lower range limits the utility of finding a low serum prolactin (Toledano *et al.*, 2007).

1.3.1 Prolactinomas

The prevalence of PAs varies according to the mode of detection: 1-25% by autopsy (Ezzat *et al.*, 2004); 10% by MRI screening (Hall *et al.*, 1994); and 0.1% by clinical presentation (Daly *et al.*, 2006; Fernandez *et al.*, 2010). General practice surveys show that prolactinomas are the commonest clinically relevant PA, accounting for 57-66% of cases (Daly *et al.*, 2006; Fernandez *et al.*, 2010).

Prolactinomas predominate in women with a female-to-male ratio of 10:1; however, men are more likely to have macroprolactinomas than microprolactinomas and to present at a more advanced stage, with mass effect symptoms such as headaches and visual disturbances (Fernandez *et al.*, 2010). Central hypogonadism is the most common complication of prolactinomas in both sexes. In men, androgen deficiency is present in 73-78% at diagnosis (Sibal *et al.*, 2002; Colao *et al.*, 2004; Gillam *et al.*, 2006), and the commonest symptoms are fatigue and low libido (Fernandez *et al.*, 2010). The rate of prolactinoma-associated hypogonadism is independent of adenoma size, highlighting that reduced gonadotrophin secretion is more often due to direct functional inhibition by prolactin rather than mass effect (Pinzone *et al.*, 2000).

Treatment is indicated in all macroprolactinomas and in microprolactinomas with hypogonadism or bothersome galactorrhoea. The combined oral contraceptive pill may be used in premenopausal women with microprolactinomas and amenorrhoea if they do not desire fertility. In all other cases, the first-line treatment of prolactinomas is medical therapy in the form of DAs (Casanueva *et al.*, 2006). This differs from other pituitary tumours where the first-line treatment, if required, is surgery. The DAs used in the treatment of prolactinomas – namely, cabergoline, bromocriptine and quinagolide – are highly effective with prolactin normalisation in 60-90% of patients (Pinzone *et al.*, 2000; Gillam *et al.*, 2006), mediated by inhibition of adenylate cyclase via D2 receptors on lactotrophs (Gillam *et al.*, 2006). Success rates are higher with cabergoline than bromocriptine (Webster *et al.*, 1994), and in microprolactinomas than macroprolactinomas (Verhelst *et al.*, 1999). Cabergoline is better tolerated than bromocriptine, which has frequent side effects including nausea, headache, dizziness and gastrointestinal upset (Webster *et al.*, 1994). Quinagolide is a less frequently used DA which, like cabergoline, has greater D2 receptor specificity and therefore fewer side effects than bromocriptine (Vinkers & van der Wee, 2007). By achieving normoprolactinaemia and tumour shrinkage, DAs may restore eugonadism without the need for sex steroid replacement (Gillam *et al.*, 2006). In a study of 51 men with prolactinomas treated with cabergoline, testosterone normalised in approximately 60% of patients by 6 months, irrespective of whether the patient had a macroprolactinoma or microprolactinoma (Colao *et al.*, 2004).

DA treatment is typically commenced at a standard dose of cabergoline of 0.25-0.5 mg once or twice weekly. Serial prolactin measurement is then used to guide DA therapy (Casanueva *et al.*, 2006). Maximal prolactin reduction by increasing DA doses may optimise tumour shrinkage as nadir prolactin on treatment is one of the strongest predictors of DA-induced tumour shrinkage (Colao *et al.*, 2000). However, most DA side effects are dose-dependent

(Colao *et al.*, 2000), and fertility may be enhanced by normal rather than low serum prolactin levels (Kauppila *et al.*, 1988; Gonzales *et al.*, 1989; Casanueva *et al.*, 2006). On balance, the normal range is usually an appropriate serum prolactin target in patients with prolactinomas (Casanueva *et al.*, 2006; Melmed *et al.*, 2011).

The side effect profile of DAs is not insignificant, with 3% of cabergoline-treated patients and 12% of bromocriptine-treated patients ultimately ceasing therapy due to intolerable side effects (Webster *et al.*, 1994). The most common side effects include nausea mediated by the 5-hydroxytryptamine type 1 (5-HT₁) receptor and postural hypotension mediated by the D₁ receptor (Barake *et al.*, 2018). Rare complications include cardiac valvulopathy due to prolonged, high-dose treatment with ergot-derived DAs (i.e., cabergoline, bromocriptine) that interact with the 5-HT_{2B} receptor (Elenkova *et al.*, 2012; Caputo *et al.*, 2015), and overt psychiatric disorders including psychosis, mania and major depression that may occur irrespective of DA type, dose or duration (Ioachimescu *et al.*, 2019).

DA-induced psychosis is a well-known but rare phenomenon. A cohort study of 600 patients treated with DAs for prolactinoma or acromegaly found that eight (1.3%) patients developed psychosis irrespective of the tumour type and despite the absence of a personal or family history of mental illness. Bromocriptine and lisuride were both implicated and doses as low as 7.5 mg daily of bromocriptine were causative, although there was evidence of intraindividual dose response effects. Psychosis resolved in each case upon ceasing or reducing the DA agent and the three patients who resumed DA therapy experienced relapses (Turner *et al.*, 1984). In further support of the drug rather than the underlying pituitary disorder as the cause, psychosis has been induced when DAs have been administered for other indications, including Parkinson's disease and the treatment of antipsychotic-induced galactorrhoea (Boyd, 1995). DA-induced psychosis has also been

described in the puerperium when these drugs are not uncommonly used to terminate breastmilk production (Boyd, 1995; Snellen *et al.*, 2016). DAs may act as a trigger for psychosis in susceptible individuals as dopamine is established as a central mediator of psychosis, and contemporary antipsychotics virtually all exert their therapeutic effect through dopamine antagonism (Boyd, 1995; Snellen *et al.*, 2016). On the other hand, there are a number of prospective studies which have shown no worsening of psychiatric symptoms when antipsychotic-induced hyperprolactinaemia has been treated with either bromocriptine (Beumont *et al.*, 1975; Perovich *et al.*, 1989; Lee *et al.*, 2010) or cabergoline (Cavallaro *et al.*, 2004).

Psychosis represents the extreme end of the psychological spectrum of DA-induced side effects. Few studies have explored the potential for DAs to cause milder mood or anxiety symptoms. A study of nine women before, and 6 and 12 months after, starting bromocriptine for prolactinoma showed no increase in depressive, anxious or aggressive tendencies either at baseline compared to normative data, or on treatment compared to paired baseline results (Rocco *et al.*, 1993). Another study of 93 patients with various pituitary tumours, 36 of whom had prolactinomas, also found no increase in the rate of mental illness compared to controls; however, only 10.7% of the overall sample was treated medically and the assessments occurred soon after tumour diagnosis which might have had a confounding effect on mental wellbeing (Korali *et al.*, 2003). Larger studies of pure prolactinoma cohorts taking DAs of various types and doses for varying durations are required to evaluate the potential for mood disturbances in DA-treated prolactinoma patients.

A recently recognised side effect of DAs is the development of ICDs such as gambling, hypersexuality, compulsive shopping and binge eating. This is thought to be mediated by

stimulation of reward pathways in the mesolimbic system which bears dopamine receptors. The vast majority of DA-induced ICDs have been described in neurology, where these medications are used in high doses for the treatment of Parkinson's disease and restless legs syndrome (Moore *et al.*, 2014). Little is known about the nature of ICDs in the setting of prolactinomas where endocrine factors, specifically sex hormone fluctuations, may influence behaviour (Andela *et al.*, 2015). A case series highlighting the severity and possible mechanisms of hypersexuality in these patients is presented in Chapter 3. In the publication of this case series (De Sousa *et al.*, 2017a), we proposed the term 'dopa-testotoxicosis' to highlight the male predilection and hypersexuality predominance in DA-induced ICDs in hyperprolactinaemic patients. This prompted much discussion in the literature as to the role of testosterone in causing hypersexuality in this context (Bancos *et al.*, 2017; Athanasoulia-Kaspar *et al.*, 2018; Barake *et al.*, 2018; Celik *et al.*, 2018; Dogansen *et al.*, 2019; Ioachimescu *et al.*, 2019); we have since further explored this association in Chapter 4, which represents the largest cross-sectional analysis of the risk of ICDs in hyperprolactinaemic patients compared to controls. This study also evaluated the risk of mood disturbances.

Another issue in the DA treatment of prolactinomas is tumour resistance, typically defined as failure to normalise prolactin and/or failure to induce $\geq 50\%$ tumour shrinkage (Molitch, 2005b; Gillam *et al.*, 2006). Persistent hyperprolactinaemia is observed in approximately 25–50% of bromocriptine-treated patients and 5–18% of cabergoline-treated patients, whilst $< 50\%$ tumour shrinkage is observed in 33% of bromocriptine-treated patients and 5–10% of cabergoline-treated patients (Gillam *et al.*, 2006). Biochemical and structural responses to DA therapy are usually, but not always, concordant (Molitch, 2005b; Gillam *et al.*, 2006). Surgery may be considered in patients who do not respond to DA treatment despite dose escalation and possibly switching DA agents. However, some tumours (e.g., those with cavernous sinus invasion) are not amenable to total resection, and recurrent

hyperprolactinaemia is observed in approximately 20% of patients despite prolactin normalisation in the early postoperative period (Casanueva *et al.*, 2006). The surgical management of prolactinomas in patients with DA resistance or intolerance offers the opportunity for molecular tumour studies, although the proportion of patients available for such studies is substantially less than other pituitary tumours that are treated with upfront surgery. Radiotherapy is infrequently used in the treatment of prolactinomas (Casanueva *et al.*, 2006).

Perhaps because of the success of DA therapy in the majority of patients with prolactinomas, this PA subtype has been relatively understudied in contemporary literature. Searching by Medical Subject Headings ('MeSH') terms in the online PubMed search engine (<https://www.ncbi.nlm.nih.gov/pubmed/>, accessed 27 Sept 2019), the number of publications from 2010 to 2018, inclusive, was 767 for 'prolactinoma' compared to 805 for 'Cushing's disease' and 1540 for 'acromegaly'. This is despite prolactinomas being the commonest of these PA subtypes. Even the subgroup alone of patients with prolactinomas that are inadequately treated due to DA intolerance or resistance is likely comparable to the total prevalence of CD (56 per million) and the total prevalence of acromegaly (125 per million) (Daly *et al.*, 2006). It is difficult to ascertain the precise proportion of prolactinoma patients that is inadequately treated by DAs because studies tend to subdivide patients according to gender and/or whether they have microprolactinomas or macroprolactinomas. The more recent studies focusing on patients with DA resistance are also limited by the ascertainment bias of only including patients who have sufficiently tolerated DA treatment in order to be deemed as DA-resistant. If we consider one of the original studies of cabergoline treatment that was performed in women with hyperprolactinaemic amenorrhoea, up to 8% of the treated women either ceased DA therapy due to intolerable side effects or did not achieve normoprolactinaemia (Webster *et al.*, 1994). This study

notably excluded women with macroprolactinomas and those who had previously discontinued DA treatment due to side effects. As a conservative estimate, 8% of the total prolactinoma prevalence of up to 625 per million (Daly *et al.*, 2006) amounts to 50 per million as the prevalence of patients with inadequately treated prolactinomas. Prolactinomas should thus be considered as much of a research priority in pituitary endocrinology as CD and acromegaly.

Prolactinomas also merit further study as lactotrophs are one of the leading cells of origin amongst aggressive pituitary tumours (APT) and pituitary carcinomas (PC). A European Society of Endocrinology survey of clinicians treating patients with APT/PC found that the predominant cell subtype was corticotrophs (45% of APT, 48% of PC), followed by lactotrophs (20% of APT, 38% of PC) (McCormack *et al.*, 2018). Similarly, a review of 72 published PC cases found that hormone immunohistochemistry (IHC) was most commonly positive for adrenocorticotrophic hormone (ACTH) (35%), followed by prolactin (24%) (Yoo *et al.*, 2018). Appendix 1 further examines the molecular and clinical aspects of APT/PC.

1.3.2 *Hyperprolactinaemia in association with Cushing's syndrome*

Although a rare cause of hyperprolactinaemia overall, pituitary Cushing's is frequently associated with prolactin excess. Hyperprolactinaemia is observed in 23-50% of patients with CD, but it is not seen in adrenal Cushing's, suggesting that hyperprolactinaemia is an intrinsic feature of corticotrophinomas (Yamaji *et al.*, 1984). As illustrated in Chapter 5 and by others (Crock *et al.*, 1988; Schulte *et al.*, 1988; Zovickian *et al.*, 1988; Tabarin *et al.*, 1992; Loli *et al.*, 1998; Daousi *et al.*, 2010), patients with CD are typically found to have local prolactin hypersecretion ipsilateral to the side of the corticotrophinoma. This apparent peritumoural prolactin production may be a paracrine effect of β -endorphin or galanin, both of which are secreted by corticotrophinomas and can stimulate lactotrophs (Schulte *et al.*,

1988; Freeman *et al.*, 2000). In addition, patients with CD demonstrate enhanced lactotroph responsiveness to galanin compared to controls (Invitti *et al.*, 1993). Such stimulation of lactotrophs, together with the hyperplasia-adenoma sequence apparent in the pituitary (Horvath *et al.*, 1999; Villa *et al.*, 2011), may explain the predominance of prolactinomas amongst secondary tumours in CD patients (Ratliff & Oldfield, 2000). An alternative explanation for the hyperprolactinaemia observed in pituitary Cushing's is direct prolactin hypersecretion by corticotrophinomas; however, this seems less likely as corticotrophinomas rarely co-stain for prolactin (Crock *et al.*, 1988; Tabarin *et al.*, 1992; Loli *et al.*, 1998). The aforementioned stalk effect may be the cause of hyperprolactinaemia in some cases of CD, although this is not expected to account for the majority of cases as corticotrophinomas are typically microadenomas where infundibular compression is unlikely (Yamaji *et al.*, 1984).

Irrespective of the mechanism, the phenomenon of ipsilateral hyperprolactinaemia in CD challenges the validity of measuring prolactin during inferior petrosal sinus sampling (IPSS) to aid the diagnosis of CD. Proponents of the use of prolactin measurement argue that this improves the diagnostic accuracy of ACTH measurement alone. This is based on the assumption of symmetrical prolactin secretion. The aim is to correct ACTH measurements for cannula proximity to the pituitary venous effluent in order to avoid results that are falsely negative for CD because of distant cannula placement (McNally *et al.*, 1993; Findling *et al.*, 2004; Sharma *et al.*, 2011; Grant *et al.*, 2012; Sharma & Nieman, 2013; Qiao *et al.*, 2015). Chapter 5 demonstrates how the peritumoural hyperprolactinaemia of CD may impact upon the various proposed prolactin-corrected ACTH IPSS equations in the evaluation of ACTH-dependent CS.

Hypercortisolism is intermittent in up to 20% of CS (Meinardi *et al.*, 2007); this is termed ‘cyclical CS’, or ‘cyclical CD’ in the case of pituitary Cushing’s. Cyclical CD, which is the focus of Chapter 8, may also be associated with peritumoural hyperprolactinaemia (De Sousa *et al.*, 2017c). The frequency of this finding has not been reported. The patient with cyclical CD studied in Chapter 8 did not exhibit peripheral hyperprolactinaemia and IPSS was not performed; thus, it is unknown whether he had local hyperprolactinaemia. There may nonetheless be pathogenic links between such cases and lactotrophs as this patient was found to have a suspicious variant in the *RXRG* gene which has been proposed as a candidate gene in familial prolactinomas (Melo *et al.*, 2016).

1.3.3 *Hyperprolactinaemia in association with carotid aneurysms*

Marked hyperprolactinaemia may very rarely occur in the context of aneurysms of the internal carotid artery (ICA). Despite this rarity, this association is noteworthy in the evaluation of hyperprolactinaemia because prolactin levels corresponding to 20-fold normal have otherwise been considered “*diagnostic of a macroprolactinoma*” in international guidelines (Melmed *et al.*, 2011). Failure to recognise the association of ICA aneurysms and severe hyperprolactinaemia could lead to catastrophic consequences in clinical practice. Specifically, a sellar aneurysm could be misdiagnosed as a DA-resistant prolactinoma, with uncontrollable haemorrhage upon breaching of the aneurysm during transsphenoidal surgery.

In Chapter 6, we describe hyperprolactinaemia in the setting of carotid aneurysms with serum prolactin levels reaching up to 190-fold normal. The cases described build on previous case reports and case series of hyperprolactinaemia in association with carotid aneurysms (Verbalis *et al.*, 1982; Garg & Dash, 1985; Ooi & Russell, 1986; Fernandez-Real *et al.*, 1994; Kahn *et al.*, 1997; Heshmati *et al.*, 2001; Duarte *et al.*, 2008; Gungor *et al.*, 2015; Goldman *et*

al., 2016). In the vast majority of these cases, the aneurysms have been located in or proximal to the C6 (ophthalmic) segment of the ICA, which also gives rise to the superior hypophyseal artery (Bouthillier *et al.*, 1996). It is possible that a PrRF produced by damaged endothelial cells within carotid aneurysms reaches the lactotrophs via the superior hypophyseal arteries and exerts a stimulatory paracrine or endocrine effect. Supporting a specific role for carotid aneurysms in the development of hyperprolactinaemia, prolactin excess is not found in patients with aneurysms beyond the ICA (Barbieri *et al.*, 2011).

In the publication arising from Chapter 6 (De Sousa *et al.*, 2017d), we proposed the term ‘vasculogenic hyperprolactinaemia’ to convey the apparent biological association of hyperprolactinaemia and carotid aneurysms. The mechanism of this association has not previously been investigated, but it is clear that the degree of hyperprolactinaemia is far greater than that caused by the stalk effect. Chapter 6 outlines molecular studies undertaken in our patients with the aim of finding a prolactin secretagogue being produced by the injured carotid endothelium.

1.4 Genetic aspects of hyperprolactinaemia

The genetic causes of hyperprolactinaemia remain largely unknown. Only a small proportion of prolactinomas occur in the setting of germline genetic mutations that result in characteristic FPTS. Sporadic prolactinomas are less understood. In contrast to a number of other pituitary tumours, there are currently no consistent somatic mutations responsible for prolactinomas.

1.4.1 Germline genetic changes in patients with hyperprolactinaemia

Historically, only 5% of PAs have been considered to occur within a FPTS, with germline mutations most commonly found in *MEN1*, encoding the menin protein, and *AIP*, encoding

aryl hydrocarbon receptor-interacting protein (AIP) (Lecoq *et al.*, 2014). As described in Appendix 2, more recent evidence shows an enrichment of germline mutations in PA patients who are young or who have a personal or family history of related endocrine tumours (De Sousa *et al.*, 2017b). Unless stated otherwise, the germline mutations underpinning pituitary tumours are heterozygous loss-of-function variants with an autosomal dominant inheritance pattern.

The multiple endocrine neoplasia type 1 (MEN1) syndrome arises due to germline *MEN1* mutations that predispose patients to parathyroid, pituitary and pancreatic tumours as well as facial angiofibromas, collagenomas and lipomas; less frequent manifestations include other endocrine lesions such as adrenocortical tumours, and non-endocrine lesions such as meningiomas (Wermer, 1954; Steiner *et al.*, 1968; Lecoq *et al.*, 2014). Germline mutations in *CDKN1B*, encoding cyclin-dependent kinase inhibitor 1B (also known as p27Kip1), are a rare cause of familial PAs producing an MEN1-like phenotype labelled 'MEN4' (Pellegata *et al.*, 2006; Lecoq *et al.*, 2014). Germline *AIP* mutations account for 20% of familial isolated PA kindreds, and are clinically characterised by typically GH-secreting PAs with incomplete penetrance, young age of onset, male predilection and treatment resistance (Beckers *et al.*, 2013; Lecoq *et al.*, 2014).

The succinate dehydrogenase (SDH) genes, recognised for their role in pheochromocytoma/paraganglioma (PPGL) syndromes, gastrointestinal stromal tumours (GIST) and renal cell carcinoma (RCC) (Gill, 2012), have also recently been implicated in PAs. Germline mutations in *SDHA*, *SDHB*, *SDHC* and *SDHD* (collectively, *SDHx*) have been found in both individuals and kindreds with combinations of PAs and PPGL, now termed the 3P association syndrome (3PAs) (Xekouki *et al.*, 2012; Dwight *et al.*, 2013; Dénes *et al.*, 2015; Xekouki *et al.*, 2015). Chapter 9 details the clinical and molecular aspects of the full SDH-

deficient tumour spectrum, including the four classical SDH-deficient tumours, and highlights a novel mutation type amongst the *SDHx* genes. The index family described in this chapter includes a man with a macroprolactinoma which is suspected to relate to the familial *SDHC* mutation. However, the successful medical treatment of his prolactinoma precluded tissue studies to further investigate this possibility. This typically successful DA response highlights one of the barriers in the genetic evaluation of hyperprolactinaemia.

Rare syndromic causes of PAs include Carney complex, X-LAG and McCune-Albright syndrome, where the pituitary lesion may be either an adenoma or hyperplasia (Pack *et al.*, 2000; Collins *et al.*, 2012; Trivellin *et al.*, 2014). Carney complex is due to germline mutations in *PRKAR1A*, encoding the type 1A regulatory subunit of cyclic adenosine monophosphate-dependent protein kinase A. This multisystem disorder includes spotty skin lentigines, cardiac and other myxomas and primary pigmented nodular adrenocortical disease (PPNAD), as well as PAs in up to 20% of patients (Carney *et al.*, 1985; Lecoq *et al.*, 2014). X-LAG is a highly penetrant, X-linked dominant disorder of paediatric-onset acrogigantism due to germline or mosaic microduplications of Chr Xq26.3 that consistently involve the *GPR101* gene (Trivellin *et al.*, 2014). The seminal paper describing X-LAG also suggested a role for activating *GPR101* mutations in sporadic cases of adult-onset acromegaly (Trivellin *et al.*, 2014), although this has not been corroborated by subsequent studies (Iacovazzo *et al.*, 2016). McCune-Albright syndrome, due to postzygotic activating *GNAS* mutations, results in poly- or monostotic fibrous dysplasia, café-au-lait macules and various endocrinopathies, including GH excess in 20% of patients. Precocious puberty is another key feature of McCune-Albright syndrome, but this is due to autonomous sex steroid production at the level of the gonads rather than a pituitary source (Collins *et al.*, 2012).

Other genes implicated in the germline predisposition to pituitary tumorigenesis include *CDH23* (Zhang *et al.*, 2017), *MAX* (Roszko *et al.*, 2017), *CABLES1* (Hernandez-Ramirez *et al.*, 2017) and *DICER1* (de Kock *et al.*, 2014).

Prolactinomas feature prominently amongst these FPTS. Prolactinomas are the most common PA subtype in patients with mutations in the *MEN1* (Cebrián *et al.*, 2003) and *SDHx* (Papathomas *et al.*, 2014; Xekouki *et al.*, 2015) genes, and the second most common subtype in patients with *AIP* mutations (Beckers *et al.*, 2013). Although *MEN4* is generally regarded as an *MEN1* mimic, prolactinomas have not been described in the setting of *CDKN1B* mutations (Frederiksen *et al.*, 2019). The characteristic pituitary lesion in patients with Carney complex is somatomammotroph hyperplasia, which appears to give way to GH- and prolactin-staining PAs following large-scale copy number variation (Pack *et al.*, 2000). Hyperprolactinaemia also frequently coexists with the more clinically apparent GH excess of X-LAG and McCune-Albright syndrome. Finally, prolactinomas have also been observed in association with mutations in *CDH23* (Zhang *et al.*, 2017) and *MAX* (Roszko *et al.*, 2017; Kobza *et al.*, 2018). The defining pituitary lesion in patients with *DICER1* mutations is pituitary blastoma, which is a tumour with an embryonic appearance often resulting in CD due to ACTH excess (de Kock *et al.*, 2014). A single case of a *DICER1* mutation in association with a prolactinoma has been reported (Cotton & Ray, 2018); however, no molecular details were provided, and the tumour was a microprolactinoma in a reproductive age female, suggesting that this case may represent a phenocopy (phenotype mimicking a genetic condition) rather than a true genetic association.

Inactivating mutations in the *PRLR* gene encoding the prolactin receptor have been described in a familial syndrome of hyperprolactinaemia, oligomenorrhoea, infertility and galactorrhoea, without pituitary changes on imaging (Newey *et al.*, 2013a). The original

description of this syndrome was questioned by multiple experts. Firstly, hyperprolactinaemia-associated central hypogonadism and galactorrhoea suggest intact prolactin signalling in the hypothalamus and in breast tissue, respectively (Grossmann, 2014; Harris, 2014; Molitch, 2014). Secondly, hyperprolactinaemia as a consequence of a defect in the prolactin receptor suggests a negative feedback loop; however, evidence of this is restricted to rodent studies demonstrating short loop negative feedback involving hypothalamic neurons expressing the prolactin receptor (Molitch, 2014). Most recently, activating *PRLR* mutations have been detected in the germline DNA of patients with sporadic prolactinomas, with apparent enrichment in males and DA-resistant cases (Gorvin *et al.*, 2019). The paradoxes of *PRLR* inactivation causing both decreased and increased prolactin signalling and both loss- and gain-of-function *PRLR* mutations resulting in hyperprolactinaemia may be explained by tissue-specific differences in mutant/non-mutant prolactin receptor dimerisation, a putative second prolactin receptor independent of the *PRLR* gene, and age- and sex-related differences in prolactin regulation. Further mechanistic studies are required to explore these possibilities.

1.4.2 Somatic genetic changes in sporadic prolactinomas

Candidate gene studies of sporadic prolactinomas have shown nil or only rare somatic variants in the known pituitary tumorigenesis genes. This includes genes implicated in FPTS, such as *MEN1* (Poncin *et al.*, 1999) and *AIP* (Raitila *et al.*, 2007), and genes that are somatically mutated in other PA subtypes (Tordjman *et al.*, 1993; Reincke *et al.*, 2015; Chen *et al.*, 2018). In contrast to the germline gain-of-function *PRLR* variants described in patients with sporadic prolactinomas, somatic *PRLR* variants have not been found in the tumour DNA of prolactinomas (Gorvin *et al.*, 2019).

Broader genomic studies of prolactinoma specimens have also been unrevealing. This may relate to methodological limitations and the small, heterogeneous prolactinoma cohorts in the extant literature. Only three pangenomic studies of prolactinomas have been performed to date (Wang *et al.*, 2014; Song *et al.*, 2016; Bi *et al.*, 2017b), all employing whole exome sequencing (WES). One study focussed only on genetic variants in relation to bromocriptine resistance and did not describe whether any of the variants of interest were present in more than one tumour (Wang *et al.*, 2014). The other two studies analysed PAs of various subtypes and no variants of interest occurred recurrently in prolactinomas (Song *et al.*, 2016; Bi *et al.*, 2017b). These studies raised the possibility of copy number variants (CNV) playing a role in the development of PAs, but this finding was limited by the heterogeneous mix of PA subtypes in these cohorts. Chapter 7 reports the genomic results of a pure prolactinoma cohort with investigation of both point mutations and CNVs.

The poor understanding of the somatic molecular basis of prolactinomas is in contrast to other pituitary tumours. Consistent somatic associations include *GNAS* mutations in somatotrophinomas and less commonly NFPAs (Tordjman *et al.*, 1993; Song *et al.*, 2016; Bi *et al.*, 2017a), and *CTNNB1* (encoding β -catenin) and *BRAF* mutations in adamantinomatous and papillary craniopharyngiomas, respectively (Brastianos *et al.*, 2014).

Corticotrophinomas have most recently gained attention because of newly found associations with somatic mutations in: *USP8* causing constitutive epidermal growth factor receptor (EGFR) signalling (Reincke *et al.*, 2015; Song *et al.*, 2016); *NR3C1* encoding the glucocorticoid receptor and causing loss of glucocorticoid negative feedback (Wells *et al.*, 2015; Song *et al.*, 2016); and *USP48* and *BRAF*, both enhancing promoter activity and hence transcription of the *POMC* gene encoding proopiomelanocortin (POMC) (Chen *et al.*, 2018). A notable omission from previous genomic studies of corticotrophinomas is cyclical CD.

Multiple mechanisms of cyclical hypercortisolism have been proposed, including: episodic haemorrhage; fluctuating proliferation and death of tumour cells; persistence of negative feedback; and, in cyclical CD only, altered hypothalamic control of the pituitary, via dopaminergic fluctuations for example (Meinardi *et al.*, 2007). However, the molecular basis of cyclical CS neither is known, nor has it been studied, apart from the finding of *PRKARA1A* mutations in a subset of adrenal Cushing's related to PPNAD (Powell *et al.*, 2008). Given the inherent cyclical nature of the disorder (Meinardi *et al.*, 2007) and the strong temporal patterns of the hypothalamic-pituitary-adrenal (HPA) axis in normal and diseased states (Moreira *et al.*, 2018), we performed WES in a case of cyclical CD as described in Chapter 8 to explore possible mutations in genes relating to timekeeping.

1.4.3 Next generation sequencing in endocrinology

The shared history of endocrinology and genetics is exemplified by insulin being the first protein to have its peptide sequence fully deduced (Sanger & Tuppy, 1951a, 1951b; Sanger & Thompson, 1953a, 1953b) – a feat that earned Frederick Sanger his first Nobel Prize in Chemistry in 1958. The contemporary role of genetic testing in endocrinology is discussed in detail in Appendix 3. Next generation sequencing (NGS) has revolutionised the approach to the genetic diagnosis of endocrinopathies. NGS involves DNA/RNA fragmentation and amplification of these DNA/RNA fragments into thousands of copies that can be sequenced simultaneously. NGS enables parallel sequencing of multiple genes resulting in high throughput data and, most valuably, allows hypothesis-free testing. NGS has proven crucial to the study of disorders with genetic heterogeneity such as disorders of sexual development (Appendix 4) and monogenic diabetes (Appendix 5). It has also been instrumental in the discovery of the role of hitherto unknown genes in various endocrinopathies. For example, the genetic cause of familial hyperaldosteronism type 2

(FH2) was unknown until the discovery of novel activating *CLCN2* mutations in 2018 through the use of WES (Scholl *et al.*, 2018). Prior to this, in 2017, we reported exome sequencing results in four FH2 kindreds (Appendix 6); however, our results were negative as we employed a bioinformatic pipeline based on biologically plausible genes rather than performing a true hypothesis-free genetic analysis. NGS has also been integral to the discovery of several pituitary tumorigenesis genes, including *USP8* in corticotrophinomas (Reincke *et al.*, 2015) and *BRAF* and *CTNNB1* in craniopharyngiomas (Brastianos *et al.*, 2014).

At the clinical level, NGS facilitates batch genetic testing of mixed patient cohorts to encompass various genes matched to various phenotypes, such as mixed PA subtypes (Appendix 2). This has led to NGS being adopted in routine clinical practice, including whole genome sequencing (WGS) and WES, which provides information at the DNA level, and RNA-Seq, providing information at the RNA level. Whereas WES targets exons and exon-intron boundaries, WGS includes coding DNA regions as well as intronic, promoter and intergenic regions.

1.5 Thesis aims

The overarching aim of this thesis was to perform clinical and molecular studies based on challenging cases arising in clinical practice in order to improve the medical care of future patients with hyperprolactinaemia and related endocrinopathies.

Chapters 2-5 (De Sousa *et al.*, 2017a; De Sousa *et al.*, 2017c; De Sousa *et al.*, 2019a; De Sousa *et al.*, 2020a) report clinical studies evaluating newly or recently recognised pitfalls in the assessment and management of hyperprolactinaemia. Given the rarity of the individual disorders, this research involved multicentre and interstate collaborations, patient database recruitment and retrospective data to accumulate sufficient evidence to address the study hypotheses.

Chapters 6-9 (De Sousa *et al.*, 2017d; De Sousa *et al.*, 2019b; De Sousa *et al.*, 2020b) report mechanistic studies looking into the molecular basis of hyperprolactinaemia and related endocrine tumours, with the long-term aims of optimising diagnosis and potentially finding new treatment targets. Gene sequencing was a key methodology in these studies. NGS was carried out in the form of WES in Chapters 7-9 and in the form of RNA-Seq in Chapter 9. Recognising the hitherto poor understanding of the molecular basis of hyperprolactinaemia, these pangenomic investigations allowed us to extend beyond biologically plausible genes. Traditional methods were also employed, including reverse transcriptase-polymerase chain reaction (RT-PCR) in Chapter 6 and Sanger sequencing confirmation in Chapter 9.

Chapter 2: Serum prolactin overestimation and risk of misdiagnosis

2.1 Introduction

The first step in assessing the hyperprolactinaemic patient is assessing the degree of hyperprolactinaemia. The magnitude of prolactin elevation guides the differential diagnosis of hyperprolactinaemia and typically parallels tumour diameter in prolactinomas. Severe hyperprolactinaemia (e.g., >10-fold normal) is almost always due to macroprolactinomas (Biller *et al.*, 1999; Casanueva *et al.*, 2006; Karavitaki *et al.*, 2006; Vilar *et al.*, 2008; Melmed *et al.*, 2011) or pregnancy/lactation (Hu *et al.*, 2018). Causes of mild hyperprolactinaemia (e.g., <four- to six-fold normal) include microprolactinomas, dopamine interference (e.g., infundibular compression or transection, antipsychotics, metoclopramide), primary hypothyroidism, polycystic ovary syndrome, and prolactin co-secretion in acromegaly or CD. Mild, transient increases in prolactin may follow stress, pain, coitus, exercise, sleep, meals or seizures (Casanueva *et al.*, 2006; Melmed *et al.*, 2011; Vilar *et al.*, 2014).

Following clinical observation of prolactin interassay discordance in local practice, we conducted a clinical audit of patients presenting with hyperprolactinaemia and a laboratory audit of split serum samples where prolactin was measured on both the Roche and Siemens platforms.

2.2 Methods

We performed a clinical audit of consecutive patients with serum prolactin discordance between the Roche and the Siemens platforms. The Siemens Centaur® platform and either the Roche Cobas® or Roche Modular E170® platforms were employed in each case. PEG precipitation was performed to assess for macroprolactinaemia. Based on our clinical

observations, we then measured serum prolactin by the Siemens Centaur® and Roche Cobas® platforms using split laboratory samples.

2.3 Results

The clinical audit consisted of 18 patients (12 women, 6 men, age 26-79 yr, mean 51 yr). Prolactin levels as measured by the Roche and Siemens platforms in the patients of the clinical audit are shown in Table 2.1. Macroprolactinaemia was excluded by PEG precipitation in 6/18 patients. PEG precipitation was not performed in the remaining 12 patients as prolactin was normal or near-normal on repeat testing on the Siemens platform (8 patients) or macroprolactinaemia had previously been excluded (4 patients).

Table 2.1. Serum prolactin interassay discordance in clinical practice

| Pt | Cause of hyperPRL | Roche absolute level | Roche ULN | Roche normalised level | Siemens absolute level | Siemens ULN | Siemens normalised level | % Roche increment, absolute ^a | % Roche increment, normalised ^b |
|----|---|----------------------|-----------|------------------------|------------------------|-------------|--------------------------|--|--|
| 1 | PRLoma | 25,233 | 500 | 50.5 | 20836 | 375 | 55.6 | 21% | -9% |
| 2 | PRLoma | 2,000 | 400 | 5.0 | 1588 | 375 | 4.2 | 26% | 18% |
| 3 | Other pituitary mass | 3,341 | 630 | 5.3 | 2431 | 620 | 3.9 | 37% | 35% |
| 4 | NFPA | 701 | 500 | 1.4 | 489 | 375 | 1.3 | 43% | 8% |
| 5 | Normal or transient idiopathic hyperPRL | 222 | 630 | 0.4 | 148 | 620 | 0.2 | 50% | 48% |
| 6 | PRLoma | 13,051 | 400 | 32.6 | 8650 | 375 | 23.1 | 51% | 41% |
| 7 | PRLoma | 2,475 | 400 | 6.2 | 1632 | 375 | 4.4 | 52% | 42% |
| 8 | PRLoma | 5,065 | 500 | 10.1 | 3258 | 619 | 5.3 | 55% | 92% |
| 9 | PRLoma | 18,852 | 500 | 37.7 | 12109.1 | 620 | 19.5 | 56% | 93% |
| 10 | Idiopathic hyperPRL or escitalopram | 3,466 | 500 | 6.9 | 2060 | 619 | 3.3 | 68% | 108% |
| 11 | Idiopathic hyperPRL | 1,344 | 500 | 2.7 | 759 | 620 | 1.2 | 77% | 120% |
| 12 | Normal | 780 | 500 | 1.6 | 437 | 619 | 0.7 | 78% | 121% |
| 13 | Idiopathic hyperPRL | 939 | 500 | 1.9 | 434 | 620 | 0.7 | 116% | 168% |
| 14 | Flupentixol | 3,378 | 500 | 6.8 | 1538 | 619 | 2.5 | 120% | 172% |
| 15 | Normal | 598 | 500 | 1.2 | 225 | 375 | 0.6 | 166% | 99% |
| 16 | NFPA | 1,037 | 500 | 2.1 | 328 | 619 | 0.5 | 216% | 291% |
| 17 | Normal or sertraline | 2,140 | 500 | 4.3 | 143 | 619 | 0.2 | 1397% | 1753% |
| 18 | Normal or metoclopramide | 3,895 | 500 | 7.8 | 139 | 620 | 0.2 | 2702% | 3375% |

HyperPRL, hyperprolactinaemia; NFPA, non-functioning pituitary adenoma; PRLoma, prolactinoma; Pt, patient number in ascending order of absolute Roche increment; ULN, upper limit of normal; %, percentage increase comparing Roche against Siemens; ^acalculated as (Roche absolute level – Siemens absolute level)/Siemens absolute level; ^bcalculated as (Roche normalised level – Siemens normalised level)/Siemens normalised level

Clinical confounders were absent in all but three patients. Patient 1 commenced cabergoline after the Roche measurement and possibly one day prior to the Siemens measurement. Patient 17 ceased low-dose sertraline in the interval between testing on the Roche and Siemens platforms. Patient 18 took 20 mg metoclopramide the day prior to both the Roche and Siemens measurements, but cumulative metoclopramide use may have differed in the preceding weeks.

In the 15 patients with no clinical confounders, absolute prolactin level by Roche compared to Siemens was 81% higher (range 26-216%) and normalised prolactin level (absolute level/upper limit of normal) was 97% higher (range 8-291%). The normalised prolactin increment by Roche was more pronounced in women (Roche 125% higher) than men (Roche 42% higher) and in patients with prolactinomas (Roche 117% higher) than patients with no final diagnosis of prolactinoma (Roche 57% higher).

The interassay discordance was often clinically significant. For example, baseline prolactin by Roche was 10-fold normal in Patient 8, suggesting a macroprolactinoma, whereas her Siemens result of five-fold normal was more consistent with the 7 mm pituitary tumour subsequently detected on MRI. If this patient had a macroadenoma, the mixed findings of mild and severe hyperprolactinaemia would have made it difficult to distinguish between macroprolactinoma and NFPA with stalk effect hyperprolactinaemia. In another patient with schizophrenia, hyperprolactinaemia at seven-fold normal by Roche prompted investigation for a concomitant prolactinoma. MRI showed a normal pituitary gland and repeat prolactin by Siemens was only 2.5-fold elevated, in keeping with known antipsychotic use. Overall, 7/18 patients had unnecessary endocrine reviews and/or MRI, with incidental findings in 3/6 MRI reports.

We next measured serum prolactin by the Siemens and Roche platforms using split laboratory samples ($n=40$) across a range of serum prolactin (5-5051 mIU/L). Passing & Bablok regression returned an intercept of 10.31 and a gradient of 1.52 (95% CI 1.46-1.60), representing a consistent increase in serum prolactin of approximately 50% by Roche compared to Siemens (Figure 2.1). Reference intervals for the two assays were similar. Our review of the original Roche data revealed no technical error in reference interval calculation.

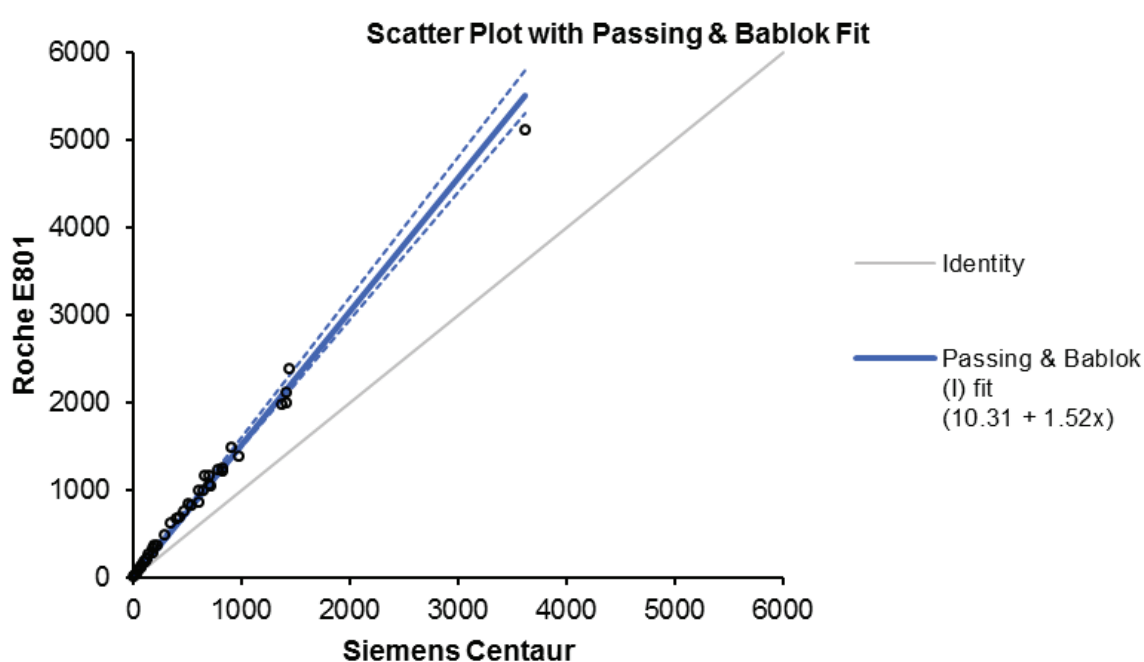


Figure 2.1. Comparative performance of prolactin by Roche Cobas vs. Siemens Centaur

Passing & Bablok fit is shown for the 40 split laboratory samples

2.4 Discussion

Our clinical audit of 18 patients and assay comparison of 40 split samples showed that serum prolactin is consistently overestimated by Roche compared to Siemens, in both absolute values (mIU/L) and in relative values (i.e., compared to the upper limit of normal). This is relevant to laboratories and to clinicians that typically measure prolactin to investigate

menstrual disturbances in women, low testosterone in men, infertility or pituitary masses. The potential diagnostic and therapeutic implications of prolactin overestimation are outlined in Table 2.2. It is worth also noting the costs of further investigation due to misleadingly high serum prolactin levels, including, but not limited to, pituitary MRI scans costing approximately AUD 600.

Table 2.2. Potential implications of serum prolactin overestimation

| True result | Overestimated result | True diagnosis | False diagnosis | Potential implications |
|---------------|----------------------|---|--|---|
| Normal PRL | Mild hyperPRL | Normal | MicroPRLoma or other pituitary mass with stalk effect hyperPRL | Unnecessary pituitary MRI Unnecessary endocrine review Incidental findings Unnecessary DA therapy with risk of side effects |
| | | Adequately controlled PRLoma on DA therapy | DA resistance or escape | Unnecessary increase in DA dose with increased risk of side effects Unnecessary referral for surgery/radiotherapy |
| | | Other cause of infertility or menstrual disturbance | Occult microPRLoma | Unnecessary pituitary MRI Incidental findings Unnecessary/ineffective DA therapy Inappropriate deferral of investigations for other reproductive pathology |
| Mild hyperPRL | Severe hyperPRL | Drug-induced hyperPRL | PRLoma or other pituitary mass with stalk effect hyperPRL | Unnecessary pituitary MRI Unnecessary endocrine review Incidental findings |
| | | Pituitary mass with stalk effect hyperPRL | MacroPRLoma | Unnecessary/ineffective DA therapy Inappropriate delay in surgical intervention |

DA, dopamine agonist; hyperPRL, hyperprolactinaemia; macroPRLoma, macroprolactinoma; microPRLoma, microprolactinoma; MRI, magnetic resonance imaging; PRL, prolactin; PRLoma, prolactinoma

The cause of prolactin overestimation by the Roche assay is unclear. We excluded errors in reference interval calculation; however, progressive positive bias with successive reagent lot numbers and antibody variability over time remain possible. Several other factors could contribute to interassay discordance. When tested on different days, the commencement or cessation of drugs that interrupt the tonic inhibition of prolactin secretion by dopamine could respectively lead to higher or lower prolactin levels on the second test. Heterophile

antibodies with varying assay interactions are also possible. The latter was suspected in two patients in the clinical audit who had markedly higher serum prolactin on the Roche vs. Siemens assays with absolute increases of 1397% in Patient 17 and 2702% in Patient 18. However, both patients were intermittently taking dopamine interfering medications and were thus excluded from the final analysis. We also found no consistent relationship between the prolactin increment by Roche and age, between test interval, and whether the Roche or Siemens test was performed earlier in the day (data not shown). Transient stimuli of prolactin secretion (e.g., stress, coitus) cannot be excluded, but the consistency of higher Roche prolactin levels in all 58 cases, including split samples, argues against this.

Whether prolactin is overestimated by the Roche platform or underestimated by the Siemens platform could not be distinguished in the 40 split samples of the assay comparison. In the clinical audit, it was possible to deduce the likely true prolactin result in 7/15 cases, all of which favoured the Siemens prolactin result being correct. For example, a perimenopausal patient had a robust gonadotrophin response which was consistent with her normal serum prolactin by Siemens as opposed to her two-fold elevation in prolactin by Roche. Another two patients were diagnosed with drug-induced hyperprolactinaemia where serum prolactin is typically two- to three-fold elevated as found by the Siemens platform in these patients, rather than six- to seven-fold elevated as found by the Roche platform. Two women only had slight menstrual irregularity and normal pituitary MRI studies that favoured their serum prolactin values near the upper limit of normal by Siemens compared to two- to three-fold elevations by Roche. The last two patients were being serially followed after surgery for a prolactinoma in one patient and cessation of prolactinoma DA treatment in the other patient who had developed disruptive hypersexuality on treatment. The two patients both had gradually increasing serum prolactin levels on the Siemens assay as expected due to their known tumour remnants, but their latest prolactin result by Roche caused sharp

inflections in their trajectories. This was discordant with clinical findings in both cases as the tumour remnants were stable on serial imaging and cabergoline had been restarted in the postoperative patient in the lead up to the latest test on the Roche platform. Overall, these informative cases indicated serum prolactin overestimation by Roche.

Our findings of prolactin interassay discordance may be overcome by a higher Roche reference interval as prolactin should be interpreted relative to the upper limit of normal rather than as an absolute value. Determining new reference intervals will require large numbers of healthy controls and patients with varying degrees of hyperprolactinaemia. In the meantime, clinicians should be aware of the potential for prolactin overestimation and the utility of repeat testing on different platforms. In the case of mild hyperprolactinaemia by the Roche platform and normoprolactinaemia by other platforms, patients may be spared from unnecessary endocrine reviews and MRI studies. In true hyperprolactinaemia, separating patients with mild vs. severe hyperprolactinaemia will narrow the diagnostic possibilities.

2.5 Conclusion

Serum prolactin is overestimated on the Roche platform compared to the Siemens platform. Laboratories should review Roche reference intervals for serum prolactin, and clinicians should consider repeating serum prolactin on another platform if the serum prolactin is incongruent with the clinical scenario. Given this potential pitfall in prolactin measurement, prolactin levels in both clinical practice and research should be considered in reference to the upper limit of normal by a given assay.

Chapter 3: Dopa-testotoxicosis: disruptive hypersexuality in hypogonadal men with prolactinomas treated with dopamine agonists

3.1 Introduction

True, symptomatic hyperprolactinaemia is typically treated with DAs such as cabergoline and quinagolide. DA therapy is generally considered to be highly effective and safe. However, it has been recently recognised that patients on DAs may develop ICDs such as gambling, hypersexuality, compulsive shopping and binge eating. The risk of DA-induced ICDs is well recognised by neurologists who use DAs in the treatment of Parkinson's disease and restless legs syndrome (Moore *et al.*, 2014), but the nature of this risk is unclear in the endocrine setting.

Herein we report eight men who developed profound hypersexuality in the setting of DA therapy for prolactinomas and central hypogonadism. We propose synergy between DA therapy and restoration of the eugonadal state, encapsulated by the suggested term 'dopa-testotoxicosis'. Whilst hypersexuality may certainly occur with DA therapy in the absence of pre-existing hypogonadism (Moore *et al.*, 2014), we propose that the severity, frequency and male predominance of hypersexuality in the prolactinoma setting specifically relates to the compounding effect of testosterone normalisation with DA therapy, often following years of hypoandrogenism prior to treatment. This may explain, in part, the anecdotally higher risk of dopamine agonist-induced hypersexuality in men.

3.2 Methods

We conducted a case series describing the clinical experience of dopa-testotoxicosis among five endocrinologists at two centres. Each case of dopa-testotoxicosis was encountered in regular clinical practice with hypersexuality identified during routine follow-up

appointments. All data were collected retrospectively. A waiver of informed consent was approved by the Royal Adelaide Hospital Human Research Ethics Committee in accordance with the National Health and Medical Research Council guidelines.

3.3 Results

We observed eight patients with hypersexuality during DA therapy, all of whom were male. Clinical data are summarised in Table 3.1. All patients had presented with prolactinomas and central hypogonadism with no past history of psychiatric disease. All but one man (Patient 5) were post-pubertal. Both microprolactinomas and macroprolactinomas were found and serum prolactin ranged from four- to 76-fold normal. Two young men (Patients 4 and 5) notably had normal pre-treatment testosterone levels. However, their testosterone values rose from the lower half of the reference range at baseline to the upper half with DA therapy, suggesting these patients had relative hypogonadism prior to treatment. The other men had frankly low testosterone levels prior to DA therapy. Six men received no androgen replacement and subsequent increases in testosterone were solely attributable to DA commencement. DA therapy was commenced between the third and eighth decades of life after a period of low libido of at least 1 year. Tumour shrinkage was frequent but not invariable. In one man (Patient 2), the prolactinoma was ectopically situated in the sphenoid sinus.

Table 3.1. Local data of eight men with dopa-testotoxicosis

| Pt | Age at Dx (decade) | Pubertal status | Tumour size (mm) | PRL at Dx* (mIU/L) | Testo at Dx* (nmol/L) | Duration of low libido (yr) | DA | Time to HS after starting DA (months) | PRL at HS onset* (mIU/L) | Testo at HS onset* (nmol/L) | Tumour shrinkage | Features of ICD | Social implications | Outcome |
|----|--------------------|-----------------|------------------|--------------------|-----------------------|-----------------------------|-----------------------|---------------------------------------|--------------------------|-----------------------------|------------------|---|---|---|
| 1 | 5th | Post | 14 | 15,316 | 4.0 | Constant | CBG 0.5-1 mg wkly | 24 | 327 | 13 | Yes | New onset libido, visited sex workers who introduced patient to amphetamines | Family discord, admissions for drug detoxification | HS and illicit drug use resolved with CBG reduction, eugonadal after tumour resection and CBG cessation with no HS |
| 2 | 8th | Post | 32 | 22,196 | 0.7 | 10 | CBG 0.5 mg twice wkly | 6 | 149 | 9.7 | No | ↓ sexual frequency unwelcomed by partner, sexual advances made towards female friend | Discord in patient's marriage and friend's marriage | HS resolved with recurrence of depression, reduced energy and low libido when CBG ceased, no HS when switched to testosterone alone |
| 3 | 4th | Post | >10 | 28,662 | 6.3 | >15 | CBG 0.5 mg wkly | <1 | 233 | 16.6 | Yes | ↑ sexual frequency to four times daily, partner suspected extramarital affairs, pathological gambling | Marital discord requiring marriage counselling | HS resolved with recurrence of low libido after 8 years despite ongoing CBG and normal PRL/Testo, renewed marital discord due to low libido |

| | | | | | | | | | | | | | | |
|---|-----|------------|-------------|-------|-----|----------|--|----|--------|------|-----|---|--|---|
| 4 | 3rd | Post | Not visible | 1,526 | 15 | >1 | BRC 1.25-2.5 mg twice daily, then QGL 75 mcg daily | 18 | 270 | 21 | N/A | Spent hours on internet dating and poker sites nightly | Lost >\$10,000 over 3 months | Stopped gambling and reduced internet dating frequency when QGL halved |
| 5 | 4th | Incomplete | 4 | 4,728 | 9.7 | Constant | CBG 0.25 mg twice wkly | 3 | Normal | 26.9 | Yes | New onset libido, high risk homosexual activity, engaged in theft and amphetamine/marijuana use through sexual encounters | Family discord, estranged from parents, imprisoned for theft/arson | All high-risk behaviour reduced when CBG reduced, resolved after a few years despite ongoing CBG and normal PRL/Testo |
| 6 | 6th | Post | 8 | 3,600 | 1.1 | >5 | CBG 0.5 mg wkly, then BRC 0.625 mg wkly then 0.625 mg twice wkly | <1 | 494 | 7.0 | No | Incessant sexual thoughts interfering with work, ↑ sexual frequency to multiple times daily | Interference with relationship and work | HS resolved when DA ceased despite ongoing testosterone replacement, eugonadal after tumour resection with no HS |
| 7 | 7th | Post | 12 | 2,991 | 4.9 | >1 | CBG 0.5 mg twice wkly | <1 | 31 | 12 | No | ↑ sexual frequency unwelcomed by partner | Reduced hours of work, distress in partner | HS resolved 2 wk after CBG ceased, no HS when switched to testosterone alone |
| 8 | 4th | Post | 15 | 6,425 | 3.8 | 1-2 | CBG 0.5 mg twice wkly | 6 | 78 | 11 | Yes | ↑ sexual frequency unwelcomed by partner, engagement in prostitution | Distress in partner | Unable to reduce DA as patient not distressed by HS |

BRC, bromocriptine; CBG, cabergoline; DA, dopamine agonist; Dx, diagnosis of prolactinoma; HS, hypersexuality; ICD, impulse control disorder; N/A, not applicable; PRL, prolactin; Pt, patient; QGL, quinagolide; Testo, testosterone; wk, week; wkly, weekly; * male reference intervals: PRL 45-375 mIU/L; Testo 8.0-30.0 nmol/L

Each man developed hypersexuality with onset ranging from days to years after commencing DA therapy. Prolactin and testosterone consistently improved to be very close or within the reference range by the time of symptom onset. Cabergoline, bromocriptine and quinagolide were all implicated at low to standard doses for prolactinoma therapy. The social consequences of hypersexuality included family and marital discord, major financial losses from online dating, reduced work performance, and illicit activity prompted by high-risk sexual behaviour. Frequent sexual thoughts were of concern to the patient, partners and/or family members in all cases. Two men (Patients 3 and 4) also developed pathological gambling with social and financial ramifications.

Hypersexuality resolved in three men after DA cessation (Patients 2, 6 and 7) and in two men after DA dose reduction (Patients 1 and 5). Hypersexuality did not recur in the four men who achieved normal testosterone levels off DA therapy, whether it was achieved by androgen replacement (Patients 2 and 7) or after pituitary tumour resection (Patient 1 and 6). Spontaneous resolution of hypersexuality was noted after 8 years in one man (Patient 3) despite ongoing treatment with cabergoline, and after a few years in another man (Patient 5) who had earlier experienced some reduction in his high-risk behaviour when cabergoline was reduced. Another man (Patient 4) noted some reduction in his hypersexual behaviour when quinagolide was halved. The remaining patient (Patient 8) was not agreeable to reducing his DA therapy as he was not distressed by his hypersexuality, although he engaged in sexual activity with prostitutes and his behaviour distressed his partner.

3.4 Discussion

The risk of ICDs with DA therapy is less recognised in endocrinology than in neurology settings. In a study of DA-associated ICDs reported to the United States Food and Drug Authority (FDA), 62% of events occurred in the treatment of Parkinson's disease and 24% in

restless legs syndrome, whereas only 3.5% of events occurred in individuals treated for hyperprolactinaemia (Moore *et al.*, 2014). This is in part due to the preferential affinity for the mesolimbic D3 receptors exhibited by the DA agents, pramipexole and ropinirole, that are commonly used in neurology settings. Moreover, the treatment of Parkinson's disease and restless legs syndrome requires higher DA doses than in prolactinoma therapy and a dose effect is plausible. Notwithstanding, we believe DA-associated ICDs may present differently in the prolactinoma setting, and that endocrinologists may be less aware, resulting in underreporting of this toxicity.

It is likely that restoration of eugonadism, in absolute or relative terms, reflecting a state of relative testosterone excess, contributed to the hypersexuality observed in our study. This was beyond what would be predicted by the use of bromocriptine, cabergoline and quinagolide which lack D3 receptor specificity (Moore *et al.*, 2014). It is notable that overt hypogonadism at baseline was not necessary for the development of dopa-testotoxicosis, as two men (Patients 4 and 5) developed hypersexuality despite normal baseline testosterone levels. However, in both cases, testosterone significantly increased from the lower half to the upper half of the reference range with DA initiation, suggesting that they too likely experienced a state of relative testosterone excess which precipitated their symptoms. In addition, testosterone is pulsatile and circadian (Spratt *et al.*, 1988), and our measurements would not represent the full signature of normal testosterone secretion, such as nocturnal pulses.

DA therapy was undoubtedly contributory as men with hypogonadism treated by other means do not appear to be at risk of hypersexuality if serum testosterone is maintained within the normal range. Furthermore, in four of our patients, hypersexuality resolved upon DA cessation and did not recur with restoration of normal testosterone levels through

curative prolactinoma resection or androgen replacement. This observation reinforces the notion of synergy between dopamine agonism and rising testosterone levels in production of the hypersexuality effect. DAs may indeed have an independent effect on sexual behaviour as hypersexuality accounted for 29% of ICDs in the aforementioned FDA study which mostly included neurology patients who were presumably eugonadal (Moore *et al.*, 2014). Furthermore, a longitudinal study of 51 men with prolactinomas treated with cabergoline for 6 months found that nocturnal penile tumescence improved with prolactin normalisation even when testosterone was still low (De Rosa *et al.*, 2004). We hypothesise that the reward-seeking behaviour induced by DAs, together with restoration of eugonadism following longstanding hypogonadism, resulted in the disruptive hypersexuality described here, hence the term 'dopa-testotoxicosis'.

Bancos *et al.* (2014) recently found a trend towards higher rates of ICDs amongst DA-treated prolactinoma patients compared to controls with NFPA not treated with DAs. Though this difference was not statistically significant overall, there were striking gender differences such that DA-treated men were 9.9-fold more likely to develop an ICD compared to their NFPA counterparts. Unlike the neurology setting where pathological gambling is the commonest ICD as shown by the aforementioned FDA study (Moore *et al.*, 2014), the most frequent ICD in the study by Bancos *et al.* (2014) was hypersexuality. Moreover, hypersexuality was the only ICD to be significantly increased when DA-treated patients and DA-naïve controls were compared irrespective of gender (12.99% vs. 2.86%, $P=0.03$). The authors suggested that supraphysiological testosterone levels were unlikely to have contributed to this phenomenon as only four patients with an ICD in the DA group were on androgen replacement and serum testosterone was not elevated in any of the men with hypersexuality (Bancos *et al.*, 2014). We similarly found that none of the men in our cohort had testosterone levels above the upper limit of normal; however, in contrast to Bancos *et*

al., we argue that eugonadism may reflect relative testosterone excess in these men who are often hypogonadal for years prior to their diagnosis. Rising testosterone levels may account for the overrepresentation of hypersexuality in DA-treated prolactinoma patients compared to patients with restless legs syndrome and Parkinson's disease when comparing the findings of Bancos *et al.* (2014) and the FDA study (Moore *et al.*, 2014).

Another study looking at 17 men and 62 women treated with cabergoline for prolactinomas found that 25% of patients reported increased libido while on DA therapy; however, it was not stated whether this was disruptive to the personal lives of the patients (Athanasoulia *et al.*, 2012b). There is evidence of a possible dose effect, with increased impulsivity observed with increasing weekly cabergoline doses in a study of hyperprolactinaemic patients, although dose-dependence was only demonstrated in one of nine impulsivity scores in this study (Barake *et al.*, 2014). We have demonstrated that hypersexuality may occur even at standard doses as the men on cabergoline in our case series had an average weekly dose of 0.75 mg. Furthermore, one of our patients experienced disruptive hypersexuality on as little as 0.625 mg of bromocriptine weekly rather than the usual daily dosing for this agent. This man was also administered transdermal testosterone, but hypersexuality was only present when he was on a DA, regardless of the type, and remitted when DAs were intermittently ceased despite ongoing androgen replacement. In each of our patients, the testosterone level closest to the onset of hypersexuality was higher than the baseline level and DA dose reduction would expectedly cause a parallel reduction in testosterone levels, therefore we cannot distinguish the relative contributions of DA therapy and testosterone levels. It is possible that in some men the DA effect is more important and in others the testosterone effect is more relevant; individual factors are likely to influence the relative importance of these effects.

There are previous case reports of disruptive or pathological hypersexuality developing in male prolactinoma patients on DAs (Falhammar & Yarker, 2009; Gupta & Zimmerman, 2011; Martinkova *et al.*, 2011; Nannenga *et al.*, 2011; Bancos *et al.*, 2014). Though the data are vague, these cases are suggestive of dopa-testotoxicosis and are summarised in Table 3.2. As in our study, disruptive hypersexuality has been documented across a range of ages from 14 to 66 years, in men with either micro- or macroprolactinomas, and with use of cabergoline or bromocriptine. There is a single case report of a woman with a prolactinoma who developed mania on quinagolide which included *“increased sexual thoughts”*; however, this was one of many reported symptoms and it did not predominate in the clinical picture (Vinkers & van der Wee, 2007). The persistence of hypersexuality in Patient 3 of our study even after switching to quinagolide therefore represents the first report of this phenomenon with quinagolide, thus extending the risk of hypersexuality to all DA agents used in the treatment of prolactinomas.

Table 3.2. Published reports of 12 men likely affected by dopa-testotoxicosis

| Age (yr) | Tumour size | Hypogonadal | Duration of low libido | DA | PRL normalised | Testo normalised | Tumour shrinkage | HS behaviour | Other ICD | Outcome | Ref |
|----------|-------------|-------------|------------------------|--|----------------|------------------|------------------|---|-----------|--|-----------------------------------|
| 66 | Macro | N/S | N/S | CBG 1 mg w/ky | N/S | N/S | N/S | Extreme HS behaviour, loss of job, divorce, promiscuity | N/S | N/S | (Bancos <i>et al.</i> , 2014) |
| 33 | Micro | N/S | N/S | CBG 0.5 mg w/ky | N/S | N/S | N/S | HS behaviour on survey, no details | N/S | N/S | (Bancos <i>et al.</i> , 2014) |
| 60 | Macro | Yes | N/S | CBG 1 mg w/ky | N/S | N/S | N/S | HS behaviour on survey, no details | N/S | HS improved after CBG reduced | (Bancos <i>et al.</i> , 2014) |
| 38 | Macro | N/S | N/S | BRC 52.5 mg w/ky | N/S | N/S | N/S | HS behaviour on survey, no details | N/S | HS improved after BRC reduced to 35 mg w/ky | (Bancos <i>et al.</i> , 2014) |
| 39 | Not visible | N/S | N/S | CBG, dose N/S | N/S | N/S | N/S | HS behaviour on survey, no details | N/S | HS resolved after pituitary surgery and CBG cessation | (Bancos <i>et al.</i> , 2014) |
| 47 | Micro | N/S | N/S | CBG 0.5 mg w/ky | N/S | N/S | N/S | HS behaviour on survey, not interfering with life | N/S | N/S | (Bancos <i>et al.</i> , 2014) |
| 66 | Micro | N/S | N/S | CBG 2 mg w/ky | N/S | N/S | N/S | HS behaviour on survey, no details | N/S | CBG reduced, response N/S | (Bancos <i>et al.</i> , 2014) |
| 53 | Macro | No | None | BRC 15 mg daily | Yes | N/A | Yes | Extramarital affair to satisfy desired frequency of sexual activity | No | HS resolved after BRC reduced to 3 mg daily | (Martinkova <i>et al.</i> , 2011) |
| 50 | Micro | Yes | 12 months | CBG 0.5 mg twice w/ky | Yes | Yes | No | Marital discord, divorce | Gambling | ICDs resolved 12 wk after stopping CBG | (Falhammer & Yarker, 2009) |
| 61 | Macro | N/S | N/S | BRC, dose N/S, then CBG 1 mg daily | Yes | N/S | Yes | Infidelity, marital discord | No | Ongoing HS with reduced CBG, HS resolved 6 wk after stopping | (Gupta & Zimmerman, 2011) |
| 51 | Macro | N/S | N/S | BRC 25 mg daily | N/S | N/S | N/S | NS | Gambling | ICDs resolved after stopping BRC | (Nannenga <i>et al.</i> , 2011) |
| 14 | Macro | N/S | N/S | BRC 22.5 mg daily, then CBG 2.25 mg w/ky | N/S | N/S | N/S | Promiscuous, volatile sexual relationships, two suicide attempts | No | HS resolved after stopping DAs | (Nannenga <i>et al.</i> , 2011) |

BRC, bromocriptine; CBG, cabergoline; HS, hypersexuality; ICD, impulse control disorder; Macro, macroadenoma; Micro, microadenoma; N/A, not applicable; N/S, not stated; PRL, prolactin; Ref, reference; wk, week; wkly, weekly

The hypersexuality experienced by patients in the literature has been unequivocally disruptive with multiple cases of threatened or completed marriage breakdowns (Falhammar & Yarker, 2009; Gupta & Zimmerman, 2011) and suicidal attempts in one case (Nannenga *et al.*, 2011). Together with the present study, these cases suggest that dopa-testotoxicosis should be considered in all men started on DAs for prolactinoma regardless of patient age, tumour size, DA agent or dose, or the presence or absence of other ICDs. It may also occur in the rare instance of ectopic prolactinoma as demonstrated in the present series. Vigilance should be heightened when patients reach normal prolactin and testosterone levels as the onset of hypersexuality appeared to coincide with biochemical normalisation in our patients, although there is little biochemical data in previously published cases. Other uncertainties in the cases described in the literature include whether patients had completed puberty, the duration of low libido, whether tumour shrinkage was achieved, and the co-existence of other ICDs. Improved awareness of the condition and further research is required to elucidate risk factors for dopa-testotoxicosis.

As testosterone contributes to libido in women, a similar syndrome of hypersexuality due to the interaction of DAs and a rise in testosterone following DA-induced restoration of gonadal function may exist. However, female patients treated with DAs for prolactinoma appear to be rarely affected by hypersexuality, which may reflect the greater surge in testosterone in hypogonadal men restored to the eugonadal state compared to hypogonadal women. Bancos *et al.* (2014) noted three female patients with hypersexuality in the setting of cabergoline or bromocriptine therapy of prolactinomas; however, the degree of their symptomatology was not described. There is one case report in the literature of a female prolactinoma patient with DA-associated hypersexuality (Sandyk *et al.*, 1987), although this occurred in the setting of overt psychosis in contrast to the otherwise intact mental state seen in the men in our study. We have not observed dopa-testotoxicosis in female patients

in our institutions and there are still no case reports of women with the degree of disruptive hypersexuality observed in the men in this case series. It remains to be seen whether women are at risk of dopa-testotoxicosis. Commercial assays generally lack sufficient sensitivity to discriminate normal from low testosterone levels in women, thus it may be especially challenging to ascertain the contribution of testosterone fluctuations in symptomatic women.

Whilst the predisposition to dopa-testotoxicosis may not be fully understood, it is clear that DA cessation is a highly effective strategy as hypersexuality promptly resolved in three men in our study and in case reports in the literature when DA therapy was stopped (Falhammar & Yarker, 2009; Gupta & Zimmerman, 2011; Nannenga *et al.*, 2011; Bancos *et al.*, 2014). Resolution was also achieved with reducing the DA dose in two of our patients and in some cases in the literature (Martinkova *et al.*, 2011; Bancos *et al.*, 2014), consistent with previous evidence of dose-dependent impulsivity (Barake *et al.*, 2014). However, resolution with dose reduction was not observed in other reported cases (Gupta & Zimmerman, 2011) and some men in our series experienced hypersexuality even on low-dose DA therapy. In Patient 3, hypersexuality ultimately resolved spontaneously after 8 years despite ongoing exposure to cabergoline with normal prolactin and testosterone levels. In Patient 5, hypersexuality resolved after a few years despite continuing on low-dose cabergoline with normal prolactin and testosterone levels. It is tempting to draw an analogy here with the physiology of puberty where the surge in libido during teenage years reaches a plateau in adulthood. Nonetheless, the hypersexuality experienced by these men was disruptive with long-term social consequences, arguing against a wait-and-see approach in dopa-testotoxicosis. This is particularly pertinent given the availability of transsphenoidal surgery as an acceptable alternative to medical therapy in the management of prolactinomas.

The option of DA cessation can only be considered when patients are forthcoming with symptoms, which may necessitate direct questioning by the treating clinician. The consequences of DA cessation include ongoing hyperprolactinaemia which appears to have no direct effect in of itself, tumour persistence or expansion, and ongoing hypogonadism with detrimental effects including osteopenia. For these reasons, some of the patients in our study were referred for pituitary surgery to eliminate mass effects by their tumours and/or commenced on low-dose androgen replacement primarily to maintain bone density. Theoretically, low-dose androgen replacement alone should circumvent the mid-range serum testosterone levels and reward pathway stimulation thought to underpin dopa-testotoxicosis. Consideration of pituitary surgery even when hormone normalisation and tumour shrinkage is achieved with DA therapy is contrary to usual practice and close communication with neurosurgeons may be required to highlight the rationale if this is considered.

Endocrinologists should be aware of the specific risk of dopa-testotoxicosis in male prolactinoma patients with hypogonadism treated with DAs, regardless of the particular agent selected, age of the patient or baseline hormone and tumour characteristics. Patients should be educated regarding this risk to allow for self-monitoring and open discussions with their partners. Given the personal nature of sexual behaviour, patients may still be reluctant to report hypersexuality to their treating clinicians and therefore patients should be specifically questioned about this complication on follow-up. Written questionnaires filled by patients prior to follow-up appointments may facilitate a gentle introduction to the issue during consultations. This may be especially helpful for patients and/or clinicians from cultures where discussing sexual behaviour is considered inappropriate.

Based on the clinical observations of this case series, we subsequently conducted a large cross-sectional analysis of DA-treated hyperprolactinaemic patients vs. controls in order to determine the prevalence of hypersexuality and other DA-induced ICDs and to evaluate proposed risk factors, such as male gender, that could hone patient surveillance. This study is described in Chapter 4.

3.5 Conclusion

DA-associated hypersexuality appears to be overrepresented amongst male patients with prolactinomas compared to patients with Parkinson's disease and restless legs syndrome. We hypothesise that this distinct drug toxicity is due to synergy between mesolimbic reward pathway stimulation by DAs and restoration of the eugonadal state induced by prolactin normalisation and tumour shrinkage. The next chapter explores the prevalence of DA-induced ICDs in hyperprolactinaemic patients compared to controls and the effect of male gender amongst other possible risk factors.

Chapter 4: Impulse control disorders in dopamine agonist-treated hyperprolactinaemia: prevalence and risk factors

4.1 Introduction

The recent recognition of DA-induced ICDs in hyperprolactinaemic patients follows on from the experience of neurologists who have for long observed the association of ICDs with DA treatment in Parkinson's disease and restless legs syndrome. In addition to our case series (De Sousa *et al.*, 2017a) described in Chapter 3, there are now several case reports (Falhammar & Yarker, 2009; Gahr *et al.*, 2011; Thondam *et al.*, 2013; Premaratne *et al.*, 2014; Bulwer *et al.*, 2017; Athanasoulia-Kaspar *et al.*, 2018) and emerging studies (Martinkova *et al.*, 2011; Bancos *et al.*, 2014; Celik *et al.*, 2018; Dogansen *et al.*, 2019) highlighting this risk.

There are five dopamine receptors (D1-5), encoded by separate genes; physiologically these receptors exhibit overlapping roles in locomotion, cognition, emotion, affect and neuroendocrine secretion. D1, D2 and D3 receptors all contribute to impulse control. The D2 receptor is most important in lactotrophs as an inhibitor of prolactin release. Dopamine receptors are also common in the periphery, notably in the vasculature where they may play a role in the postural hypotension seen with DA use and in the kidney (Missale *et al.*, 1998).

Functioning as both a neurotransmitter and a hormone, dopamine brings together the nervous and endocrine systems with various DA agents prescribed by neurologists and endocrinologists. Ropinirole and pramipexole are favoured in the treatment of Parkinson's disease and restless legs syndrome due to their activity at the D2 and D3 receptors, both expressed in the nigrostriatal dopaminergic pathway involved in motor function. The ergot-derived DAs, cabergoline and bromocriptine, and the non-ergot derived DA, quinagolide, are

used in the treatment of hyperprolactinaemia because of their relative selectivity for the D2 receptor, which predominates in the tuberoinfundibular dopaminergic pathway that tonically inhibits prolactin secretion. Dopamine receptors, predominantly D3, also exist in the mesocorticolimbic dopaminergic pathway of the reward system (Ioachimescu *et al.*, 2019). Off target activation of this reward system appears to be the mechanism of DA-induced ICDs in both neurology and endocrine patients.

The defining feature of ICDs is failure to resist impulses to engage in a pleasurable activity that is harmful to self or others (American Psychiatric Association, 2000). The latest version of the *Diagnostic and Statistical Manual of Mental Disorders (DSM-V)* includes the general group of ICDs within the 'disruptive, impulse-control, and conduct disorders', defined as difficulty in self-regulating emotions and behaviours, leading to distress or impaired function (American Psychiatric Association, 2013). However, *DSM-V* does not provide a uniform approach to diagnosing ICDs, with gambling disorder and binge-eating listed separately within 'substance-related and addictive disorders' and 'feeding and eating disorders', respectively (American Psychiatric Association, 2013). For complex reasons including concerns about inappropriately defining high sexual desire as a pathology (Reid & Kafka, 2014), hypersexuality was ultimately omitted from *DSM-V* despite prior validation of diagnostic criteria, including: excessive time consumed by sexual thoughts and behaviours; inability to control sexual thoughts and behaviours; use of sex to cope with unpleasant affective states; preoccupation with sexual pursuits despite harm to self or others; and functional impairment (Kafka, 2010). These criteria are reflected in the Hypersexuality Behaviour Inventory (HBI), which remains clinically useful in diagnosing hypersexuality (Reid *et al.*, 2011). Other ways in which impulsivity may manifest are outlined in the Questionnaire for Impulsive-Compulsive Disorders in Parkinson's Disease (QUIP), which focuses on the ICDs of pathological gambling, hypersexuality, compulsive buying and compulsive eating, as well

as compulsive behaviours relating to medication use, punding (preoccupation with meaningless motor activities – e.g., cleaning, arranging objects), hobbyism (preoccupation with a specific activity – e.g., writing, repairing machinery) and walkabout (excessive wandering by car or foot without purpose) (Weintraub *et al.*, 2009).

ICDs were initially considered rare in the hyperprolactinaemia setting, with only 3.5% of drug-induced ICD reports to the FDA from 2002 to 2012 occurring in hyperprolactinaemic patients, compared to 61.7% of patients having Parkinson's disease and 23.8% of patients having restless legs syndrome (Moore *et al.*, 2014). The rarity of ICDs in hyperprolactinaemia has been attributed to the 5-20 times lower doses and lower D3 selectivity of the DA agents used in hyperprolactinaemia (Bancos *et al.*, 2014). Recent studies of select hyperprolactinaemic patient samples have shown ICD prevalence to be 8-25% (Martinkova *et al.*, 2011; Bancos *et al.*, 2014; Celik *et al.*, 2018; Dogansen *et al.*, 2019), stimulating interest in a risk factor-based approach to patient monitoring (Barake *et al.*, 2018; Ioachimescu *et al.*, 2019). Though it is tempting to draw upon the neurology experience, a key distinction in the hyperprolactinaemia setting is the typical restoration of eugonadism coinciding with the commencement of DA therapy. This has sparked interest in the possible contribution of testosterone to the risk of ICDs, especially hypersexuality as highlighted by our case series (De Sousa *et al.*, 2017a) and subsequent literature (Bancos *et al.*, 2017; Athanasoulia-Kaspar *et al.*, 2018; Barake *et al.*, 2018; Celik *et al.*, 2018; Dogansen *et al.*, 2019; Ioachimescu *et al.*, 2019) debating our proposed concept of 'dopa-testotoxicosis'.

Because studies to date have generally been small or uncontrolled, the prevalence of DA-induced ICDs in hyperprolactinaemic patients remains uncertain and postulated risk factors, including male gender and testosterone levels, are yet to be fully elucidated (Bancos *et al.*, 2017; Barake *et al.*, 2018). We hypothesised that ICDs in patients treated with DAs would be

more common than in community controls, and that demographic and clinical characteristics of patients would be associated with ICD risk. The primary aim of our study was to determine the prevalence of ICDs in DA-treated hyperprolactinaemic patients compared to healthy controls. Secondary aims were to identify ICD risk factors that could be utilised to tailor ICD surveillance in hyperprolactinaemic patients, and to quantify the impact of hypersexuality given the predominance of this ICD in the hyperprolactinaemia setting (Bancos *et al.*, 2014; De Sousa *et al.*, 2017a).

4.2 Methods

Patients and controls

This was a multicentre cross-sectional study of DA-treated hyperprolactinaemic patients ($n=113$) in three Australian tertiary referral centres (Royal Adelaide Hospital, Princess Alexandra Hospital in Brisbane and St Vincent's Hospital in Sydney) and healthy volunteers serving as controls ($n=99$). The study was approved by the Royal Adelaide Hospital Research Ethics Committee (HREC/16/RAH/494) and all participants provided written informed consent.

Patients were recruited from April 2017 to December 2018. Inclusion criteria were diagnosis of pathological hyperprolactinaemia after exclusion of physiological and drug-induced hyperprolactinaemia and current DA treatment for ≥ 1 month. Exclusion criteria were age < 18 yr, current antipsychotic medication use, prior brain or pituitary radiotherapy, and inability to consent or engage in neuropsychological assessment due to intellectual impairment, mental illness, non-English speaking background or any other reason. Eligible patients were identified using local pituitary clinic databases and contacted by telephone and mail. Of 51 eligible patients at the primary site, 42 (82.4%) completed the study, whilst four (7.8%) were uncontactable, four (7.8%) declined participation, and two (3.9%) did not

return the required paperwork. Additional patients were consecutively recruited during clinics and admissions with similarly high acceptance rates.

Clinical data were collated using medical records and information from patient questionnaires. Eugonadism was defined as regular menses in women and normal serum testosterone in men. As this was an observational study with various hormone assays employed as in usual clinical practice, prolactin levels were compared by dividing absolute values by the upper limit of normal for a given assay as supported by our findings in Chapter 2. Weekly cabergoline equivalent dose was calculated as cabergoline 1 mg weekly = quinagolide 75 mcg daily as previously defined (Primeau *et al.*, 2012). Cumulative cabergoline equivalent dose was calculated by weekly cabergoline equivalent dose multiplied by duration of DA therapy in weeks, noting that patients are typically on a stable DA dose over years, although this calculation would not reflect interim changes in DA type, dose or compliance.

Healthy controls were selected as the comparator arm because of the frequent medical comorbidity, older age and difficulty in controlling for gender when NFPA controls have been employed in other studies (Bancos *et al.*, 2014; Celik *et al.*, 2018). The importance of comparing patients with age- and gender-matched controls is underscored in *DSM-V* which explains that impulsivity is more common in younger individuals and in men more than women (American Psychiatric Association, 2013). Volunteers were recruited by hospital and laboratory staff email asking for participation in an anonymised study relating to mood, wellbeing and behaviour. Exclusion criteria specific to the control group were active medical problems (other than asthma, allergic rhinitis and minor joint problems), use of prescription medications (other than asthma inhalers, intranasal sprays and non-steroidal anti-inflammatory drugs) and previous medical attention for an ICD.

Neuropsychological assessment

Patients completed a written questionnaire consisting of summary demographic and medical history questions, and five validated neuropsychological tools as follows below.

1. Depression Anxiety Stress Scale, DASS21: a 21-item questionnaire with three subscales assessing the severity of depression, anxiety and stress symptoms (Lovibond & Lovibond, 1995), with good reliability and validity in clinical and community samples (Antony *et al.*, 1998; Henry & Crawford, 2005).
2. Questionnaire for Impulsive-Compulsive Disorders in Parkinson's disease – Shortened version, QUIP-S: a comprehensive instrument validated for the diagnosis of DA-induced ICDs in patients with Parkinson's disease. Sensitivity is similar between the 13-item QUIP-S and the full QUIP (94 vs. 96%, respectively) (Weintraub *et al.*, 2009).
3. Hypersexual Behaviour Inventory, HBI: a 19-item questionnaire regarding control, consequences and coping associated with sexual thoughts, feelings and behaviours. It has high internal consistency and shows reliability over time, with scores ≥ 53 diagnostic of hypersexuality (Reid *et al.*, 2011). The tool was originally designed and validated in men, and has more recently been used in female populations (Dhuffar & Griffiths, 2014; Klein *et al.*, 2014).
4. Hypersexual Behaviour Consequences Scale, HBCS: a detailed 22-item questionnaire assessing hypersexuality consequences in affected patients. This complements the HBI, with high internal consistency and reliability over time. Comparison of the HBCS between hypersexual and non-hypersexual psychiatric patients and the HBCS against other hypersexuality assessment tools has shown discriminant and convergent validity, respectively (Reid *et al.*, 2012).

5. Social Desirability Response Set Scale, SDRS5: a 5-item measure indicating a participant's tendency to give socially desirable responses with similar reliability as more lengthy social desirability scales but less participant burden (Hays *et al.*, 1989). This measure was included to screen for confounding as prolactinoma patients have been shown to respond in a more socially desirable manner compared to healthy controls (Athanasoulia *et al.*, 2012a), possibly reflecting the evolutionary functions of prolactin in promoting parental behaviours as observed in animal and human studies (Grattan, 2015).

Controls completed an anonymised online version of the demographic questions and five-part neuropsychological questionnaire.

Semi-structured psychological interview

Patients who tested positive for an ICD by QUIP-S or HBI were offered a follow-up semi-structured telephone interview with a clinical psychologist to assess patient knowledge of the risk of DA-induced ICDs prior to study participation and to identify any relationships between ICD symptoms and DA dosing or cessation.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 25.0. Missing data were coded as negative where appropriate. For example, GH deficiency was defined as the presence of a low IGF1 level or requirement for GH treatment and patients without available data to the contrary were considered to be GH sufficient. Cases were excluded where missing data could not be assumed to be negative. Categorical variables were defined by frequency expressed as percentages, and continuous variables by mean \pm standard deviation (*SD*) unless otherwise stated. Chi-square tests with continuity corrections and unpaired t-tests were employed for categorical and continuous variables, respectively, for comparisons between

all patients and controls and for comparisons between patients with and without ICDs. The Chi-square test was substituted for the 2-tailed Fisher's exact test when $\geq 20\%$ of expected cell counts were < 5 . Statistical significance was set at $P < 0.05$. Risk factors for the ICDs shown to be more frequent in patients than controls were first assessed by univariate analysis comparing patients with and without ICDs. Logistic regression models were then developed to assess the relationship between patient characteristics and screening positive for the ICDs that were overrepresented in patients vs. controls. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated.

4.3 Results

General characteristics

Demographic and clinical data of the 113 patients (56 males, 57 females, age 38.1 ± 15.9 yr) are outlined in Table 4.1. The cause of hyperprolactinaemia was a prolactinoma in 107/113 (95%) patients and other sellar masses in the remainder. Most patients had hypogonadotropic hypogonadism at diagnosis; a minority demonstrated other pituitary hormone deficiencies. At the time of neuropsychological assessment, 109/113 (96.5%) patients were taking cabergoline and four (3.5%) were taking quinagolide; no patients were taking bromocriptine.

Table 4.1. Demographic and clinical data of study participants

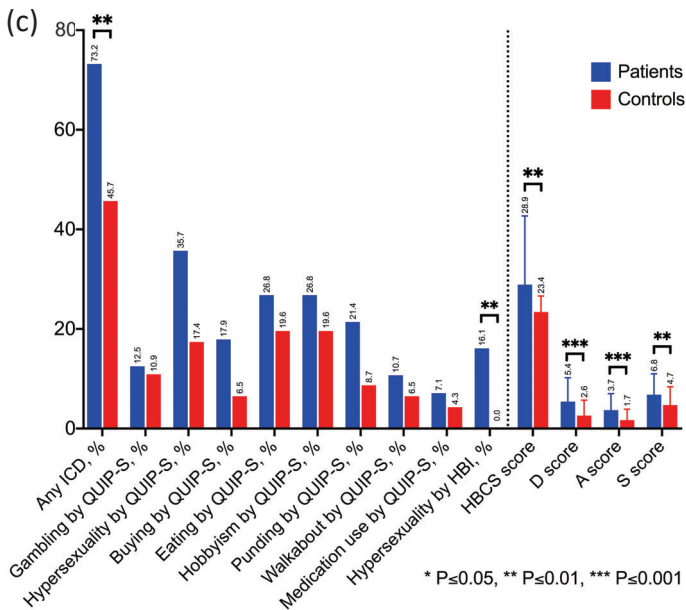
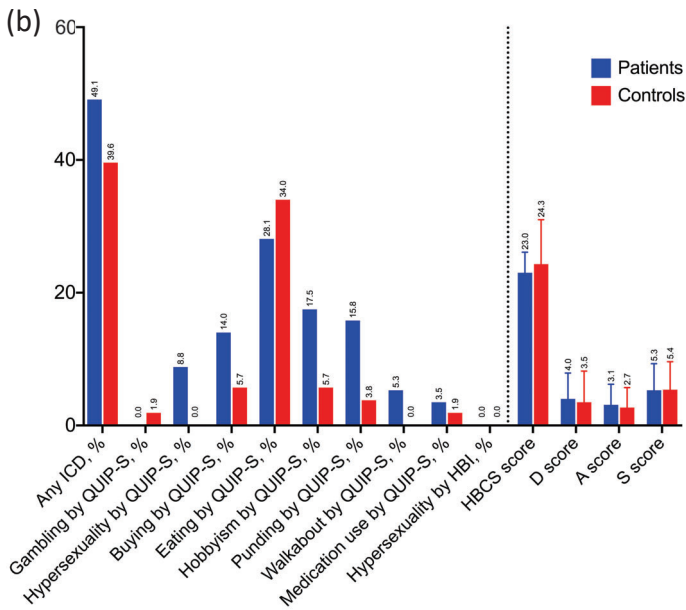
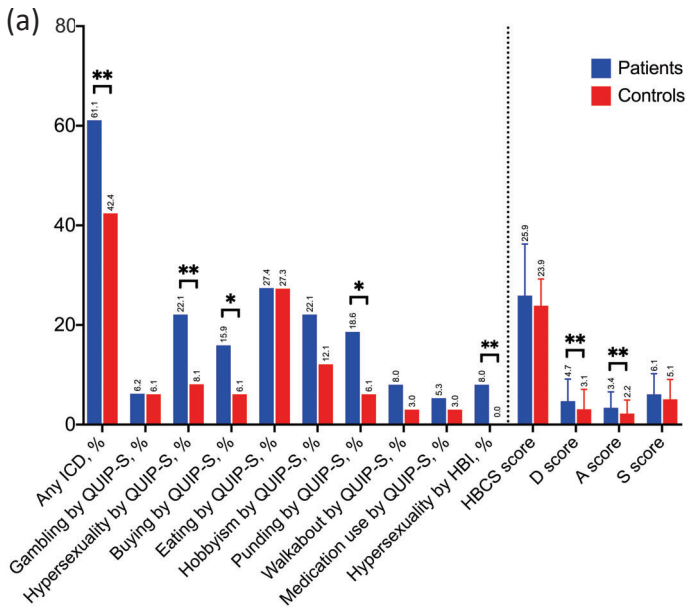
| | Patients (n=113) | Controls (n=99) | P |
|-------------------------------------|------------------|-----------------|-------|
| Gender, % male | 49.6 | 46.5 | ns |
| AT DIAGNOSIS | | | |
| Age, yr | 38.1±15.9 | | |
| Tumour diameter, mm | 20.8±17.0 | | |
| Hardy's score, % | | | |
| 0 | 0.9 | | |
| I | 27.4 | | |
| II | 27.4 | | |
| III | 31.0 | | |
| IV | 0.0 | | |
| Unknown | 13.3 | | |
| Tumour consistency, % | | | |
| Solid | 57.5 | | |
| Cystic | 8.0 | | |
| Mixed | 16.8 | | |
| Unknown | 17.7 | | |
| Visual field defects, % | 15.9 | | |
| Prolactin, xULN* | 56.4±144.3 | | |
| Testosterone, nmol/L* | 5.2±3.7 | | |
| Pituitary hormone deficiency, % | | | |
| LH/FSH | 78.8 | | |
| GH | 6.2 | | |
| TSH | 3.5 | | |
| ACTH | 1.8 | | |
| AT ASSESSMENT | | | |
| Age, yr | 45.5±16.5 | 41.2±10.1 | 0.020 |
| Employment status, % | | | |
| Full-time | 54.9 | 73.7 | 0.007 |
| Part-time | 15.0 | 26.3 | ns |
| Home duties | 7.1 | 0.0 | 0.008 |
| Retired | 15.0 | 0.0 | 0.000 |
| Unemployed | 8.0 | 0.0 | 0.004 |
| Marital status, % | | | |
| Single | 20.4 | 15.2 | ns |
| Married | 73.5 | 79.8 | ns |
| Divorced | 4.4 | 5.1 | ns |
| Widowed | 1.8 | 0.0 | ns |
| Has children, % | 61.1 | 62.6 | ns |
| Current/former smoker, % | 31.9 | 12.1 | 0.001 |
| Any alcohol consumption, % | 57.5 | 78.8 | 0.002 |
| Tumour duration, yr | 7.5±7.3 | | |
| DA duration, yr | 6.5±7.1 | | |
| Non-ICD DA side effects, % | 24.8 | | |
| Wkly CBG equivalent dose, mg | 969.0±1100.5 | | |
| Cumulative CBG equivalent dose, g | 307.3±419.1 | | |
| On sex hormone replacement, % | 27.4 | | |
| Prolactin, xULN | 0.8±1.8 | | |
| Testosterone, nmol/L | 13.7±8.4 | | |
| Prolactin fall, xULN (median, IQR)^ | 11.2, 4.5-38.6 | | |
| Testosterone rise, nmol/L^ | 8.3±7.6 | | |
| Prior pituitary surgery, % | 15.9 | | |

ACTH, adrenocorticotrophic hormone; CBG, cabergoline; DA, dopamine agonist; FSH, follicle-stimulating hormone; GH, growth hormone; ICD, impulse control disorder; IQR, interquartile range; LH, luteinising hormone; ns, non-significant; TSH, thyroid-stimulating hormone; ULN, upper limit of normal; wkly, weekly; * various normal ranges due to observational nature of study with different hormone assays employed depending on the referring clinician; ^ since time of diagnosis

Compared to patients, controls were more likely to be employed full-time, reflecting our method of workplace-based recruitment. Controls were also less likely to be current/former smokers and more likely to drink alcohol. Controls were slightly younger (41.2 ± 10.1 vs. 45.5 ± 16.5 yr, $P=0.020$) and there was no difference in sex ratio.

Neuropsychological dysfunction

Neuropsychological dysfunction in patients and controls was determined by the composite questionnaire as shown in Figure 4.1. DA-treated hyperprolactinaemic patients were more likely than controls to test positive by QUIP-S for any ICD (61.1 vs. 42.4% , $P=0.01$), hypersexuality (22.1 vs. 8.1% , $P=0.009$), compulsive buying (15.9 vs. 6.1% , $P=0.041$) and punding (18.6 vs. 6.1% , $P=0.012$). Hypersexuality as defined by the more stringent HBI tool was also more common in patients (8.0 vs. 0.0% , $P=0.004$), with all HBI-positive patients also scoring positive for hypersexuality by QUIP-S. Multiple ICDs were found in 32.7% of patients vs. 15.2% of controls ($P=0.005$). Patients scored higher than controls for depression (4.7 ± 4.4 vs. 3.1 ± 4.0 , $P=0.005$) and anxiety (3.4 ± 3.2 vs. 2.2 ± 2.7 , $P=0.005$) by DASS21. Trends towards higher patient scores were observed for stress by DASS21 (6.1 ± 4.2 vs. 5.1 ± 4.0 , $P=0.080$) and for hypersexuality consequences by HBCS (25.9 ± 10.4 vs. 23.9 ± 5.4 , $P=0.072$).



* P≤0.05, ** P≤0.01, *** P≤0.001

Figure 4.1. ICD frequency and mean HBCS and DASS21 scores in patients vs. controls

A. All patients vs. all controls.

B. Female patients vs. female controls.

C. Male patients vs. male controls.

A, anxiety score by DASS21; D, depression score by DASS21; HBCS, Hypersexual Behaviour Consequences Scale; HBI, Hypersexual Behaviour Inventory; ICD, impulse control disorder; QUIP-S, Questionnaire for Impulsive-Compulsive Disorders in Parkinson's Disease Shortened Version; S, stress score by DASS21

Because of the gender dimorphism in ICD risk observed in Chapter 3 and as reported by others (Bancos *et al.*, 2014; Dogansen *et al.*, 2019), we analysed patients and controls stratified by gender (Figure 4.1). In female patients vs. female controls, there were trends towards higher prevalence of hypersexuality by QUIP-S (8.8 vs. 0.0%, $P=0.058$) and punning (15.8 vs. 3.8%, $P=0.075$), with no differences in other ICD frequencies or depression, anxiety, stress or hypersexuality consequences scores. Men showed greater divergence, with male patients vs. male controls showing higher prevalence of any ICD (73.2 vs. 45.7%, $P=0.008$) and hypersexuality by HBI (16.1 vs. 0.0%, $P=0.004$), as well as higher depression (5.4 ± 4.8 vs. 2.6 ± 3.1 , $P=0.001$), anxiety (3.7 ± 3.3 vs. 1.7 ± 2.2 , $P=0.000$), stress (6.8 ± 4.2 vs. 4.7 ± 3.7 , $P=0.009$) and hypersexuality consequences (28.9 ± 13.8 vs. 23.4 ± 3.2 , $P=0.005$) scores. A trend towards more frequent hypersexuality by QUIP-S was apparent in male patients vs. male controls (35.7 vs. 17.4% , $P=0.066$).

ICD risk factors in DA-treated patients

We analysed risk factors for each of the ICDs shown to be more common in patients compared to controls (i.e., any ICD, hypersexuality by QUIP-S or HBI, compulsive buying and punning), postulating that different risk factors may pertain to different ICDs. This was first performed by univariate analysis. Compared to patients screening negative for all ICDs, patients screening positive for at least one ICD had a higher proportion of men, greater alcohol use, and higher depression, anxiety, stress and hypersexuality consequences scores (Table 4.2). Hypersexuality by QUIP-S was associated with male gender, having children, lower Hardy's tumour score at diagnosis, higher testosterone at assessment in men, eugonadism at assessment in men and women, and higher hypersexuality scores by HBI and HBCS. Hypersexuality by HBI was associated with male gender, divorce, full-time employment, comorbid mental illness, higher testosterone at diagnosis in men, lower

agreeableness by SDRS5, and higher scores for depression, stress and hypersexuality consequences. Compulsive buying was associated with younger age at diagnosis and at assessment, GH deficiency at diagnosis, and higher depression, anxiety and stress scores. Punding was associated only with higher anxiety and stress scores.

Table 4.2. Differences in patients screening positive vs. negative for ICDs

Only those ICDs shown to be higher in patients vs. controls were investigated and only statistically significant differences on univariate analysis are shown

| Parameter [^] | Any ICD | Hypersexuality by QUIP-S | Hypersexuality by HBI | Buying by QUIP-S | Punding by QUIP-S |
|---|--|---|--|--|--|
| <i>n</i> , positive/negative | 69/44 | 25/88 | 9/104 | 18/95 | 10/103 |
| Male, % | 59.4 vs. 34.1, <i>P</i> =0.015 | 80.0 vs. 40.9, <i>P</i> =0.001 | 100.0 vs. 45.2, <i>P</i> =0.001 | ns | ns |
| Divorced, % | ns | ns | 22.2 vs. 2.9, <i>P</i> =0.050 | ns | ns |
| Has children, % | ns | 80.0 vs. 55.7, <i>P</i> =0.049 | ns | ns | ns |
| Employed full-time, % | ns | ns | 88.9 vs. 51.9, <i>P</i> =0.039 | ns | ns |
| ≥2 SD/d alcohol, % | 15.9 vs. 2.3, <i>P</i> =0.027 | ns | ns | ns | ns |
| Alcohol, SD/d | 1.0±1.0 vs. 0.5±0.5, <i>P</i> =0.027 | ns | ns | ns | ns |
| Has mental illness, % | ns | ns | 33.3 vs. 6.7, <i>P</i> =0.032 | ns | ns |
| Age at Dx | ns | ns | ns | 30.9±12.7 vs. 39.5±1.1, <i>P</i> =0.018 | ns |
| Testosterone at Dx, nmol/L (<i>n</i> =48) | ns | ns | 8.4±4.8 vs. 4.8±3.3, <i>P</i> =0.026 | ns | ns |
| Hardy's score at Dx (<i>n</i> =98) | ns | 1.7±0.8 vs. 2.1±0.8, <i>P</i> =0.035 | ns | ns | ns |
| GH-deficient at Dx, % | ns | ns | ns | 22.2 vs. 3.2, <i>P</i> =0.012 | ns |
| Age at Ax | ns | ns | ns | 36.5±12.5 vs. 47.3±16.6, <i>P</i> =0.004 | ns |
| Testosterone at Ax, nmol/L (<i>n</i> =55) | ns | 17.3±8.9 vs. 11.8±7.5, <i>P</i> =0.018 | ns | ns | ns |
| Hypogonadism at Ax, % (<i>n</i> =95) | ns | 12.5 vs. 40.8, <i>P</i> =0.022 | ns | ns | ns |
| SDRS5 score (<i>n</i> =111) | ns | ns | 0.9±0.8 vs. 1.9±1.5, <i>P</i> =0.005 | ns | ns |
| D score | 5.6±3.9 vs. 3.4±4.9, <i>P</i> =0.010 | ns | 7.6±4.8 vs. 4.5±4.3, <i>P</i> =0.022 | 7.4±4.2 vs. 4.2±4.3, <i>P</i> =.004 | ns |
| A score | 4.1±3.2 vs. 2.3±2.8, <i>P</i> =0.003 | ns | ns | 5.6±3.3 vs. 3.0±3.0, <i>P</i> =0.001 | 5.7±3.7 vs. 2.9±2.8, <i>P</i> =0.000 |
| S score | 7.3±4.0 vs. 4.2±3.8, <i>P</i> =0.000 | ns | 9.3±3.4 vs. 5.8±4.1, <i>P</i> =0.013 | 9.2±4.8 vs. 5.5±3.8, <i>P</i> =0.000 | 9.1±4.3 vs. 5.4±3.8, <i>P</i> =0.000 |
| HBI score | ns | 42.6±18.5 vs. 22.2±5.1, <i>P</i> =0.000 | N/A | ns | ns |
| HBCS score | 28.2±12.8 vs. | 37.0±17.8 vs. | 56.8±14.1 vs. | ns | ns |

| | | | | | |
|--|----------------------|----------------------|----------------------|--|--|
| | 22.3±1.1, P=0.000 | 22.7±2.4, P=0.001 | 23.2±3.3, P=0.000 | | |
|--|----------------------|----------------------|----------------------|--|--|

A, anxiety score by DASS21; Ax, assessment; D, depression score by DASS21; Dx, diagnosis; GH, growth hormone; HBCS, Hypersexual Behaviour Consequences Scale; HBI, Hypersexual Behaviour Inventory; ICD, impulse control disorder; N/A, not applicable; ns, non-significant; QUIP-S, Questionnaire for Impulsive-Compulsive Disorders in Parkinson's Disease Shortened Version; S, stress score by DASS21; SD, standard drinks; SDRS5, Social Desirability Response Set Scale; ^ n given if subset analysis performed due to missing data or if parameter not applicable in some cases (e.g., testosterone levels only obtained in men), otherwise n=113

Statistically significant differences were not observed in other employment/marital status, smoking history, cause of hyperprolactinaemia, DA or pituitary tumour duration, DA type, weekly or cumulative CBG equivalent dose, degree of hyperprolactinaemia or other pituitary hormone perturbations at diagnosis or at assessment, degree of prolactin fall or testosterone rise between diagnosis and assessment, tumour diameter or cerebrovascular change on MRI at diagnosis or at assessment, visual field deficits at diagnosis or at assessment, previous pituitary surgery, sex steroid or antidepressant use at assessment, or central nervous system comorbidity.

In addition to the male bias in any ICD and hypersexuality by QUIP-S and HBI (Table 4.2), we found that the proportion of men was greater in patients screening positive vs. negative for gambling (100.0 vs. 46.2%, $P=0.006$). No sex difference was seen in patients with and without compulsive eating, hobbyism, walkabout or compulsive medication use.

Logistic regression analysis

Logistic regression models were generated to identify predictive factors that remained independent upon multivariate analysis. This showed that screening positive for any ICD was significantly associated with an increase in stress score (OR 1.23, 95% CI 1.10-1.37). Hypersexuality by QUIP-S ($n=80$) was significantly associated with male gender (OR 13.85, 95% CI 2.89-66.49), eugonadism at assessment (OR 7.85, 95% CI 1.45-42.42), and lower Hardy's tumour score at diagnosis (OR 11.60, 95% CI 1.87-71.88 for I vs. III; OR 4.59, 95% CI 1.03-20.47 for II vs. III). Hypersexuality by HBI was significantly associated with mental illness (OR 6.86, 95% CI 1.28-36.72) and higher stress score (OR 1.22, 95% CI 1.02-1.48). Compulsive buying was significantly associated with younger age at assessment (OR 0.95, 95% CI 0.91-0.99 for each increasing year of age) and higher stress score (OR 1.26, 95% CI 1.10-1.46). Punding was significantly associated with a higher stress score (OR 1.26, 95% CI 1.11-1.45).

Separate logistic regression models were created for men with available testosterone levels. Hypersexuality by QUIP-S in applicable men ($n=55$) showed a trend towards a higher risk of hypersexuality with higher testosterone at assessment (OR 1.11, 95% CI 1.00-1.23). Hypersexuality by HBI, only found in 6/48 applicable men, showed a weak trend towards higher testosterone at diagnosis (OR 1.25, 95% CI 0.97-1.61).

Patient insights

Free prose responses in patient questionnaires illustrated the extremity of the impact of DA-induced side effects (Table 4.3).

Table 4.3. Questionnaire excerpts of DA side effects in hyperprolactinaemic patients

| |
|---|
| Regarding hypersexuality |
| <ul style="list-style-type: none"> • <i>"I am fighting a constant internal battle between being the nice guy all love and respect, and an inner 'captain caveman' that keeps trying to come out. This is very much dependent on medication levels... One of the biggest issues for me is since treatment began I have not taken sexual rejection well. It considerably causes me to question my self-worth."</i> • <i>"I have separated from my wife of (decades) after being on Dostinex. This has been helpful in helping me to understand it a little better, even though obviously choices I made were mine."</i> • <i>"I feel I am sex obsessed and this potentially makes me vulnerable to female sexual predators. I have found it difficult to extricate myself from situations where I am the target of such an individual due to my own obsession which was not present prior to treatment."</i> |
| Regarding other ICDs |
| <ul style="list-style-type: none"> • <i>"I have become addicted to buying tools and cars to a point of almost financial ruin. I get a high when buying and then become very low when reality kicks in to then find the money to pay for purchases. Previously I never had this behaviour but it's very consistent these days."</i> |
| Regarding non-ICD side effects |
| <ul style="list-style-type: none"> • <i>"I do feel my moods are very low. I found it hard to be happy or get excited about something. I don't feel I know what is happiness. Before I got this I used to be a very happy-go-lucky person."</i> • <i>"Self-harm side effects have included punching my head; bashing my head on ground, benches and walls; biting myself very hard on forearms; very frustrated; as often as 4 times every 7 days during the taking of medication."</i> |

Semi-structured psychological interview

Of 69 patients who screened positive for an ICD by the questionnaire, 51 (73.9%) participated in the psychological interview. 17/51 (33.3%) patients reported ICD symptom fluctuation with dose changes and/or worsening ICD symptoms on the day of or the day

after cabergoline dosing. Of 11 patients who interrupted DA treatment, 8 (72.8%) experienced resolution or improvement of ICD symptoms. Amongst all interviewees, 19/51 (37.3%) knew about the relationship between DAs and ICDs before participating in the study. 30/51 (58.8%) had the opportunity to discuss their ICD symptoms with their treating endocrinologist; 18/30 (60.0%) found this to be helpful.

4.4 Discussion

This study represents the largest cross-sectional analysis of the risk of ICDs in hyperprolactinaemic patients compared to controls, demonstrating significantly higher rates of any ICD, multiple ICDs, hypersexuality, compulsive buying and punding in DA-treated patients. Risk factors that remained predictive after logistic regression were male gender, eugonadism at assessment, lower Hardy's tumour score at diagnosis and psychiatric comorbidity for hypersexuality, and younger age for compulsive buying. DA dose was not predictive of ICD risk. Higher testosterone at assessment appeared to have a permissive effect on the development of hypersexuality as diagnosed by QUIP-S. The burden of hypersexuality consequences was substantial, with significantly higher HBCS scores in patients screening positive vs. negative for hypersexuality. This is noteworthy as increased sexual thoughts and behaviours following DA treatment of hyperprolactinaemia could otherwise simply reflect a normal return of libido with reversal of hypogonadism.

The prevalence of ICDs in DA-treated hyperprolactinaemic patients was much higher than the few previously published studies (Table 4.4). The low ICD rates in other studies may be explained by the exclusion of patients with a psychiatric history (Barake *et al.*, 2014; Celik *et al.*, 2018; Dogansen *et al.*, 2019), short follow-up times following DA commencement (Celik *et al.*, 2018), the inclusion of patients who have ceased DA treatment and may not accurately recall their experiences during treatment (Bancos *et al.*, 2014), and the use of

more focused tools in screening for ICDs (Bancos *et al.*, 2014; Celik *et al.*, 2018; Dogansen *et al.*, 2019). The known inverse association between age and impulsivity (American Psychiatric Association, 2013) could partly explain the high ICD prevalence in our study where patient age was 46 ± 16 yr compared to the study by Bancos *et al.* (2014) where patient age was 55 ± 14 yr. However, our patients were older than studies by Dogansen *et al.* (2019) (36 ± 12 yr), Celik *et al.* (2018) (40 ± 10 yr) and Martinkova *et al.* (2011) (41 ± 11 yr). Two studies were undertaken in Turkey (Celik *et al.*, 2018; Dogansen *et al.*, 2019), where gambling is prohibited (Dogansen *et al.*, 2019), although all of the tested ICD subsets were more frequent in our patients. Cultural differences in reporting impulsivity could be contributory, particularly given the high ICD prevalence in our controls. The male bias in developing ICDs and our relatively high proportion of men at 49.6% of study patients is also noteworthy. Tertiary endocrine centres typically show female-to-male prolactinoma ratios that are much lower than the community ratio of 10:1 because of the greater invasiveness of prolactinomas in men and consequent referral bias (Fernandez *et al.*, 2010; Athanasoulia *et al.*, 2012a). We found that men similarly comprised 51.0% of eligible patients in our primary site 30-year pituitary database.

Table 4.4. ICD prevalence in studies of DA-treated hyperprolactinaemic patients

| Study | n | Tool | Any ICD | Gambling | Hypersexuality | Buying | Eating | Hobbyism | Punding | Walkabout | Medication use | Predictive factors |
|-----------------------------------|------------------------------|--|---------|----------|----------------|--------|--------|----------|---------|-----------|----------------|--|
| (Martinkova <i>et al.</i> , 2011) | 20 pts | MIDI | 10 | 5% | 5% | 0% | 5% | - | - | - | - | - |
| (Bancos <i>et al.</i> , 2014) | 77 pts, 70 NPPA ctrls | MIDI, South Oaks Gambling Screen, modified HS & P questionnaires | 25% | 6% | 13% | 5% | - | - | 9% | - | - | Male sex |
| (Celik <i>et al.</i> , 2018) | 25 pts, 31 NPPA ctrls, 32 HC | MIDI, BIS-11, SCL-90-R, BDI, BAI | 8% | 0% | 8% | 0% | - | 0% | 0% | - | - | Nil independent (age, sex, cumulative DA dose, smoking & alcohol predictive in combination) |
| (Dogansen <i>et al.</i> , 2019) | 308 pts | Modified QUIP, BIS-11 | 17% | 3% | 10% | 5% | 6% | - | - | - | - | Male, alcohol, smoking, nadir PRL, others (incl T rise) only on univariate analysis |
| Present study | 113 pts, 99 HC | QUIP-S, HBI, HBCS, DASS21, SDRSS | 61% | 6% | 22% | 16% | 27% | 22% | 19% | 8% | 5% | Male, eugonadism, lower Hardy's tumour score, mental illness, younger age, others (incl T) only on univariate analysis |

BART, Balloon Analog Risk Task; BAI, Beck Anxiety Inventory; BDI, Beck Depression Inventory; BIS, Barratt Impulsiveness Scale; ctrls, controls; DA, dopamine agonist; DASS21, Depression, Anxiety and Stress Scale; EDT, Experiential Discounting Task; GH, growth hormone; HBCS, Hypersexual Behaviour Consequences Scale; HBI, Hypersexual Behaviour Inventory; HC, healthy controls; ICD, impulse control disorder; incl, including; MIDI, Minnesota Impulse Disorders Interview; NEPA, non-functioning pituitary adenoma; PRL, prolactin; pts, patients; QUIP, Questionnaire for Impulsive-Compulsive Disorders in Parkinson's disease; QUIP-S, Questionnaire for Impulsive-Compulsive Disorders in Parkinson's disease – Shortened version; SDRSS, Socially Desirable Response Set Five-Item Survey; SCL-90-R, Symptom Checklist-90-R; T, testosterone; - not tested

Though not encompassing the overall DA-induced ICD risk, we proposed the term ‘dopa-testotoxicosis’ in the case series described in Chapter 3 in order to highlight the male predilection and hypersexuality predominance in hyperprolactinaemic patients, putatively due to synergy between restoration of eugonadism and D3 receptor stimulation (De Sousa *et al.*, 2017a). The present study found no association between hypersexuality and testosterone rise from time of tumour diagnosis to time of neuropsychological assessment, although statistical power was limited by only 41/48 men with available testosterone levels exhibiting a DA-induced testosterone rise. Testosterone appears to be permissive in the development of any ICD and hypersexuality based on the male predominance shown here and by others (Weintraub *et al.*, 2010; Bancos *et al.*, 2014; De Sousa *et al.*, 2017a; Grall-Bronnec *et al.*, 2018; Dogansen *et al.*, 2019). Whilst DA-treated hyperprolactinaemic patients do not reach supraphysiological testosterone levels (Bancos *et al.*, 2014; De Sousa *et al.*, 2017a), we and Dogansen *et al.* (2019) have shown greater ICD risks with relative testosterone increases into the normal range. Dogansen *et al.* (2019) found a higher testosterone percentage rise at the preceding visit in hypersexual men, although this was not significant upon multivariate analysis. We found an independent trend towards higher testosterone at assessment in hypersexual men, and eugonadism at assessment was one of the few predictive factors in logistic regression modelling. The trend towards increased hypersexuality in female patients vs. female controls in our study does not refute the concept of dopa-testotoxicosis as testosterone also contributes to female libido. Nonetheless, testosterone is not sufficient for the development of hypersexuality as this ICD only occurs in a minority of hyperprolactinaemic men rendered eugonadal by DA therapy, and hypersexuality is not associated with androgen replacement in post-pubertal males (Bancos *et al.*, 2017). Testosterone is also not necessary for the development of hypersexuality as it frequently occurs in the neurology setting (Grall-Bronnec *et al.*, 2018)

where DA therapy is not expected to cause testosterone fluctuations. Notably, DA therapy is also neither necessary nor sufficient as ICDs may occur in healthy controls and not all DA-treated patients develop ICDs.

Other factors contribute to the risk of DA-induced ICDs (Table 4.4). The independent inverse association between age and compulsive buying is especially pertinent as hyperprolactinaemic patients tend to be younger than patients with Parkinson's disease or restless legs syndrome. This is a new association in the hyperprolactinaemia setting and there are other differences in ICD risk factors between this and previous studies, which may partly have a sociocultural basis. Smoking was predictive of the risks of any ICD and of hypersexuality in the study by Dogansen *et al.* (2019) that had a high proportion of current smokers (24%), whereas only 8% of our patients were current smokers and we did not find a statistically significant association between current/former smoking and ICD risk. Alcohol use was common in our patients (58%) and we found a higher ICD risk only when grouping patients who consumed ≥ 2 standard drinks of alcohol daily, whilst Dogansen *et al.* (2019) found an association with any alcohol use, which reflected a small minority (5%) of their patients. We also observed an association between a lower Hardy's tumour score and increased ICD risk; the reason for this is unclear.

We found higher DASS21 subset scores in patients vs. controls, and in patients with vs. without different ICDs. We also found a higher prevalence of mental illness in patients who tested positive vs. negative for hypersexuality by HBI. Parkinson's disease studies have similarly reported higher rates of depression and anxiety in patients with vs. without DA-induced ICDs (Weintraub *et al.*, 2010; Grall-Bronnec *et al.*, 2018). This comorbidity is unsurprising as *DSM-V* highlights the link between the various ICDs and an externalising spectrum, characterised by greater disinhibition, less constraint and negative emotionality

(American Psychiatric Association, 2013). It is possible that depression, anxiety and stress leads to ICDs as a method of coping with these negative affective states. Alternatively, ICDs may induce hyper-emotionality and consequent stress. Celik *et al.* (2018) found no difference in depression or anxiety scores by the Beck Depression Inventory and Beck Anxiety Inventory, respectively, in prolactinoma patients compared to NFPA patients or healthy controls; however, this may reflect the smaller size of that study, with only two prolactinoma patients screening positive for ICDs. Notwithstanding the constraints of sample size, the authors found higher rates of obsession, interpersonal sensitivity and paranoia in DA-treated prolactinoma patients compared to their two control arms, highlighting the breadth of neuropsychiatric changes that DAs may incur (Celik *et al.*, 2018).

The present study was not a formal prevalence study as it used a convenience sample and had limited size, and there may be some recruitment biases due to referral patterns in tertiary centres. Nevertheless, the study is likely to be representative of ICD patterns in patients typically treated in tertiary endocrine clinics. Investigation of rare ICDs and some associations may need a large DA registry study. With respect to our control group, there appeared to be a higher ICD rate amongst hospital and laboratory staff compared to other control groups (Valenca *et al.*, 2013; Napier & Persons, 2018), which might have underestimated differences in ICD prevalence between patients and controls. Our reliance on healthy controls rather than a diseased population may also be considered a weakness of our study as the presence of a PA may influence DA responses. Our method of recruiting controls via hospital and laboratory staff email may have introduced an additional selection bias. However, healthy controls were intentionally selected to better approximate the demographics and general health status of prolactinoma patients compared to NFPA patients. As in previous studies (Bancos *et al.*, 2014; Celik *et al.*, 2018; Dogansen *et al.*, 2019), another limitation of our study is that we relied on neuropsychological tools validated

outside of the hyperprolactinaemia setting. QUIP-S was designed for patients with Parkinson's disease. The present study and that by Dogansen *et al.* (2019) broadens the experience of this tool in hyperprolactinaemic patients, but further data are required to validate it in distinguishing between patients with disruptive hypersexuality vs. those with a normal return in libido with DA treatment. We were able to compare hypersexuality diagnosis by QUIP-S and HBI, finding that fewer patients tested positive by HBI but with greater impact as determined by mean HBCS score (56.8 ± 14.1 in HBI-positive vs. 37.0 ± 17.8 in QUIP-positive).

In addition to validating neuropsychological tools for clinical use in hyperprolactinaemic patients, it may be illuminating to study other impulsive activity that may apply to the younger hyperprolactinaemic population compared to the neurology setting – for example, exercise and video game use. Caffeine consumption may also be more relevant in the hyperprolactinaemia setting compared to patients with Parkinson's disease who tend to be older and less likely to be employed and patients with restless legs who may be advised to avoid coffee as this may worsen their symptoms. Higher caffeine consumption has been found in DA-treated hyperprolactinaemic patients compared to untreated hyperprolactinaemic and normoprolactinaemic controls (Barake *et al.*, 2014), and in Parkinson's disease patients with vs. without ICDs (Bastiaens *et al.*, 2013). There are also other ICDs such as kleptomania (American Psychiatric Association, 2013) that have not been addressed by the present or other studies in the hyperprolactinaemia setting.

Prospective studies will be valuable in capturing patients who, because of the development of ICDs or other side effects, cease DA therapy, leading to exclusion in cross-sectional studies of only currently treated patients. At the primary site for our study, 48 hyperprolactinaemic patients in our patient database were ineligible as they were no longer taking a DA, including

11 patients (23%) who ceased DA therapy due to side effects that were mostly neuropsychological effects. In addition, prospective prolactin and testosterone measurement by standardised assays will better evaluate the possible relationships between ICD risk and hormone levels. Chapter 2 highlights the particular importance of using a single platform for serial measurements of prolactin. Other directions of future research should include recruitment of prolactinoma patients beyond tertiary centres and comparison of prolactinoma patients with both healthy and diseased controls. Given the lack of interaction between ICD risk and DA dose and duration, future research should also consider whether DA-induced psychological side effects relate to the underlying psychological structure of individual patients. Screening tools that identify such at-risk patients may in turn guide a personalised approach to prolactinoma management with avoidance of DA therapy as the usual first-line treatment in susceptible individuals.

An alternative consideration is to routinely offer upfront surgery in preference to long-term DA therapy given that any risk of iatrogenic mood and behavioural changes due to DA therapy could be considered unacceptable by some patients and clinicians. This may be especially pertinent in younger patients expected to need prolonged DA treatment, which is associated with a low but cumulative dose-dependent risk of cardiac valvulopathy (Caputo *et al.*, 2015), and those with surgically accessible pituitary tumours where gross total resection is feasible. Before such an approach can be recommended, prospective trials are required to determine the success of contemporary surgical management of prolactinomas, noting that older studies are likely flawed by selection of the most aggressive prolactinomas rather than the non-aggressive tumours that predominate in hyperprolactinaemic patients in general, including those patients who simply cannot tolerate DA therapy because of ICDs or other side effects.

Mechanistic research is required to inform the pathogenesis of DA-induced ICDs. The leading theory of pathogenesis is that DA therapy causes increased dopaminergic signalling in the reward pathway, producing greater reward for a given stimulus, and hence increasing stimulus-seeking behaviours (Barake *et al.*, 2018). Alternatively, DA therapy could downregulate dopamine receptor expression, thereby lessening the reward for a given stimulus and increasing stimulus-seeking behaviours to obtain the same reward as unaffected individuals. Of these competing theories, the former is supported by a recent study showing a known activating dopamine receptor polymorphism in the *DRD3* gene to be independently predictive of the risk of DA-induced ICDs in Parkinson's disease patients (Krishnamoorthy *et al.*, 2016). ICDs could also reflect altered decision-making given the high expression of dopamine receptors in cortical regions responsible for executive function (Missale *et al.*, 1998). A personalised pharmacogenomic approach to treating hyperprolactinaemic patients may transpire through molecular research in these areas. This would complement ongoing studies on the DA-induced central nervous system side effects associated with polymorphisms in other dopaminergic pathway genes such as *DRD1*, *DRD2*, *TPH2* and *DAT* (Grall-Bronnec *et al.*, 2018) and drug transport genes including *ABCB1* encoding P-glycoprotein 1 which transports substrates across the blood-brain barrier (Athanasoulia *et al.*, 2012b). In view of our finding of the role of testosterone as a cofactor in the development of hypersexuality, genetic studies could also investigate the role of genes in the hypothalamic-pituitary-gonadal axis.

This growing area of research may be of interest to diabetes-focused endocrinologists given the 2009 FDA approval of a quick-release formulation of bromocriptine for the treatment of type 2 diabetes mellitus (Barake *et al.*, 2018). In addition, mechanistic research may be valuable in guiding the development of novel DAs that circumvent the risk of ICDs. Part of the rationale for the study in Chapter 7 investigating the genomic basis of prolactinomas was

to identify molecular changes that could be targeted by agents other than DAs, recognising that a significant proportion of hyperprolactinaemic patients do not tolerate DAs due to ICDs and other side effects. Moreover, although surgical resection is a current treatment alternative for patients with prolactinomas, postoperative remnants or recurrences occur frequently as found in 10 of the 12 operated patients reported in the prolactinoma cohort in Chapter 7.

In summary, we have shown a significantly higher prevalence of ICDs in DA-treated hyperprolactinaemic patients compared to healthy controls. The greatest risk appears to be hypersexuality with a strong male bias and a permissive effect from normal testosterone levels. This risk of DA-induced ICDs is not addressed in hyperprolactinaemia guidelines (Casanueva *et al.*, 2006; Melmed *et al.*, 2011). Moreover, only 37% of patients were aware of the relationship between DAs and ICDs before their involvement in the present study and patient awareness may be lower outside of tertiary pituitary centres. Increased awareness is required amongst endocrinologists and patients, especially in view of the multiple successful class actions against pharmaceutical companies for failing to warn patients of ICD risks in the setting of Parkinson's disease and restless legs syndrome. We recommend considering all DA-treated hyperprolactinaemic patients to be at risk of developing ICDs, educating patients regarding this risk at the time of DA commencement, directly asking patients about ICD symptoms at follow-up, and potentially using written questionnaires as in the current study to overcome communication barriers in this sensitive area. If a DA-induced ICD develops, patients should be assessed for concurrent ICDs, depression and anxiety, and DA therapy should be ceased with consideration of treatment alternatives. We recommend caution if following a wait-and-see approach given the severe, long-lasting consequences that may occur with transient ICDs (De Sousa *et al.*, 2017a). Reducing or switching DA agents should also be performed cautiously as this is not substantiated by current and previous evidence

showing ICD development regardless of DA doses (Bancos *et al.*, 2014; Celik *et al.*, 2018; Dogansen *et al.*, 2019) and agents (Martinkova *et al.*, 2011; De Sousa *et al.*, 2017a). Heightened awareness of DA-induced ICDs should improve treatment safety.

4.5 Conclusion

Building on the clinical observations outlined in Chapter 3, this cross-sectional analysis shows that DA treatment of hyperprolactinaemia poses a high, previously underestimated risk of ICDs, especially in the form of hypersexuality in eugonadal men. An improved understanding of the mechanisms of hyperprolactinaemia is required to identify molecular targets other than the dopamine receptor in order to provide pharmacological alternatives for patients who develop ICDs or other dopamine receptor-mediated side effects.

Chapter 5: Prolactin correction for adequacy of petrosal sinus cannulation may diminish diagnostic accuracy in Cushing's disease

5.1 Introduction

Although not requiring treatment in and of itself, hyperprolactinaemia is frequently seen in CD. In this chapter, we demonstrate abnormalities in prolactin secretion in patients with CD and show how this can confound the interpretation of IPSS results if ACTH is interpreted in relation to prolactin.

IPSS is primarily employed to differentiate pituitary from ectopic sources of ACTH excess in patients with ACTH-dependent CS. A secondary benefit in CD is the possibility of corticotroph lateralisation, which may aid surgical planning. ACTH central-to-peripheral (c/p) ratios ≥ 2 before or ≥ 3 after corticotrophin-releasing hormone (CRH) administration diagnose CD with sensitivity and specificity approaching 100% according to early reports (Oldfield *et al.*, 1991), although more recent data suggest sensitivity and specificity of 94% (Newell-Price *et al.*, 2006). False negative results for CD may arise due to failure to cannulate the petrosal sinuses or anatomical variation leading to venous admixture between the pituitary and periphery. There may also be venous admixture or unequal sampling between petrosal sides, leading to incorrect lateralisation or failure of lateralisation (McNally *et al.*, 1993). The accuracy of lateralisation with an ACTH intersinus gradient ≥ 1.4 is approximately 70% (Oldfield *et al.*, 1991).

Sinus serum prolactin measurement has been proposed as an addition to IPSS to improve the diagnostic accuracy of ACTH measurement alone. This is based on the assumption that there is symmetrical secretion of prolactin into each inferior petrosal sinus (IPS) (McNally *et al.*, 1993; Grant *et al.*, 2012). Several prolactin-based equations have been recommended in

the literature to correct for cannula proximity to the pituitary venous effluent. Elimination of false negative results is generally considered to be the primary role of prolactin measurement in IPSS (Findling *et al.*, 2004; Sharma *et al.*, 2011; Sharma & Nieman, 2013; Qiao *et al.*, 2015). Prolactin measurement is used in preference to other hormones as the distribution of lactotrophs is diffuse, prolactin secretion is not suppressed by cortisol, and prolactin assays are widely available (Sharma & Nieman, 2013).

Prolactin-based equations in IPSS fall into three broad categories: firstly, prolactin c/p and intersinus gradients to determine the adequacy of catheter placement; secondly, prolactin correction of ACTH c/p ratios to differentiate CD vs. EAS; and thirdly, prolactin correction of ACTH intersinus gradients to determine the side of ACTH excess in CD (Table 5.1). Some groups utilise basal prolactin levels as CRH may stimulate prolactin to variable degrees (Sharma & Nieman, 2013), whilst others utilise stimulated prolactin levels concurrent with ACTH as catheters may dislodge during IPSS (Mulligan *et al.*, 2011).

Table 5.1. Published equations employing prolactin measurement in IPSS interpretation

| PRL c/p and intersinus gradients to determine adequacy of catheter placement | | |
|--|---|--|
| | Equation | Rule |
| 1A | Peak* stimulated Rt PRL/Lt PRL (McNally <i>et al.</i> , 1993) | <1 indicates poor cannulation of Rt IPS, >1 indicates poor cannulation of Lt IPS (McNally <i>et al.</i> , 1993) |
| 1B | Basal c/p PRL on the uncorrected stimulated ACTH dominant side (Findling <i>et al.</i> , 2004; Sharma <i>et al.</i> , 2011; Sharma & Nieman, 2013) | ≥1.8 indicates adequate IPS cannulation (Findling <i>et al.</i> , 2004; Sharma <i>et al.</i> , 2011; Sharma & Nieman, 2013); <1.2 indicates poor cannulation (Findling <i>et al.</i> , 2004) |
| 1C | c/p PRL at each time point (Mulligan <i>et al.</i> , 2011; Qiao <i>et al.</i> , 2015) | ≥1.8 indicates adequate IPS cannulation (Qiao <i>et al.</i> , 2015); <1.3 indicates poor cannulation and thus indicates CD when the uncorrected ACTH c/p ratio is <2 pre-CRH and <3 post-CRH (i.e., indicates false negative ACTH result) (Mulligan <i>et al.</i> , 2011) |
| PRL correction of ACTH c/p ratios to differentiate CD vs. EAS | | |
| | Equation | Rule |
| 2A | If equation 1A is <1, correct Rt side by dividing peak* Rt ACTH by peak* stimulated Rt PRL/Lt PRL ratio; if equation 1A is >1, correct Lt side by multiplying peak* Lt ACTH by peak* stimulated Rt PRL/Lt PRL ratio; then calculate ACTH c/p ratio using concurrent pACTH (McNally <i>et al.</i> , 1993) | ≥3 indicates CD (McNally <i>et al.</i> , 1993) |
| 2B | [peak^ stimulated c/p ACTH] / [ipsilateral basal c/p PRL] (Findling <i>et al.</i> , 2004; Sharma <i>et al.</i> , 2011; Grant <i>et al.</i> , 2012; Sharma & Nieman, 2013) | >0.8 indicates CD and <0.6 indicates EAS (Findling <i>et al.</i> , 2004; Grant <i>et al.</i> , 2012); or ≥1.3 indicates CD, ≤0.7 indicates EAS and 0.7-1.3 indeterminate (Sharma <i>et al.</i> , 2011; Sharma & Nieman, 2013) |
| 2C | [peak^ c/p ACTH at any time point] / [ipsilateral concurrent c/p PRL] (Qiao <i>et al.</i> , 2015) | >0.8 indicates CD and <0.6 indicates EAS (Qiao <i>et al.</i> , 2015) |
| PRL correction of ACTH intersinus gradients to determine side of adenoma in CD | | |
| | Equation | Rule |
| 3A | [dominant cACTH] / [non-dominant cACTH], using PRL-corrected cACTH from equation 2A (McNally <i>et al.</i> , 1993) | ≥1.4 indicates lateralisation of CD towards ACTH dominant side (McNally <i>et al.</i> , 1993) |
| 3B | Peak^ [dominant cACTH / ipsilateral concurrent cPRL] / [non-dominant cACTH / ipsilateral concurrent cPRL] at any time point (Mulligan <i>et al.</i> , 2012; Qiao <i>et al.</i> , 2015) | ≥1.4 indicates lateralisation of CD towards ACTH dominant side (Mulligan <i>et al.</i> , 2012; Qiao <i>et al.</i> , 2015) |

ACTH, adrenocorticotrophic hormone; c, central; CD, Cushing's disease; CRH, corticotrophin-releasing hormone; EAS, ectopic ACTH syndrome; IPS, inferior petrosal sinus; Lt, left; p, peripheral; PRL, prolactin; Rt, right; * refers to peak absolute value; ^ refers to peak ratio

The objective of this study was to assess the effect of using prolactin measurements to confirm petrosal sinus cannulation and correct for any dilution of petrosal blood from non-petrosal sinus sources.

5.2 Methods

Patients and IPSS procedure

We retrospectively studied thirteen consecutive patients (10 women, 3 men, median age 49 yr) with clinical and biochemical ACTH-dependent CS diagnosed in accordance with the 2008 Endocrine Society guidelines (Nieman *et al.*, 2008). Following informed consent, patients underwent IPSS with prospective measurement of ACTH and prolactin at two tertiary referral centres in Australia: Royal Adelaide Hospital and Royal North Shore Hospital. IPSS was performed between 2001 and 2017. Concurrent venous samples were taken from the periphery and each IPS at baseline and 2, 5, 10 and 15 min after CRH administration. Baseline samples consisted of two samples in 7/13 patients and a single sample in 6/13. Basal ACTH and prolactin levels were derived from the averages in patients with two baseline samples. The 15-min ACTH and prolactin values were not available in one patient. Retrograde flow of contrast dye into the contralateral IPS on venography confirmed bilateral IPS cannulation in all but one patient where cannulation appeared likely successful in the right IPS and likely failed in the left IPS.

Preoperative findings

Each patient met diagnostic criteria for CD on IPSS with uncorrected ACTH c/p ratios ≥ 2 before and ≥ 3 after CRH. The side of ACTH excess could be determined by IPSS with peak ACTH intersinus gradients ≥ 1.4 in all patients. MRI demonstrated no adenoma in three

patients, a central tumour in one patient and a lateral tumour in nine patients. IPSS/MRI lateralisation was concordant in 5/9 patients and discordant in 4/9.

Surgical outcomes

Of 11 operated patients, transsphenoidal surgery resulted in cure in 7/11, improvement of hypercortisolism in 1/11 and no response in 2/11. Cure status is uncertain in the final patient as she remains eucortisolaemic after undergoing surgery during a period of eucortisolaemia in cyclical CD. An adenoma was identified on histopathology in 7/11 operated patients. All adenomas stained for ACTH, except for one adenoma which stained only for prolactin and follicle-stimulating hormone. One patient died due to sepsis before his scheduled surgery and another is awaiting surgery. Clinical data are summarised in Table 5.2.

Table 5.2. Characteristics of patients with Cushing's syndrome who underwent IPSS

| Pt | Age (yr) | Sex | ACTH dominant side | PRL dominant side | MRI tumour side | Surgical tumour side | Postoperative biochemical remission | Adenoma at histopathology | Positive IHC |
|----|----------|-----|--------------------|-------------------|-----------------|----------------------|-------------------------------------|---------------------------|-------------------|
| 1 | 40 | F | R | R | L | Not stated | Y | Y | ACTH, PRL, FSH |
| 2 | 31 | F | L | L | L | Not stated | N | Y | PRL, FSH |
| 3 | 55 | F | L | L | L | L | Y | N | N/A ^a |
| 4 | 76 | M | L | L | Not seen | N/A ^b | N/A ^b | N/A ^b | N/A ^b |
| 5 | 20 | F | R | R | L | L | Partial | Y | ACTH, FSH, GH, LH |
| 6 | 45 | M | L | L | Not seen | R | Y | Y | ACTH |
| 7 | 49 | F | R | R | L | Midline | Y | Y | ACTH, galanin |
| 8 | 54 | M | L | L | Not seen | Not stated | N | N | N/A ^a |
| 9 | 63 | F | R | R | R | R | Uncertain ^c | N | N/A ^a |
| 10 | 63 | F | L | L | Midline | N/A ^d | N/A ^d | N/A ^d | N/A ^d |
| 11 | 26 | F | R | R | R | R | Y | Y | ACTH |
| 12 | 38 | F | R | R | R | R | Y | Y | ACTH |
| 13 | 54 | F | R | R | L | L | Y | N | N/A ^a |

ACTH, adrenocorticotropic hormone; F, female; FSH, follicle-stimulating hormone; GH, growth hormone; IHC, immunohistochemistry; L, left; LH, luteinising hormone; M, male; MRI, magnetic resonance imaging; N, no; No, patient number; N/A, not applicable; PRL, prolactin; R, right; Y, yes; ^a adenoma not found at histopathology; ^b never underwent surgery; ^c remains eucortisolaemic 2 weeks after surgery performed during eucortisolaemic period in cyclical CD; ^d surgery not yet performed

IPSS ACTH and prolactin analyses

IPSS results were reanalysed employing published prolactin-corrected equations (Table 5.1). Unless specified otherwise, the dominant side of ACTH or prolactin production was considered to be the side of the highest intersinus gradient for that hormone in either the basal or stimulated state.

5.3 Results

All patients exhibited ACTH and prolactin intersinus gradients ≥ 1.4 , with concordance between the ACTH dominant and prolactin dominant sides in each case. Mean prolactin values on the ACTH dominant and non-dominant sides paralleled that of ACTH (Figure 5.1). In 10/13 patients, the peak ACTH value occurred after CRH administration.

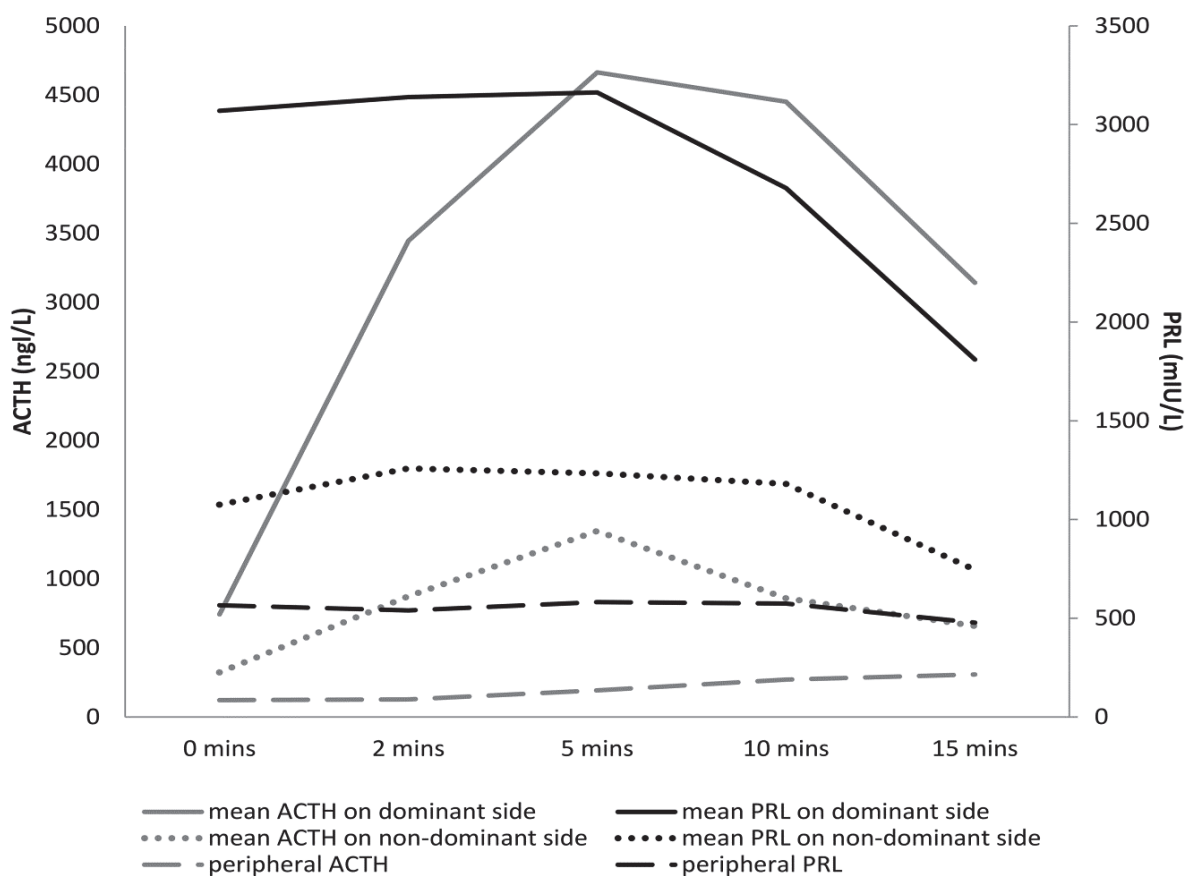


Figure 5.1. Mean ACTH and prolactin values on ACTH dominant and non-dominant sides

ACTH, adrenocorticotrophic hormone; PRL, prolactin

Prolactin gradients to determine adequacy of catheter placement

The ratio of peak stimulated right-to-left prolactin (equation 1A, Table 5.1) was >1 in all patients with right-sided ACTH dominance, suggesting inadequate sampling of the left pituitary. The ratio was <1 in all patients with left-sided ACTH dominance, suggesting inadequate sampling of the right pituitary (McNally *et al.*, 1993).

The ratio of basal c/p prolactin (equation 1B) was ≥ 1.8 in all patients on the ACTH dominant side, satisfying criteria for correct catheter placement (Findling *et al.*, 2004; Sharma *et al.*, 2011; Sharma & Nieman, 2013). However, if the ACTH non-dominant side was assessed as suggested for exploration by Sharma *et al.* (2011), then the thresholds recommended by Findling *et al.* (2004) would suggest that 6/13 patients were not cannulated on the ACTH non-dominant side due to basal prolactin c/p ratios <1.2 . The thresholds recommended by Sharma and colleagues (Sharma *et al.*, 2011; Sharma & Nieman, 2013) would suggest that 7/13 patients were not cannulated due to a ratio of 1.4 in an additional patient.

Assessing every time point (equation 1C), 4/13 patients had all prolactin c/p ratios <1.3 on one side suggesting poor catheter placement on this side (Mulligan *et al.*, 2011). Using the higher threshold of <1.8 (Qiao *et al.*, 2015), 6/13 patients appeared to have poor catheter placement on one side. In every case, it was the ACTH non-dominant side that appeared to have not been cannulated according to prolactin-corrected criteria.

Prolactin correction of ACTH to diagnose CD

The peak stimulated ACTH c/p ratios were lowered by correction from the ipsilateral basal prolactin c/p (equation 2B) in every patient. Whether or not patients still met a diagnosis of CD depended on the thresholds used to classify patients as having CD, EAS or indeterminate results. Using the threshold of >0.8 to diagnose CD (Findling *et al.*, 2004; Grant *et al.*, 2012),

all patients were still diagnosed with CD. However, using the intermediate zone defined as 0.7-1.3 by Sharma and colleagues (Sharma *et al.*, 2011; Sharma & Nieman, 2013), 2/13 patients were classified as indeterminate. Using the same thresholds and the highest ACTH ratio from the ACTH non-dominant side in this equation as also done by Sharma and colleagues (Sharma *et al.*, 2011; Sharma & Nieman, 2013), 3/13 patients were classified as indeterminate and 1/13 was classified as ectopic ACTH syndrome (EAS). The latter patient ultimately had transsphenoidal surgery confirming a diagnosis of CD through biochemical remission and an ACTH-staining adenoma on histopathology. Applying the same ratio but using concurrent ACTH and prolactin values and the peak ratio at any time before or after CRH administration (equation 2C), the corrected ACTH c/p ratios were again lower than uncorrected ratios, but CD was still diagnosed in all patients using the ratio of >0.8 to indicate CD (Qiao *et al.*, 2015).

McNally *et al.* (1993) advise correction of the ACTH c/p ratio only on the prolactin non-dominant side (equation 2A). If the peak stimulated right-to-left prolactin ratio is <1 , the right ACTH value should be corrected by dividing by this ratio. If the ratio is >1 , the left ACTH value should be corrected by multiplying by this ratio. In either case, the ACTH result is increased after correction. As the prolactin non-dominant side was the same as the ACTH non-dominant side in our patients, ACTH c/p ratios were only corrected on the side of the lower ACTH values. Using the standard uncorrected stimulated ACTH c/p ratio of ≥ 3 with the non-dominant data, 6/13 patients did not meet a diagnosis of CD on this side. Using equation 2A to correct the ACTH c/p ratio on the non-dominant side, 11/13 patients now had ratios ≥ 3 , thereby increasing the sensitivity of IPSS on the ACTH non-dominant side.

Prolactin correction of ACTH to lateralise adenomas in CD

Similarly to equation 2A, McNally *et al.* (1993) advise only correcting the ACTH value on the prolactin non-dominant side before calculating the ACTH intersinus gradient (equation 3A). As explained above, this correction increased the ACTH value on the non-dominant side and therefore it generally lowered the ACTH intersinus gradient compared to the uncorrected gradient. After correction and using a lateralisation threshold of ≥ 1.4 (McNally *et al.*, 1993), the intersinus gradient was lowered in 12/13 patients with ACTH no longer lateralising in 2/13 patients and the side of ACTH excess reversed in 4/13 patients.

Mulligan *et al.* (2012) instead advise correcting the ACTH intersinus gradient by the concurrent prolactin intersinus gradient (equation 3B), with a peak value of ≥ 1.4 defined as lateralisation. Using this correction, 11/13 patients had lower corrected ACTH intersinus gradients compared to their uncorrected gradients and 6/13 patients exhibited reversal of lateralisation.

Tumours were lateralised by surgical findings in 7/13 patients, six of whom had a clear therapeutic response. Surgical and IPSS findings were concordant in 4/7 of these patients. This was irrespective of whether or not prolactin was used to correct the ACTH intersinus gradient, although which patients had concordant surgical and IPSS findings differed depending on whether uncorrected or corrected gradients were used.

5.4 Discussion

Applying published prolactin-corrected equations to our local IPSS data from 13 patients with known CD led to misleading results. The erroneous interpretations that can be reached include lack of adequate cannulation, incorrect lateralisation and, most concerning, incorrect diagnosis of EAS. The use of prolactin in IPSS appeared to be fundamentally flawed

because of prolactin intersinus gradients ≥ 1.4 favouring the ACTH dominant side in all 13 patients.

The phenomenon of parallel ACTH/prolactin secretion in CD is likely explained by peritumoural prolactin production. Contralateral lactotroph suppression due to local hyperprolactinaemia may also occur. Other studies have similarly demonstrated prolactin intersinus gradients favouring the ACTH dominant or tumour side in CD (Crock *et al.*, 1988; Schulte *et al.*, 1988; Zovickian *et al.*, 1988; Tabarin *et al.*, 1992; Loli *et al.*, 1998; Daousi *et al.*, 2010). This local prolactin excess may explain the peripheral hyperprolactinaemia found in 23-50% of CD patients (Yamaji *et al.*, 1984; Crock *et al.*, 1988). The mechanism of peritumoural prolactin production may be a paracrine effect of β -endorphin or galanin, both of which are secreted by corticotrophinomas and can stimulate lactotrophs (Schulte *et al.*, 1988; Freeman *et al.*, 2000). This lactotroph stimulation, coupled with the hyperplasia-adenoma sequence apparent in the pituitary (Horvath *et al.*, 1999; Villa *et al.*, 2011), may underlie the not infrequent finding of prolactinomas as a secondary tumour in patients with CD. A review of 660 surgically treated CD patients found that prolactinomas were the commonest secondary tumour (Ratliff & Oldfield, 2000). We too found an incidental prolactinoma in a patient with persistent CD following pituitary surgery, and we recently encountered an incidental prolactinoma in another patient with CD (data not shown). The alternative explanation – co-secretion of prolactin from corticotrophinomas – is less likely as we found only 1/7 identifiable adenomas in our cohort stained for both prolactin and ACTH. Co-staining in CD patients has been found to be uncommon in other studies, even when prolactin excess has been observed on the tumour side (Crock *et al.*, 1988; Tabarin *et al.*, 1992; Loli *et al.*, 1998). This includes one study where the adenomatous cells from one such patient did not produce prolactin basally or upon CRH stimulation during *in vitro* studies (Tabarin *et al.*, 1992).

Adequacy of petrosal sinus cannulation

Six studies have recommended comparing central and peripheral prolactin values to determine the adequacy of cannula placement (McNally *et al.*, 1993; Findling *et al.*, 2004; Mulligan *et al.*, 2011; Sharma *et al.*, 2011; Sharma & Nieman, 2013; Qiao *et al.*, 2015). The repeated demonstration of asymmetrical prolactin secretion in CD by us and others (Crock *et al.*, 1988; Schulte *et al.*, 1988; Zovickian *et al.*, 1988; Wittert *et al.*, 1990; Tabarin *et al.*, 1992; Loli *et al.*, 1998; Daousi *et al.*, 2010) strongly challenges the validity of using the prolactin intersinus gradient to determine cannulation adequacy. For example, equations 1A and 1C will lead the non-tumour side to appear inadequately cannulated in most cases. Using equation 1B in our data, IPS cannulation appeared adequate, although this was only because the equation stipulates the use of the IPS and time point of maximal hormonal secretion. If the tumour side was not cannulated and the only available data were from the non-tumour side in CD, then reliance on the prolactin c/p gradient may suggest that this non-tumour side was also not cannulated because of lactotroph suppression. Six of our patients had basal prolactin c/p ratios <1.8 suggesting poor catheter placement on one side according to equation 1B and this was always on the ACTH non-dominant side. Furthermore, prolactin c/p gradients should exist in both CD and EAS as the pituitary is always the source of prolactin. Other than the non-tumour side in CD, the absence of this gradient should be considered to simply reflect further distance of the cannula from the pituitary. In disagreement with Mulligan *et al.* (2011), we argue that the absence of a prolactin c/p gradient should not be taken to imply a diagnosis of CD.

Differentiation of CD from EAS

Six studies have outlined the use of prolactin-corrected ACTH c/p ratios to differentiate CD from EAS (McNally *et al.*, 1993; Findling *et al.*, 2004; Sharma *et al.*, 2011; Grant *et al.*, 2012;

Sharma & Nieman, 2013; Qiao *et al.*, 2015). Because of peritumoural prolactin production in CD, prolactin corrections will reduce the difference between central and peripheral ACTH on the tumour side. Our data showed lower prolactin-corrected ACTH c/p ratios using equation 2B compared to uncorrected ratios. Some adjustment for this is made by using lower diagnostic c/p ACTH thresholds to indicate CD (Table 5.1) compared to uncorrected ACTH criteria. Still, in our cohort, corrected ACTH c/p ratios did not satisfy the more rigorous prolactin-corrected CD criteria proposed by Sharma and colleagues (Sharma *et al.*, 2011; Sharma & Nieman, 2013) in 2/13 patients, leading to potential misdiagnosis.

Equation 2A advises prolactin correction of the ACTH c/p ratio only on the side with lower prolactin values. As this is usually the non-tumour side in CD, this makes the ACTH c/p ratio larger on this side which may improve capture of CD in cases where the tumour side has not been cannulated. When looking only at the ACTH non-dominant side in our patients, 11/13 had prolactin-corrected stimulated ACTH c/p ratios consistent with CD, whereas only 6/13 had uncorrected ACTH c/p ratios consistent with CD. Prolactin correction may therefore increase IPSS sensitivity if data are only available from the non-tumour side in CD; however, specificity has not been assessed as we had no EAS patients and other studies have had few EAS cases.

As prolactin is produced by the pituitary in both CD and EAS, poor catheter placement in either disease will cause prolactin c/p gradients to approach 1 and lead to increases in corrected ACTH levels, potentially masking the diagnosis of EAS. As there are no published cases of surgically confirmed EAS and poor catheter placement bilaterally, it is unclear if prolactin correction could cause a false positive result.

Lateralisation of corticotrophinomas

Three studies have discussed the use of prolactin-corrected ACTH intersinus gradients to lateralise the source of ACTH excess in CD (McNally *et al.*, 1993; Mulligan *et al.*, 2012; Qiao *et al.*, 2015). However, peritumoural prolactin production implies that prolactin correction should diminish the ACTH difference between petrosal sides. The only case where the prolactin-corrected intersinus gradient is expected to be higher and towards the tumour side is when the tumour side is not adequately cannulated. Most of our patients had higher uncorrected than corrected intersinus gradients, and 4/13 or 6/13 patients had a reversed ACTH intersinus gradient depending on the correction method used. Another study similarly found that the uncorrected and corrected ACTH intersinus gradients were discordant in 3/11 CD patients, and corticotrophinoma side at surgery was predicted by the uncorrected rather than the corrected intersinus gradient in each case (Zovickian *et al.*, 1988). We found that 4/7 patients with corticotrophinoma lateralisation at surgery had concordance between their surgical and IPSS findings (Table 5.2), regardless of whether prolactin corrections were performed or not. Furthermore, the side of uncorrected ACTH and prolactin dominance was discordant with MRI findings in 4/9 patients with a visible lateralised tumour, and yet all four of these patients experienced complete or partial remission following transsphenoidal surgery. These data indicate that preoperative lateralisation results were scarcely better than chance to predict the side of the adenoma, consistent with previous data (Oldfield *et al.*, 1991), and prolactin failed to improve the success of preoperative lateralisation. IPSS studies in healthy volunteers have demonstrated significant ACTH intersinus gradients, representing physiological ACTH secretory asymmetry, perhaps due to a dominant petrosal sinus and/or asymmetrical pituitary function (Yanovski *et al.*, 1993; Kalogeras *et al.*, 1996). Incorrect lateralisation may also arise due to hypoplastic or plexiform petrosal sinuses, which, if present on the corticotrophinoma side and associated with anomalous drainage,

may even result in an overall false negative result for CD (Doppman *et al.*, 1999). These findings contest the recommendation to use either uncorrected or corrected ACTH intersinus gradients to guide hemihypophysectomy as proposed by others (Mulligan *et al.*, 2012).

Limitations of the extant literature

The evidence base of prolactin use in IPSS is fraught with heterogeneity and sometimes limited detail in the equations, time points and diagnostic thresholds used. Some studies also employed different stimulation methods with McNally *et al.* (1993) using CRH and TRH and Qiao *et al.* (2015) using desmopressin alone. In addition, a definitive diagnosis of CD and the side of the corticotrophinoma may be difficult to determine, hampering the ability to confirm or deny the utility of prolactin-related equations. A combination of histopathology showing an ACTH-staining adenoma and/or surgical cure was used to confirm CD in most studies (McNally *et al.*, 1993; Mulligan *et al.*, 2011; Grant *et al.*, 2012; Qiao *et al.*, 2015), whilst others confirmed CD by both positive histopathology and surgical cure (Mulligan *et al.*, 2012) or positive histopathology alone (Sharma *et al.*, 2011; Sharma & Nieman, 2013). Another issue is the paucity of EAS cases. Amongst the studies cited in Table 5.1, 18 patients with definitive surgical diagnoses were considered to have uncorrected IPSS results that were borderline or incorrect. In each case, applying the prolactin-related equations resulted in a diagnosis of CD, which was correct in all but one patient who had cyclical CS due to EAS with IPSS inappropriately performed during a period of eucortisolaemia (Sharma *et al.*, 2011). These cases are taken as supportive of prolactin use; however, the high pretest probability of CS being due to CD means that this may simply be due to chance.

Limitations of this study

Our study is limited by its small size and lack of EAS cases. As one of the key roles of prolactin in IPSS is to eliminate false negative results, prolactin-corrected equations are often recommended only when uncorrected ACTH ratios are negative for CD (Findling *et al.*, 2004; Sharma *et al.*, 2011; Mulligan *et al.*, 2012; Sharma & Nieman, 2013; Qiao *et al.*, 2015), whereas all patients in our cohort had uncorrected ACTH ratios consistent with CD. Nonetheless, depending on the equation used, either two or four of our patients could have been misdiagnosed as having EAS or indeterminate results through use of prolactin correction. Showing the failures and inconsistencies when applying these equations to otherwise unambiguous IPSS data from unselected patients illustrates the theoretical arguments against prolactin use. Other limitations of our study were that surgery was not performed or did not improve hypercortisolism in four patients, adenoma side was variably reported at surgery, and some adenomas crossed the midline on MRI or at surgery. The variability in corticotrophinoma side by surgical, IPSS and MRI findings in our patients illustrates the general uncertainty of lateralisation in CD.

Use of prolactin correction in low volume centres

The utility of prolactin in IPSS has been cited in CD diagnostic guidelines (Machado *et al.*, 2016), and specifically recommended in low volume centres in order to overcome higher failure rates of IPS cannulation (McNally *et al.*, 1993; Mulligan *et al.*, 2011). Applying these complex equations and interpreting the sometimes misleading results may be even more hazardous in such centres in terms of the endocrinologist's interpretation of the data to reach a diagnosis and the surgeon's operative planning, which could include hemihypophysectomy based on misleading ratios. Though prolactin measurement has been asserted to have no additional time costs during IPSS (Grant *et al.*, 2012), we argue the

contrary as ACTH measurement should be performed in whole blood in a dedicated pre-cooled K-EDTA vial whilst prolactin measurements should be made from a serum vial. The time required to collect an extra blood sample at each IPS and the periphery at every time point should not be underestimated, particularly in low volume centres with less experienced radiologists, endocrine nurses and endocrine trainees.

5.5 Conclusion

This study revealed universal co-lateralisation of prolactin and ACTH in IPSS in CD patients, with a probable biological basis. This co-lateralisation indicates that prolactin concentrations cannot be used to correct for completeness of sinus cannulation or venous admixture. Routine use of prolactin correction in IPSS is not necessary and may be misleading when uncorrected ACTH concentrations demonstrate a clear c/p gradient on at least one side. Moreover, prolactin correction does not improve lateralisation. Based on our data, we suggest that IPS prolactin measurements should not be used in the diagnosis of a pituitary vs. ectopic ACTH source or to predict corticotrophinoma side.

Chapter 6: Vasculogenic hyperprolactinaemia: severe prolactin excess in association with internal carotid artery aneurysms

6.1 Introduction

The previous chapters focus on prolactinomas and CD as well-known causes of prolactin excess. This chapter examines the lesser known association between hyperprolactinaemia and ICA aneurysms. This underrecognised cause of hyperprolactinaemia is especially noteworthy as it is one of only a few causes of severe hyperprolactinaemia.

Serum concentrations of prolactin >10-fold normal are virtually exclusively seen in patients with macroprolactinomas (Biller *et al.*, 1999; Casanueva *et al.*, 2006; Karavitaki *et al.*, 2006; Vilar *et al.*, 2008; Melmed *et al.*, 2011) or during pregnancy/lactation (Hu *et al.*, 2018). In other causes, such as interference in the inhibitory effect of dopamine due to drugs or pituitary stalk compression, prolactin rarely exceeds six-fold normal (Casanueva *et al.*, 2006).

We report two men who presented with headaches and visual field defects due to cavernous ICA aneurysms with the surprising finding of marked hyperprolactinaemia. Macroprolactin was excluded by PEG precipitation and pituitary tumours were not identified on MRI. We hypothesised that the abnormal endothelium of ICA aneurysms produces a factor responsible for paracrine or endocrine stimulation of the nearby lactotrophs leading to marked hyperprolactinaemia, analogous to the physiological hyperprolactinaemia of pregnancy. To explore this, we undertook biochemical and molecular studies directed towards identification of the stimulus underlying 'vasculogenic' hyperprolactinaemia.

A number of factors have been found to influence prolactin secretion *in vitro* and in animal studies (Freeman *et al.*, 2000). We limited our molecular studies to candidates that could feasibly account for hyperprolactinaemia both in pregnancy and in patients with ICA

aneurysms, based on current knowledge. The PrRFs studied were those that: 1. have a predominantly or purely stimulatory effect on prolactin secretion by lactotrophs that is abrogated by DA therapy; 2. are produced by vascular tissue; and 3. are upregulated in pregnancy and/or produced by placental tissue. The PrRFs fulfilling these criteria based on previous research included angiotensin II (Kalenga *et al.*, 1996; Burnstock, 1999; Freeman *et al.*, 2000), substance P (Burnstock, 1999; Freeman *et al.*, 2000; Marzioni *et al.*, 2005) and histamine (Burnstock, 1999; Freeman *et al.*, 2000; Pap *et al.*, 2007). We also studied prolactin-releasing hormone (PRLH) as it is produced by placental decidua and has been demonstrated to have a paracrine effect on prolactin secretion in the placenta (Reis *et al.*, 2002). *PRLH* gene expression has been demonstrated in the central nervous system, small and large intestine (Roland *et al.*, 1999) and pancreas (Fujii *et al.*, 1999), but not in endothelial tissue. We anticipated that, if aneurysms produce PRLH in abundance, there would be sufficient spill over of PRLH into the general circulation to be measurable in peripheral blood. As aneurysms cause endothelial cell apoptosis and shear stress (Sforza *et al.*, 2009) and endothelial cells produce RNA-containing exosomes (de Jong *et al.*, 2012), we also expected RNA from the aneurysmal tissue to be present in circulating blood. We therefore assessed the production of multiple PrRFs by the aneurysms through RT-PCR of circulating RNA, in addition to PRLH measurement by enzyme linked immunosorbent assay (ELISA).

6.2 Methods

Clinical, biochemical and radiological data were collected from two affected patients at the Royal Adelaide Hospital and Flinders Medical Centre in Adelaide, Australia. We performed RT-PCR and ELISA of proposed circulating PrRFs likely to be elevated in the setting of aneurysms. Informed consent for publication was obtained from patients.

In brief, circulating RNA was extracted (Qiagen) from the plasma of the patients at baseline and during DA therapy. Extracted RNA was reverse transcribed to cDNA (SuperScript-III) and RT-PCR was performed for the following genes: *AGT* encoding angiotensinogen (the precursor of angiotensin II), *TAC1* encoding substance P, and *HDC* encoding the enzyme responsible for conversion of histidine to histamine. Results were normalised against expression of the housekeeping gene, *PPIA*, encoding cyclophilin A. Plasma and serum samples from two pregnant women in the second trimester of pregnancy served as positive controls. Samples from two healthy middle-aged men served as negative controls.

Serum PRLH was quantified using a commercially obtained ELISA for prolactin-releasing peptide-31 (PrRP-31, Phoenix Pharmaceuticals, U.S.A.) with intraassay and interassay coefficients of variability <10% and <15%, respectively. Serum samples from the patients at baseline and during DA therapy were compared against one pregnant (positive) and two male (negative) control samples.

In addition, we conducted a literature review to identify other patients with ICA aneurysms and hyperprolactinaemia deemed to be unrelated to stalk effect (i.e., prolactin >2,000 mIU/L or >94 ng/mL). Text and/or images from previous publications were used to determine aneurysm location by the Bouthillier classification of ICA segments numbered in the direction of blood flow: C1, cervical; C2, petrous; C3, lacerum; C4, cavernous; C5, clinoid; C6, ophthalmic; and C7, communicating (Bouthillier *et al.*, 1996).

6.3 Results

Patient 1: A 50-year-old man, presented with headache and bitemporal hemianopia. Investigations revealed a serum prolactin level of 187-fold normal at 97,780 mIU/L (reference interval 100-525 mIU/L) with central hypocortisolism, hypothyroidism and hypogonadism, and bitemporal hemianopia. MRI demonstrated a 39 mm right cavernous

ICA aneurysm occupying the pituitary fossa and compressing the optic chiasm (Figure 6.1A). The pituitary was not visible. A single dose of cabergoline reduced prolactin by >50% and 5 days later, a flow-diverting stent was inserted. Prolactin gradually declined and then increased after 3 weeks. Regular cabergoline was subsequently commenced with prolactin normalisation (Figure 6.2A). After 16 months, cabergoline was ceased, leading to prolactin elevation to 28,877 mIU/L. Repeat MRI showed a persistent, but smaller, ICA aneurysm. The patient's visual fields normalised, although he continues to require hydrocortisone, thyroxine and testosterone replacement for hypopituitarism.

Patient 2: A 70-year-old man presented with headache and a symptomatic left visual field defect shown to be due to temporal hemianopia with a left afferent pupillary defect. MRI demonstrated a partially thrombosed 38 mm left cavernous ICA aneurysm, occupying the suprasellar space and extending into the pituitary fossa (Figure 6.1B). The pituitary and optic chiasm were displaced. Investigations revealed a prolactin level of 134-fold normal at 70,355 mIU/L, central hypogonadism and borderline central hypothyroidism. Monitoring of the aneurysm by serial MRI scans demonstrated remodelling with progressive thrombus formation. Initiation of cabergoline resulted in normalisation of prolactin and improvement in testosterone (Figure 6.2B). The patient's visual defects gradually resolved. His TSH also gradually increased and thyroxine was not required, although he has most recently commenced testosterone replacement as his serum testosterone did not fully normalise.

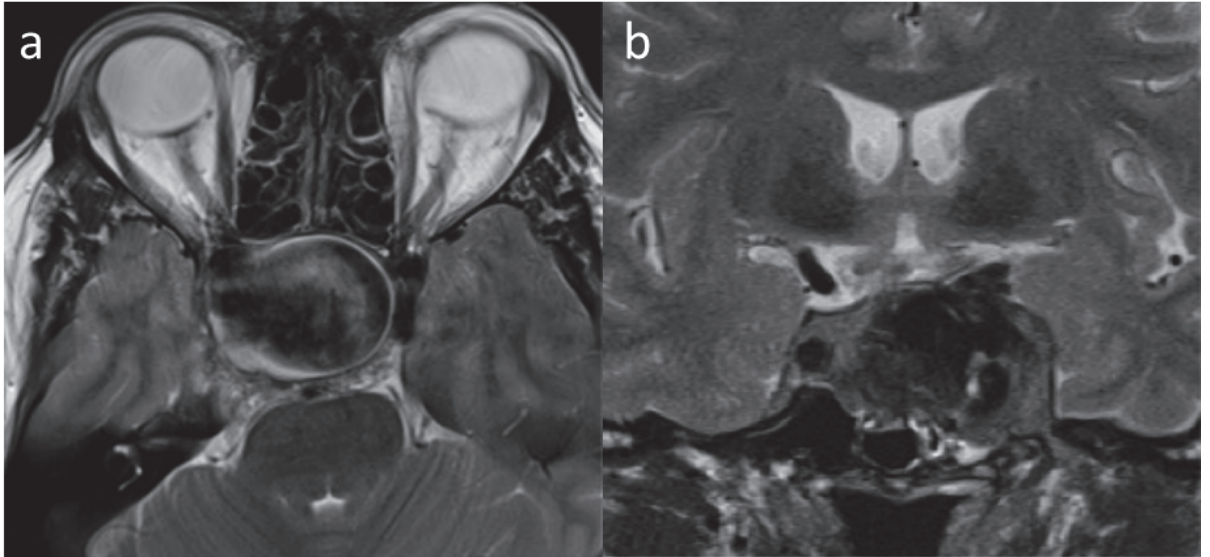


Figure 6.1 T2 MRI images of the ICA aneurysms

A. Patient 1, axial view

B. Patient 2, coronal view

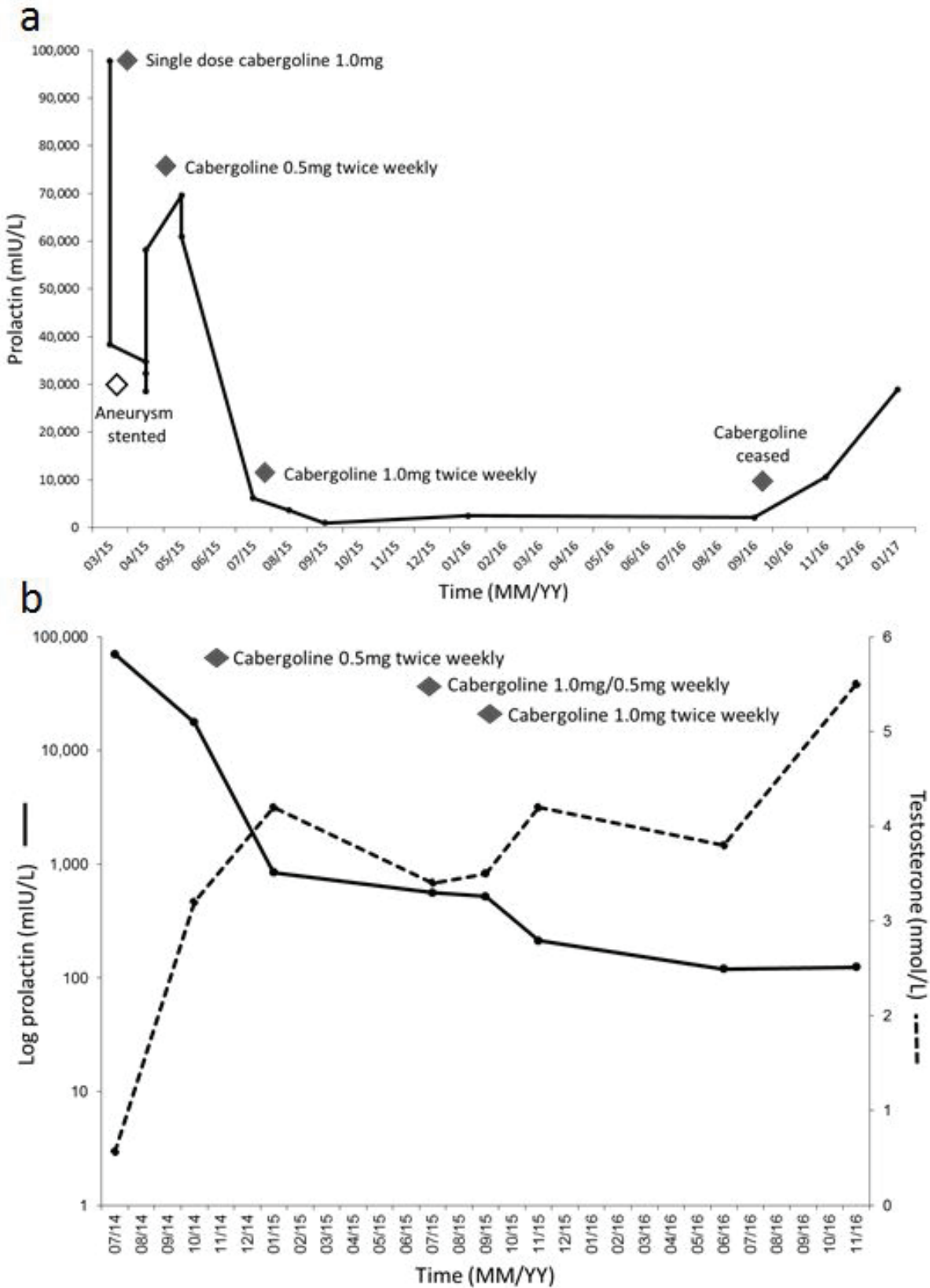


Figure 6.2 Hormonal profiles in response to therapeutic interventions

A. Prolactin profile in Patient 1

B. Prolactin (solid line) and testosterone (dashed line) profiles in Patient 2

RT-PCR analysis of circulating RNA showed no detectable levels of expression for the *AGT* or *TAC1* genes in patients or controls. *HDC* expression levels showed no differences between the patient, male control and pregnant control samples (Figure 6.3).

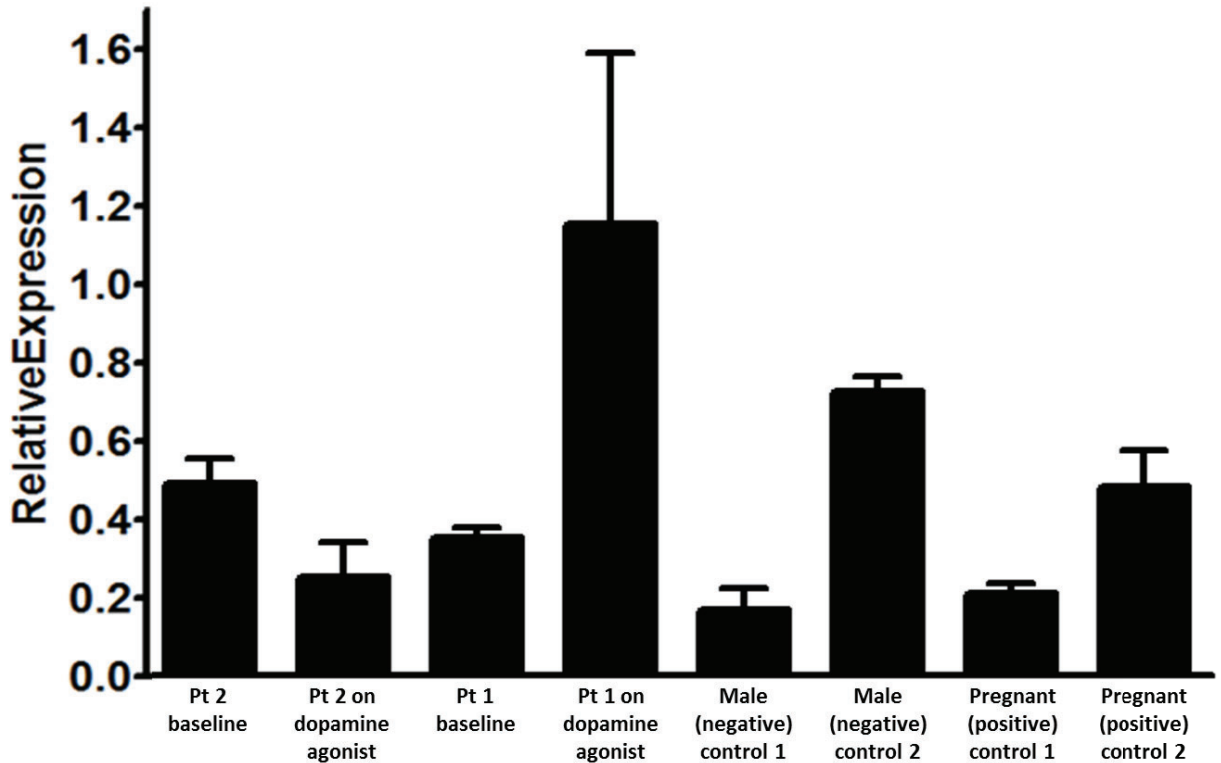


Figure 6.3 Overlapping *HDC* circulating RNA levels between patients and controls

Pt, patient

ELISA showed no difference in PrRP-31 levels between patients (<0.5-13 pmol/L) and male controls (4.6 and 12 pmol/L). Compared to these samples, PrRP-31 was up to two-fold higher in the pregnant control (25 pmol/L).

Review of the literature revealed 11 published case reports of patients with ICA aneurysms and serum prolactin levels >2,000 mIU/L (94 ng/mL), consistent with vasculogenic hyperprolactinaemia (Table 6.1) (Verbalis *et al.*, 1982; Garg & Dash, 1985; Ooi & Russell, 1986; Fernandez-Real *et al.*, 1994; Kahn *et al.*, 1997; Heshmati *et al.*, 2001; Duarte *et al.*, 2008; Gungor *et al.*, 2015; Goldman *et al.*, 2016). In 8/11 cases, the aneurysm was located in

or proximal to the C6 (ophthalmic) segment of the ICA. In one case, the aneurysm was located in the C7 (communicating) segment. The aneurysm could not be localised to a specific segment in the two remaining cases.

Table 6.1. Published cases with apparent vasculogenic hyperprolactinaemia

Cases are listed in ascending order of the reported degree of hyperprolactinaemia

| Ref | Age (Yr), sex | Presentation | Aneurysm origin, segment* | Aneurysm extension; other features | Aneurysm size (mm) | Baseline PRL, mIU/L ^a | Aneurysm surgery | DA | PRL normalisation |
|---------------------------------------|---------------|---|---------------------------|---|--------------------|----------------------------------|------------------|----|-------------------|
| (Caldas <i>et al.</i> , 1998) | 42 F | Headache, galactorrhea | ICA, C5 | U/A | U/A | U/A | Y | N | Y |
| (Heshmati <i>et al.</i> , 2001) | 65 F | Hypopituitarism | ICA, segment uncertain | N/S | N/S | 2,058 | N | N | N/S |
| (Garg & Dash, 1985) | 32 F | Galactorrhea, secondary amenorrhoea, headache, collapse | Rt ICA, C7 | Suprasellar; with SAH | N/S | 3,300 | N | Y | Y |
| (Heshmati <i>et al.</i> , 2001) | 60 F | Weight loss | Lt ICA, likely C4/5 | Intrasellar | N/S | 3,360 | N | N | N/S |
| (Verbalis <i>et al.</i> , 1982) | 59 F | Headache, Rt vision loss | Rt ICA, likely C5 | Intra/suprasellar | >30 | 3,612 | Y | N | Y |
| (Ooi & Russell, 1986) | 74 F | Fatigue, headache, confusion | Lt ICA, C4 | Intra/suprasellar | 18 | 3,826 | N | N | N/S |
| (Goldman <i>et al.</i> , 2016) | 86 F | Central hypothyroidism upon T4 withdrawal after thyroidectomy for cancer | Rt ICA, likely C4 | Intra/suprasellar | 30 | 3,885 | N | N | N/S |
| (Gungor <i>et al.</i> , 2015) | 71 M | Hypopituitarism, VF deficit | Rt ICA, C5 | Intra/suprasellar | 29 | >4,431 | Y | N | Y |
| (Kahn <i>et al.</i> , 1997) | 42 F | Galactorrhea, Lt vision loss, headache | Lt ICA, C5/6 | Intra/suprasellar; partially thrombosed | 20 | 7,665 | Y | N | Y |
| (Duarte <i>et al.</i> , 2008) | 72 F | Headache, nausea, hypertension | Rt ICA, segment uncertain | Intra/suprasellar; partially thrombosed | 20 | 29,463 | N | Y | Y |
| (Fernandez-Real <i>et al.</i> , 1994) | 52 F | Headache, reduced consciousness, diplopia, Lt ophthalmoplegia, Rt hemiparesis | Lt ICA, C5 | Intrasellar; with SAH | >25 | 35,000 | Y | N | N |

DA, dopamine agonist therapy; F, female; ICA, internal carotid artery; Lt, left; M, male; N, no; N/S, not stated; PRL, prolactin; Ref, reference; Rt, right; SAH, subarachnoid haemorrhage; T4, thyroxine; U/A, unavailable as full-text manuscript unable to be accessed; VF, visual field; Y, yes; * segment as per Bouthillier classification of the internal carotid artery; ^ prolactin multiplied by 21 if published in ng/mL

6.4 Discussion

The occurrence of marked hyperprolactinaemia and ICA aneurysms in 11 previously published cases together with the two cases presented herein likely reflects a true biological association. In contrast to the female predominance of cases in the literature (Table 6.1), our two patients were male, suggesting that ICA aneurysms can cause hyperprolactinaemia in both sexes. The presented patients are also instructive as they exhibited the highest levels of hyperprolactinaemia reported to date. The degree of hyperprolactinaemia (130- to 190-fold normal) and the absence of MRI-detectable pituitary tumours strongly support a novel mechanism for hyperprolactinaemia beyond stalk interruption and prolactinoma.

The mechanism of vasculogenic hyperprolactinaemia has not been investigated to date. We hypothesised that a vascular-derived factor from the injured carotid artery may induce severe hyperprolactinaemia through paracrine stimulation of the adjacent lactotrophs. The notion of vasculogenic hyperprolactinaemia may be extended to pregnancy where serum prolactin concentration reaches up to 15-fold by term (Hu *et al.*, 2018). This effect is proposed to be due to PrRFs produced by the highly vascular placenta. Here too, the mechanism may be paracrine as the decidua has the capacity to produce prolactin (Riddick *et al.*, 1979; Wu *et al.*, 1995) and prolactin levels within amniotic fluid are 10- to 100-fold higher than peripheral concentrations (Wu *et al.*, 1995). However, there was no evidence of upregulation of angiotensin II, substance P, histamine or PRLH in our patients to explain the development of marked hyperprolactinaemia. The negative results of our biochemical and molecular experiments may aid the formulation of future directions of research to explain this phenomenon. Detection of the causative factor in vasculogenic hyperprolactinaemia may shed light on the cause of hyperprolactinaemia of pregnancy and possibly milder forms

of prolactin excess, perhaps also related to endothelial dysfunction, such as that seen in 12-28% of patients with polycystic ovary syndrome (Delcour *et al.*, 2019).

The DA response in the cases reported here and in previously published cases of ICA aneurysm-associated hyperprolactinaemia (Garg & Dash, 1985; Duarte *et al.*, 2008) demonstrates an intact DA inhibitory effect and lactotrophs as the source of prolactin. This contrasts against a case report of proven ectopic prolactin production by a perivascular epithelioid cell tumour where DA therapy was ineffective (Korytnaya *et al.*, 2014). Mass effect on the infundibulum cannot explain the degree of prolactin elevation in vasculogenic hyperprolactinaemia, although it may underlie the other observed pituitary deficiencies. The accompanying hypogonadism reported by us and others (Verbalis *et al.*, 1982; Garg & Dash, 1985; Ooi & Russell, 1986; Fernandez-Real *et al.*, 1994; Heshmati *et al.*, 2001; Gungor *et al.*, 2015; Goldman *et al.*, 2016) may be partially explained by the inhibitory effect of prolactin, but the failure of testosterone to completely normalise in our patients despite subsequent normoprolactinaemia and the presence of other pituitary deficiencies suggests a contributory mass effect by the aneurysms.

Whilst endothelial cells can secrete prolactin, with various molecular forms of prolactin possessing either angiogenic or anti-angiogenic properties (Clapp *et al.*, 1998), hyperprolactinaemia has not been reported in patients with aneurysms beyond the ICA (Barbieri *et al.*, 2011). Still, prolactin production may be a feature unique to the cerebral vasculature. The role of these aneurysms *per se* is reinforced by the observation that surgical management of the aneurysm can reduce prolactin as shown in Patient 1 and in four previously reported patients (Verbalis *et al.*, 1982; Kahn *et al.*, 1997; Caldas *et al.*, 1998; Gungor *et al.*, 2015). We hypothesise that a putative PrRF produced by ICA aneurysms reaches the lactotrophs via the superior hypophyseal arteries and exerts a stimulatory

paracrine or perhaps endocrine effect. Using the Bouthillier classification (Bouthillier *et al.*, 1996), the aneurysms in 8/11 published cases and both our cases were in or proximal to the C6 (ophthalmic) segment, which also gives rise to the superior hypophyseal artery.

Our biochemical and molecular studies failed to show upregulation of angiotensin II, substance P, histamine and PRLH. This is perhaps not surprising as these PrRFs have not been associated with the degree of hyperprolactinaemia seen in our patients. Other arguably more potent PrRFs such as TRH and vasoactive intestinal peptide were excluded as our patients did not exhibit other features of ectopic over-production of these hormones. The cause of vasculogenic hyperprolactinaemia thus remains unknown. This reflects our limited knowledge of the regulation of prolactin in general. For instance, the physiological hyperprolactinaemia of pregnancy is driven by the direct and indirect effects of oestrogens; however, the precise pathways leading to prolactin excess are unclear (Grattan, 2015). Other than prolactinomas and ectopic prolactin production by rare non-pituitary tumours, there are no prolactin secretagogues or other aetiologies demonstrated to produce the degree of hyperprolactinaemia observed in our patients.

Endothelial tissue was not available from our patients. Obtaining tissue samples in future cases could facilitate expression studies of aneurysmal tissue to identify upregulated genes, which may shed light on novel PrRFs and explain vasculogenic hyperprolactinaemia. Serial imaging may also be valuable in confirming future cases as the presence of an ICA aneurysm may obscure identification of an underlying adenoma. This seems unlikely to have occurred in our patients as prolactinomas capable of these degrees of prolactin excess would be expected to be visible on MRI despite concomitant ICA aneurysms, and serial imaging did not disclose any pituitary lesions.

In summary, ICA aneurysms are a rare cause of severe hyperprolactinaemia that inhibits gonadotrophin secretion and responds to DA therapy. We propose the term vasculogenic hyperprolactinaemia; in part to distinguish these cases from prolactinoma, as surgery may have catastrophic consequences if an ICA aneurysm is not appreciated perioperatively. Our studies of candidate factors that may have explained the mechanism of hyperprolactinaemia did not identify the underlying cause. Further research into the mechanism of vasculogenic hyperprolactinaemia may reveal a hitherto unknown PrRF of great potency, perhaps linking this phenomenon to the hyperprolactinaemia of pregnancy.

6.5 Conclusion

We propose the term 'vasculogenic hyperprolactinaemia' to encompass the hyperprolactinaemia associated with ICA aneurysms. This may be mediated by an endothelial factor capable of paracrine stimulation of lactotrophs; however, angiotensin II, substance P, histamine and PRLH appear unlikely to be causative.

Chapter 7: The genomic landscape of sporadic prolactinomas

7.1 Introduction

Patients with prolactinomas and a family history of related tumours often have an identifiable germline mutation in FPTS genes such as *MEN1* and *AIP* and, less commonly, the *SDHx* genes as discussed in Chapter 9. However, the somatic molecular changes contributing to hyperprolactinaemia are unknown. This includes vasculogenic hyperprolactinaemia as investigated in Chapter 6 and sporadic prolactinomas which is investigated in the present chapter.

The unknown genetic basis of sporadic prolactinomas contrasts against the well-described somatic events in other pituitary tumours, namely: *GNAS* mutations in somatotrophinomas and occasional NFPAAs (Tordjman *et al.*, 1993; Song *et al.*, 2016; Bi *et al.*, 2017a); *USP8* (Reincke *et al.*, 2015; Song *et al.*, 2016) and rarely *NR3C1* (Ma *et al.*, 2015; Song *et al.*, 2016) mutations in corticotrophinomas; and *CTNNB1* and *BRAF* mutations in the vast majority of adamantinomatous and papillary craniopharyngiomas, respectively (Brastianos *et al.*, 2014). Some of these somatic events recapitulate multisystem disorders, including McCune-Albright syndrome due to *GNAS* somatic mosaicism (Collins *et al.*, 2012), and a newly-described syndromic disorder including paediatric CD due to a germline heterozygous *USP8* mutation (Cohen *et al.*, 2019). Recurrent mutational events observed at the somatic level in these pituitary tumours are now being exploited in emerging studies, including the use of EGFR inhibitors in *USP8*-mutated corticotrophinomas (ClinicalTrials.gov identifier: NCT02484755) and *BRAF* inhibitors in papillary craniopharyngiomas (Aylwin *et al.*, 2016). Understanding the genomic landscape of sporadic prolactinomas could similarly unveil novel treatment targets that would be of particular value to patients experiencing dopamine receptor-mediated side effects including the ICDs described in Chapters 3 and 4.

Gene-specific research into sporadic prolactinomas has shown nil or only rare somatic variants in biologically plausible genes. This includes genes where germline variants cause FPTS, such as *MEN1* (Poncin *et al.*, 1999) and *AIP* (Raitila *et al.*, 2007), and genes implicated in other sporadic PAs, including *GNAS* (Tordjman *et al.*, 1993), *USP8* (Reincke *et al.*, 2015) and *TP53* (Tanizaki *et al.*, 2007; Yagnik *et al.*, 2017). A somatic *HRAS* variant was previously identified in an aggressive prolactinoma, but this association was not borne out in an extension study including 72 prolactinomas (Cai *et al.*, 1994). Most recently, a recurrent germline gain-of-function *PRLR* mutation has been identified in prolactinoma patients, but no such variants have been found in the somatic setting (Gorvin *et al.*, 2019).

A limitation of single gene studies is the reliance on existing knowledge to select candidate genes. 'Orphan' genes of hitherto unknown function could be contributory to prolactinomas akin to other genetic discoveries, such as the roles of *GPR101* in X-LAG (Trivellin *et al.*, 2014) and *ARMC5* in bilateral macronodular adrenal hyperplasia (Assié *et al.*, 2013). Whole exome or genome sequencing offers an unbiased approach to novel gene discovery. Only three pangenomic studies of prolactinomas have been performed to date, all employing WES (Wang *et al.*, 2014; Song *et al.*, 2016; Bi *et al.*, 2017b). Wang *et al.* (2014) focused on point variants conferring bromocriptine resistance in a cohort of 12 prolactinomas and identified 11 candidate genes between their initial and follow-up (Gao *et al.*, 2015) studies. Bi *et al.* (2017b) investigated 41 pituitary macroadenomas, including three prolactinomas. Six genes were mutated in more than one tumour, but none of these were prolactinomas. Song *et al.* (2016) examined 125 PA, including 20 prolactinomas. Two genes were considered to be potential tumorigenesis genes, but only one prolactinoma harboured a variant in these genes. Overall, these studies did not find recurrent sequence variants amongst the prolactinomas that could constitute driver mutations. There is, however, emerging evidence

of recurrent CNVs in sporadic PAs, including the small number of prolactinomas thus far studied (Song *et al.*, 2016; Bi *et al.*, 2017a; Bi *et al.*, 2017b; Wierinckx *et al.*, 2018).

The aim of the present study was to perform WES in a pure prolactinoma cohort to identify recurrent somatic genetic events. We hypothesised that, like other pituitary tumours, somatic driver mutations and/or CNVs might also underlie the development of prolactinomas.

7.2 Methods

Patients

Twelve patients with clinically evident prolactinomas that had been surgically resected were recruited from two tertiary referral pituitary centres in Australia: Royal Adelaide Hospital and Royal Melbourne Hospital. Clinical data were collated using medical records. Tumour consistency was based on MRI appearances. The presence of postoperative remnants and tumour recurrences was determined using serial imaging and serum prolactin results.

The study was approved by the local institutional research committees (Melbourne Health: HREC/16/MH/132; Royal Adelaide Hospital: SSA/18/CALHN/445) and all participants provided written informed consent.

DNA extraction

Patients provided fresh blood samples for germline DNA extraction. Operative tumour specimens were retrieved for somatic DNA extraction. Tumour specimens had either been stored as fresh frozen ($n=6$) or formalin-fixed paraffin-embedded (FFPE; $n=6$) tissue. Duration of tumour storage ranged from 7 months to 8 years. DNA was extracted using commercially available kits (Qiagen and Bioline) according to manufacturer protocols. FFPE

samples were deparaffinised and additional DNA repair steps were performed using uracil-N-glycosylase to enzymatically remove formalin-induced cytosine deamination artefacts.

Whole exome sequencing

WES of germline and tumour DNA samples was performed using the Roche NimbleGen SeqCap EZ MedExome v3.0 target enrichment kit and the Illumina NextSeq 500 sequencing platform. The average of mean depth of coverage amongst all samples was 129X, and 97% of target bases were covered $\geq 20X$.

Filtration of sequence variants

Bioinformatic analysis was performed in the Australian Cancer Research Facility (ACRF) of the Centre for Cancer Biology, SA Pathology (Adelaide, Australia). The Burrows-Wheeler Alignment tool, BWA-MEM, was used to align short reads to GRCh37/hg19 (version b37+decoy). Small variants (typically <50 bp) were called using Genome Analysis Toolkit (GATK) HaplotypeCaller package version 3.4 (Van der Auwera *et al.*, 2013). Raw WES data were initially filtered for variants that were: high quality (by GATK internal filters); very rare (<0.2% population prevalence); potentially functional (by snpEFF impact, branching/binding predictions, or genomic evolutionary rate profiling (GERP) or combined annotation dependent depletion (CADD) scores); and not in regions of segmental duplication.

Germline variants were considered further if they had a GATK genotype quality (GQ) score >50 and depth of coverage >30X, and were not situated in a low complexity region. Drawing on existing literature, we searched for germline variants in known FPTS genes: *AIP*, *CDH23*, *CDKN1B*, *DICER1*, *GPR101*, *MAX*, *MEN1*, *PRKAR1A*, *SDHA*, *SDHB*, *SDHC*, and *SDHD* (Trivellin *et al.*, 2014; Rostomyan *et al.*, 2015; Zhang *et al.*, 2017; Pepe *et al.*, 2019).

Artefact was observed in tumour DNA results due to reasons including presumed normal tissue admixture and DNA degradation in FFPE specimens. Raw data from tumour DNA were thus reanalysed by a dedicated in-house somatic variant calling pipeline to identify variants present in tumour DNA and absent in germline DNA. To increase the reliability of somatic variant calls, this pipeline integrates four variant callers that detect insertions/deletions ('indels') and single nucleotide variants (SNV): Shimmer (v e5bafb4), Seurat (v 2.6), Strelka2 (v 2.9.0) and VarScan2 (v 2.4.0); and three callers that detect SNVs only: MuTect (v 1.1.4), SomaticSniper (v 1.0.5) and Virmid (v 1.1.1). Only somatic indels and SNVs that were detected by at least two or five variant callers, respectively, were considered to be candidate somatic sequence variants.

These candidate somatic sequence variants were shortlisted to a final list of somatic variants of interest with evidence of being highly damaging (high snpEFF impact) and absence in population genomic databases: Single Nucleotide Polymorphism Database (dbSNP), 1000 Genomes Project (1KGP), United Kingdom 10,000 Genomes Project (UK10K), Genome Aggregation Database (gnomAD), Exome Aggregation Consortium (ExAC) and Exome Sequencing Project (ESP). Pituitary expression of these final genes of interest was determined using the Genotype-Tissue Expression (GTEx) project database (<https://gtexportal.org>), comprising 53 non-diseased tissue sites including 183 pituitary samples.

All germline variants in known FPTS genes and somatic variants of interest were verified by inspection of raw sequencing data in Integrated Genomics Viewer (IGV).

Identification of somatic copy number variants

Raw WES data were interrogated for CNVs via in-house scripts, with calculation of copy number using a normalised read depth of coverage against control samples and correlation

with minor allele frequency. Coverage plots of sequence read depth and minor allele frequency were manually inspected to identify chromosomal and arm-level copy number gains and losses as well as copy number-neutral loss of heterozygosity (LOH).

Statistical analysis

IBM SPSS Statistics 25.0 was used for statistical analysis. The Mann-Whitney *U* test was used to assess differences in the median numbers of candidate somatic variants and chromosomes affected by CNVs or copy-neutral LOH per tumour according to relevant categorical clinical characteristics. *P*-values <0.05 were considered statistically significant.

7.3 Results

Clinical characteristics

The study cohort consisted of six women and six men aged 16-65 yr at prolactinoma diagnosis. Only prolactin hypersecretion was observed. Apart from one patient who presented with pituitary apoplexy, all patients were treated with DAs preoperatively. In all cases, surgical resection was by the transsphenoidal route and histopathology confirmed PAs with positive immunostaining for prolactin. Postoperative tumour remnants or recurrences were observed in 10 patients, all of whom had macroadenomas or giant adenomas at the baseline scan. The remaining two patients had microadenomas that were resected because of DA intolerance, with gross total resection achieved and no evidence of tumour recurrence to date. No patients had received other medical therapies or radiotherapy at study enrolment. Clinical characteristics of the patient cohort are further described in Table 7.1.

Table 7.1. Clinical characteristics of the patient cohort

| Pt | Age (yr), gender* | Tumour maximum diameter (mm)* | Hardy's score* | Tumour consistency* | PRL (xULN)* | Surgical indication | Postoperative remnant | Tumour recurrence# |
|----|-------------------|-------------------------------|----------------|---------------------|-----------------|---------------------|-----------------------|--------------------|
| 1 | 40 F | 16 [^] | 3 [^] | Solid [^] | 33 [^] | DA resistance | Yes | N/A |
| 2 | 16 F | 8 | 1 | Cystic | 9 | DA intolerance | No | No |
| 3 | 56 M | 8 | 3 | Solid | 10 | DA intolerance | No | No |
| 4 | 42 F | 11 | 3 | Solid | 5 | DA intolerance | Yes | N/A |
| 5 | 28 F | 60 | 3 | Solid | 278 | DA resistance | Yes | N/A |
| 6 | 53 M | 18 | 3 | Solid | 145 | DA intolerance | Yes | N/A |
| 7 | 32 M | 26 | 3 | Solid | 20 | DA resistance | Yes | N/A |
| 8 | 64 M | 52 | 3 | Mixed | 576 | Apoplexy at Dx | Yes | N/A |
| 9 | 65 M | 37 | 3 | Solid | 67 | DA resistance | Yes | N/A |
| 10 | 32 F | 16 [^] | 2 [^] | Solid [^] | 28 [^] | DA intolerance | No | Yes |
| 11 | 40 M | 41 | 3 | Solid | 215 | DA resistance | Yes | N/A |
| 12 | 61 F | 46 | 3 | Mixed | 72 | DA resistance | Yes | N/A |

DA, dopamine agonist; Dx, diagnosis; F, female; M, male; N/A, not applicable; N/S, not stated in report and images not available for review; PRL, prolactin; Pt, patient; xULN, absolute level divided by upper limit of normal; * at time of diagnosis; # only applies to tumours that were completely resected; [^] preoperative results used as results at diagnosis unavailable

Pathological characteristics

Pathological characteristics are described in Table 7.2. Most tumours were densely granulated lactotroph adenomas. Ki-67 index was only available in a minority of tumours. Few or no mitoses were observed in the remaining tumours, arguing against a significant degree of proliferation (McCormack *et al.*, 2018). Histological invasion was found in only three tumours. Fibrosis was only observed in 4/11 DA-treated tumours (Figure 7.1).

Table 7.2. Pathological characteristics of the patient cohort

| Pt | Positive IHC | Granulation pattern | Mitoses | Ki-67 index | Histological invasion | Fibrosis |
|----|-------------------|---------------------|-----------|-------------|-----------------------------|----------|
| 1 | PRL | Undetermined | Scant | U/A | No | No |
| 2 | PRL | Densely granulated | <1/10 hpf | U/A | No | No |
| 3 | PRL | Densely granulated | Nil | U/A | No | Yes |
| 4 | PRL | Undetermined | Scant | U/A | No | No |
| 5 | PRL, LH | Densely granulated | Scant | U/A | Yes - sphenoid, nasopharynx | Yes |
| 6 | PRL | Densely granulated | <1/10 hpf | U/A | Yes - dura | No |
| 7 | PRL, TSH, LH, FSH | Undetermined | Scant | U/A | No | No |
| 8 | PRL | Sparsely granulated | Nil | U/A | Yes - sphenoid | No |
| 9 | PRL | Densely granulated | Nil | <1% | No | Yes |
| 10 | PRL | Densely granulated | <1/10 hpf | 3% | No | No |
| 11 | PRL | Densely granulated | Nil | <1% | No | Yes |
| 12 | PRL | Sparsely granulated | <1/10 hpf | U/A* | No | No |

FSH, follicle-stimulating hormone; IHC, immunohistochemistry; LH, luteinising hormone; PRL, prolactin; Pt, patient; TSH, thyroid-stimulating hormone; U/A, unavailable; * topoisomerase index 5%

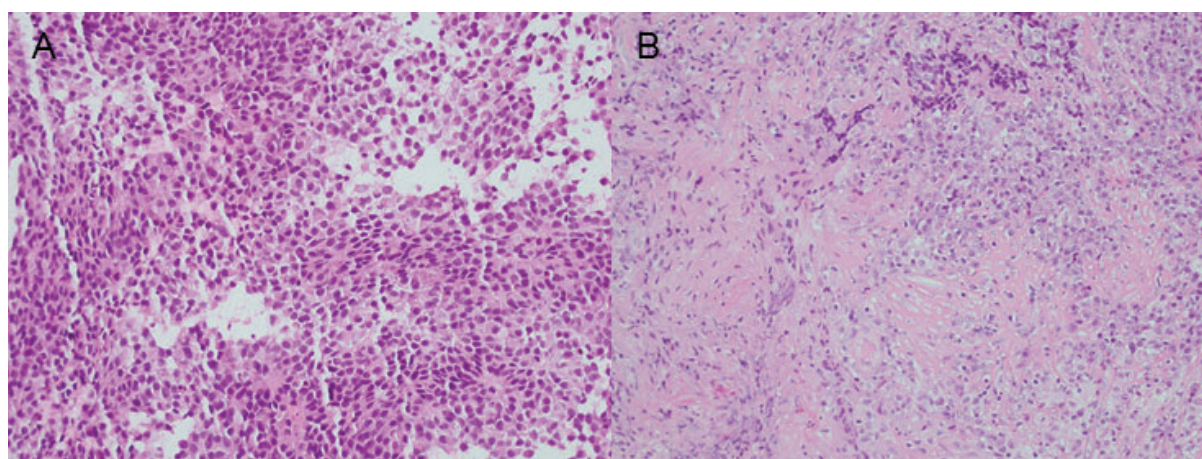


Figure 7.1. Hematoxylin & eosin appearance of prolactinomas at medium power

A. Patient 12, demonstrating no fibrosis

B. Patient 11, demonstrating marked fibrosis

Germline sequence variants

The only known FPTS gene with germline sequence variants after filtration was *CDH23*, with missense variants observed in Patient 6 (c.1103G>A (p.Arg368His), gnomAD allele frequency 0.0006, CADD 26.2, GERP 4.5) and Patient 11 (c.4510G>T (p.Ala1504Ser), gnomAD allele frequency 0.0001, CADD 25, GERP 5.06; and c.4907C>T (p.Ala1636Val), gnomAD allele frequency 0.0007, CADD 22.5, GERP 5.75).

Somatic sequence variants

Filtration of WES data revealed 138 candidate somatic variants, none of which were found in more than one tumour. Only one gene (*PHTF1*) was mutated in more than one tumour. Another two genes (*NBEAL2*, *TMEM67*) were each mutated twice in the same tumour from Patient 1. Of the 135 different genes containing candidate somatic variants, there was no overlap with genes implicated in FPTS or sporadic PA (i.e., *AIP*, *CDH23*, *CDKN1B*, *DICER1*, *GNAS*, *GPR101*, *MAX*, *MEN1*, *NR3C1*, *PRKAR1A*, *PRLR*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *TP53*, *USP8*).

Each tumour harboured multiple candidate somatic variants (median 9.5 per tumour, range 3-23). There was no significant difference in the median number of variants according to gender (male 7.5 vs. female 15.0, $P=0.107$), indication for surgery (DA intolerance 9.5 vs. other indications 14.0, $P=0.624$), tumour consistency (no cystic component 9.0 vs. cystic component 15.0, $P=0.114$) or extent of resection (no remnant 9.5 vs. remnant 14.0, $P=0.780$).

The 138 candidate somatic variants were shortlisted to 15 variants of interest (Table 7.3; Figure 7.2) that were absent in population genomic databases and highly damaging ($n=14$), or situated in a gene with another candidate somatic variant in another tumour ($n=1$,

PHTF1). The shortlist included nonsense or frameshift variants in three genes (*DRD2*, *PRL*, *TMEM67*) with known associations with the pituitary gland and the *MLH3* gene which is a tumorigenesis gene in other tissues. Using the GTEx database, we observed that the pituitary gland was in the top 10% of expressing tissues for 8/15 shortlisted genes of interest. We next used the STRING database (<https://string-db.org>) of known and predicted protein-protein interactions to look for interactions between the 15 genes of interest. The only interaction was the known link between *PRL* encoding prolactin and *DRD2* encoding the D2 receptor, which are co-expressed in multiple species and co-mentioned in medical literature.

Table 7.3. Somatic sequence variants of interest

| Gene, ID* | Chr locus | Pituitary rank by GTEX# | CNV at locus [^] | Pt. variant | VAF in tumour DNA |
|---------------------------|--------------|-------------------------|---------------------------|--|--------------------------|
| ANKK3, ENSG00000168096 | 16:4780135 | 3 | Trisomy | Pt 1: c.15delC (p.Ser5fs) | 0.33 |
| C19orf25, ENSG00000119559 | 19:1475427 | 3 | Trisomy | Pt 7: c.216dupG (p.Ile73fs) | 0.12 |
| C9orf163, ENSG00000196366 | 9:139379389 | 2 | Trisomy | Pt 1: c.491delC (p.Pro164fs) | 0.15 |
| CAST, ENSG00000153113 | 5:96077063 | 35 | Tetrasomy (2:2) | Pt 5: c.888+1G>T | 0.30 |
| DCAF10, ENSG00000122741 | 9:37860080 | 31 | Tetrasomy (2:2) | Pt 12: c.1202_1203delCT (p.Pro401fs) | 0.26 |
| DRD2, ENSG00000149295 | 11:113283323 | 1 | Monosomy | Pt 1: c.1093C>T (p.Gln365*) | 0.19 |
| KLRD1, ENSG00000134539 | 12:10460684 | 4 | Trisomy | Pt 1: c.7+1G>C | 0.21 |
| LDB2, ENSG00000169744 | 4:16597359 | 34 | Nil | Pt 9: c.3G>A (p.Met1?) | 0.37 |
| MILH3, ENSG00000119684 | 14:75514552 | 12 | Trisomy | Pt 8: c.1806delA (p.Lys602fs) | 0.27 |
| NBEAL2, ENSG00000160796 | 3:47036629 | 25 | Tetrasomy (2:2) | Pt 1: c.1407_1408delCT (p.Phe470fs) | 0.41 |
| PHTF1, ENSG00000116793 | 1:114242872 | 5 | Nil | Pt 4: n.468-7insA Pt 7: n.468-7delA | Pt 4: 0.33 Pt 7: 0.16 |
| PRL, ENSG00000172179 | 6:22290411 | 1 | Trisomy | Pt 4: c.483dupA (p.Val162fs) | 0.13 |
| SKIDA1, ENSG00000180592 | 10:21805663 | 10 | Nil | Pt 5: c.1088delC (p.Pro363fs) | 0.16 |
| SPTBN2, ENSG00000173898 | 11:66453356 | 13 | Nil | Pt 4: c.7159A>T (p.Lys2387*) | 0.35 |
| TMEM67, ENSG00000164953 | 8:94797512 | 2 | Trisomy | Pt 1: c.1194C>A (p.Tyr398*) | 0.40 |

Chr, chromosomal; CNV, copy number variant; Pt, patient number corresponding to tumour in which the variant was detected; VAF, variant allele frequency; HUGO Gene Nomenclature Committee gene symbol, gene ID per snpEFF; # pituitary rank amongst all tissue expression after sorting all 53 non-diseased tissue types by median TPM (transcripts per kilobase million) in GTEX; ^ corresponding CNV at the gene locus in the same tumour

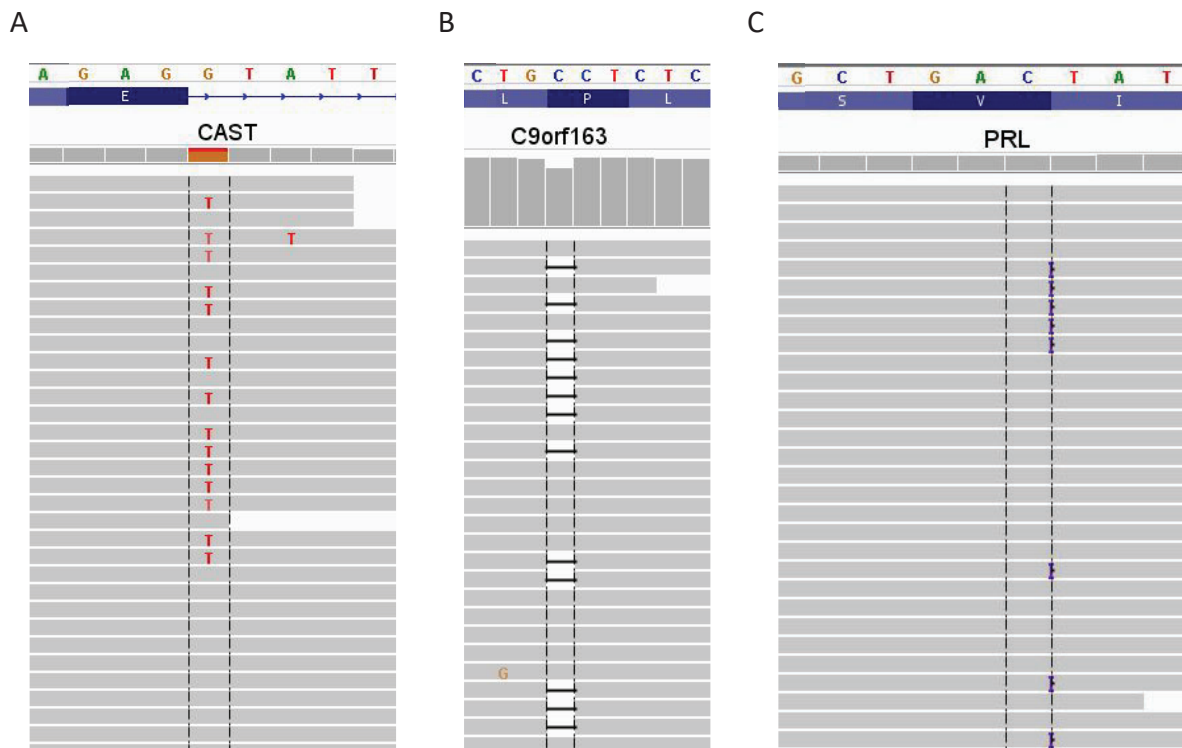


Figure 7.2. Examples of somatic point variants as shown in Integrated Genomics Viewer

- A. *CAST* substitution variant in Patient 5: c.888+1G>T
- B. *C9orf163* deletion in Patient 1: c.491delC (p.Pro164fs)
- C. *PRL* insertion in Patient 4: c.483dupA (p.Val162fs)

No patient had a germline variant in the same gene containing a somatic variant of interest in their corresponding tumour. Conversely, no candidate somatic variants were found in *CDH23* in the two patients with germline *CDH23* variants.

Somatic copy number variants

All but one tumour contained chromosomal or arm-level CNVs and/or copy-neutral LOH (median 10.5 chromosomes affected per tumour, range 0-21). There was no significant difference in the median number of chromosomes affected according to gender (male 10.0 vs. female 12.0, $P=0.377$), surgical indication (DA intolerance 8.0 vs. other indications 12.0, $P=0.514$), tumour consistency (no cystic component 10.0 vs. cystic component 17.0,

$P=0.266$) or extent of resection (no remnant 10.0 vs. remnant 10.0, $P=0.926$). CNVs manifested most commonly as whole or partial chromosomal gain (median 10 chromosomes per tumour, range 0-20) and occasionally as whole or partial chromosomal loss (median 0 chromosomes per tumour, range 0-2). Copy-neutral LOH was also seen (median 0.5 chromosomes per tumour, range 0-6).

Recurrent and single cases of chromosomal gain, loss and copy-neutral LOH are shown in Table 7.4. Examples of CNV calling are depicted in Figure 7.3. The most frequent chromosomes affected were Chr 8, 9 and 14 followed by Chr 3, 7, 12 and 20 for gains, and Chr 1 and 15 for copy-neutral LOH. No chromosomes showed recurrent losses.

Table 7.4. Somatic copy number variant analysis results

Chromosomes showing whole or partial gains, losses or copy-neutral loss of heterozygosity in either multiple or single tumours are listed

| | Copy-neutral LOH | Gain | Loss | Mixed CNV and copy-neutral LOH |
|-----------|--------------------------|----------------------------------|-----------------------|--------------------------------|
| Recurrent | Chr 1, 4, 15 | Chr 1, 3, 5-10, 12, 14, 16-22, X | nil | nil |
| Single | Chr 5, 6, 10, 11, 16, 20 | Chr 2, 4, 11, 13, 15 | Chr 11, 13, 15, 18, X | Chr 1, 4, 11 |

Chr, chromosome; CNV, copy number variant; LOH, loss of heterozygosity

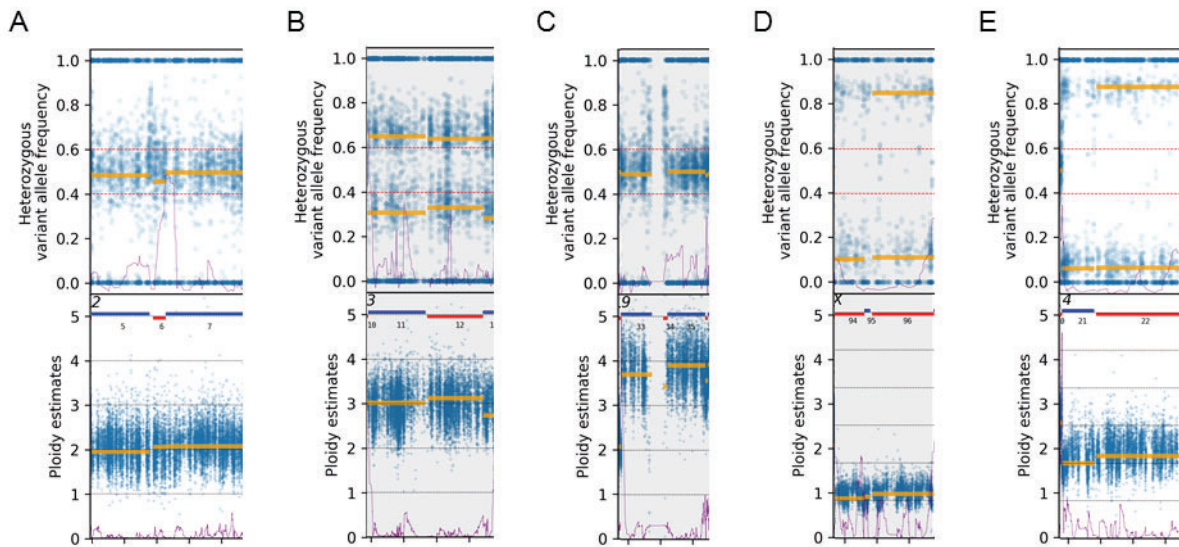


Figure 7.3. Examples of tumour DNA calls of CNVs and copy-neutral LOH

Calls of copy number variants (CNV) and copy-neutral loss of heterozygosity (LOH) were based on somatic heterozygous variant allele frequency (VAF) (top panels) and ploidy estimates using depth of coverage (bottom panels)

A. Normal disomic baseline in Chr 2 in Patient 6 represented by the usual 0.5 heterozygous VAF and ploidy count of 2

B. Chr 3 trisomy (2:1) in Patient 10 represented by separation of heterozygous VAF into VAFs of approximately 0.4 and 0.6 and increased ploidy count at 3

C. Chr 9 tetrasomy (2:2) in Patient 10 represented by usual 0.5 heterozygous VAF but increased ploidy count at 4

D. Expected Chr X monosomy in Patient 1 (female) represented by separation of heterozygous VAF and decreased ploidy count at 1

E. Chr 4 copy-neutral LOH in Patient 1 represented by separation of heterozygous VAF but normal ploidy count of 2

Each tumour was assessed for regional overlap between its CNV results and any observed variant of interest. Copy number gain was present in the corresponding tumour at the locus of 10/15 genes of interest and corresponding monosomy was observed for 1/15 genes (Table 7.3).

7.4 Discussion

The major somatic event in our cohort of 12 patients with prolactinomas was large-scale copy number variation, most commonly in the form of copy number gains. We also observed sequence variants of interest in 15 genes, including genes of putative interest in prolactinoma tumorigenesis. Although we found that pituitary expression is in the top 10% of tissues for over half of our genes of interest, these somatic variants do not appear to be classical driver mutations as none were found in more than one tumour. We also found rare missense germline variants in the recently recognised *FPTS* gene, *CDH23*. However, somatic second hits were not found in the corresponding tumours and *CDH23* is a notably large gene with 69 exons, which may increase the propensity for variants of uncertain significance. Our results recapitulate the findings of the few systematic genomic studies of prolactinomas that have been performed to date (Table 7.5), whereby copy number variation is common and recurrently mutated genes are rare.

Table 7.5. Paired tumour-germline pangenomic studies in prolactinoma patients

| Study | Cohort | Filter for GOI | GOI | Recurrent CNVs |
|---|---|--|--|--|
| (Wang <i>et al.</i> , 2014; Gao <i>et al.</i> , 2015) | DA responsive vs. resistant PRLoma (n=12) | Variants differing between responsive vs. resistant PRLoma | <i>C1orf170</i> <i>DPCR1</i> <i>DSPP</i> <i>KRTAP10-3</i> <i>MUC4</i> <i>MX2</i> <i>POTEF</i> <i>PRB3*</i> <i>PRDM2*</i> <i>PRG4</i> <i>RP1L1</i> | N/T |
| (Song <i>et al.</i> , 2016) | PA (n=120, incl 20 PRLoma) | Recurrently mutated in multiple PA | <i>GRB10*</i> <i>IARS</i> <i>KIF5A*</i> <i>SP100</i> <i>TRIP12</i> | Gains: Chr 1p13.2, 1q31.3, 3p22.3, 7q21.11, 16q12.2, 20p13, 20q13.33 Losses: Chr 1p36.31, 3p21.31, 9q34.11, 11q13.2, 11p15.5, 16p13.3 |
| (Bi <i>et al.</i> , 2017b) | Pituitary macroadenomas (n=42, incl 3 PRLoma) | Recurrently mutated in multiple PA | <i>ATAD3B</i> <i>BHLHE22</i> <i>KDM2B</i> <i>OR5M1</i> <i>TTN*</i> <i>VPS13B</i> | Gains: Chr 7, 9q, 14q Losses: Chr 1, 2, 4, 10, 11, 15q, 18 Copy-neutral LOH: Chr 11q |
| Present study | PRLoma (n=12) | Absent in population databases and strong <i>in silico</i> prediction for pathogenicity, or recurrently mutated in multiple PRLoma | <i>ANKS3</i> <i>C19orf25</i> <i>C9orf163</i> <i>CAST</i> <i>DCAF10</i> <i>DRD2*</i> <i>KLRD1</i> <i>LDB2</i> <i>MLH3</i> <i>NBEAL2</i> <i>PHTF1</i> <i>PRL*</i> <i>SKIDA1</i> <i>SPTBN2</i> <i>TMEM67*</i> | Gains: Chr 1, 3, 5-10, 12, 14, 16-22, X Losses: nil Copy-neutral LOH: Chr 1, 4, 15 |

CNV, copy number variant; DA, dopamine agonist; GOI, genes of interest; incl, including; n, number of cases that underwent whole exome sequencing; N/T, not tested; PA, pituitary adenoma; PRLoma, prolactinoma; * particular genes of interest

We observed several recurrent copy number gains and copy-neutral LOH. This is in keeping with the pituitary macroadenoma WES study by Bi *et al.* (2017b), and their follow-up NGS panel study of 114 PAs including 14 prolactinomas (Bi *et al.*, 2017a), that indicated two patterns of copy number variation: a highly disrupted group mostly consisting of functional adenomas (including prolactinomas) or atypical null-cell adenomas with copy number variation involving a mean of 39% of the genome; and a less disrupted group mostly consisting of NFPA with copy number variation involving a mean of 0.5% of the genome. Song *et al.* (2016) also found a high degree of copy number variation in their mixed cohort of PAs, with almost one-third of PAs showing copy number variation involving >80% of the genome. Our patients' tumours demonstrated recurrent gains in Chr 7, 9 and 14 similar to Bi *et al.* (2017b), and in Chr 1, 3, 7, 16 and 20 similar to Song *et al.* (2016). In contradistinction to these former studies, we observed additional recurrent gains in Chr 5-7, 10, 12, 17-19, 21, 22 and X. We also found no recurrent chromosomal losses, whereas Bi *et al.* (2017b) found losses to be particularly common in Chr 1p and 11 in hormonally active adenomas. Whilst we found recurrent copy-neutral LOH in Chr 1, 4 and 15, Bi *et al.* (2017b) only found Chr 11q LOH. Discrepancies between the different cohorts is at least partly explained by the heterogenous mixes of different PA subtypes in previous studies. Our pure prolactinoma cohort should be considered separately to these previous studies because of the potential for specific DA treatment effects in 11 of our 12 patients who were treated with a DA preoperatively. DA resistance may have led to the observed high rate of CNVs or *vice versa*.

By contrast to the high burden of CNV, we found relatively few sequence variants per tumour and a lack of recurrent sequence variants between tumours. This argues against a major role of driver mutations in the pathogenesis of prolactinomas. This differs from the experience of studying somatotrophinomas, corticotrophinomas and craniopharyngiomas, but mimics findings in other pituitary tumour subtypes. Paired tumour-normal WES studies

of seven patients with NFPA in 2013 (Newey *et al.*, 2013b) and of four patients with TSHomas in 2016 (Sapkota *et al.*, 2017) also found no recurrent variants that could be considered driver mutations. A low number of somatic mutations has been observed in previous studies of PAs compared to other neoplasms (Song *et al.*, 2016; Bi *et al.*, 2017b). Direct comparison of absolute mutation numbers between studies is limited by differing methods of variant filtration. Within studies, we and others (Song *et al.*, 2016) have found no association between prolactinoma clinical characteristics and the number of sequence variants.

Some variants within individual tumours were of interest due to their location in genes with a plausible connection to prolactinoma formation. This includes the truncating *DRD2* variant found in a patient with a 40-year history of prolactinoma that has shown DA escape over the last 3 years despite increasing doses of cabergoline, necessitating surgery and most recently radiotherapy. We speculate that this variant, found in 19% of tumour DNA, may reflect a subclone that is driving the patient's recent DA resistance. Indeed, D2 receptor expression is typically high in prolactinomas, and downregulation has been hypothesised as a mechanism of DA resistance (Evans *et al.*, 2008). Compared to DA responsive prolactinomas, resistant tumours demonstrate decreased D2 receptor density, overall reduction in *DRD2* mRNA production, and altered expression of *DRD2* mRNA isoforms with lower expression of the more efficient short isoform (Wu *et al.*, 2010). Furthermore, D2 receptor deficiency in female mice models produces lactotroph hyperplasia (Kelly *et al.*, 1997). However, we did not find *DRD2* variants of interest in our other five patients with DA resistance. Wang *et al.* (2014) reported an absence of *DRD2* sequence variants, although the sensitivity of this study was reduced by its overall low depth of coverage with only 10X coverage in 80% of the exome.

Another tumour in our cohort harboured a frameshift variant in *PRL*. This gene is well known to be highly expressed in lactotrophs (Evans *et al.*, 2008). Autocrine signalling between prolactin and the abundant prolactin receptors on lactotrophs has been postulated as the explanation for the sexual dimorphism in lactotroph hyperplasia in D2 receptor knockout mice (Kelly *et al.*, 1997). By this theory, male mice lacking the D2 receptor do not reach the prolactin threshold required for the feed-forward loop to activate and trigger lactotroph hyperplasia (Kelly *et al.*, 1997). How the stimulatory effect of prolactin in the mouse model correlates with our study is unclear as the *PRL* frameshift variant would be expected to cause a loss of function rather than a gain of function.

We also found isolated somatic variants in *TMEM67* where biallelic inactivating variants have been implicated in hypopituitarism (Brancati *et al.*, 2018), and in *MLH3* which is a mismatch repair gene with a possible role in Lynch syndrome (Wu *et al.*, 2001). Although a Lynch syndrome registry study found an increased risk of PAs (Bengtsson *et al.*, 2017), there is currently no evidence of a specific role of *MLH3* in pituitary tumorigenesis.

The remaining variants of interest were located in genes with no currently known associations with the pituitary gland. Comparison of the genes of interest in our study with results from the previously published genomic studies including prolactinomas showed little overlap: Song *et al.* (2016) found one *ANKS3* frameshift variant and two *SKIDA1* variants; and Bi *et al.* (2017b) found a *KLRD1* missense variant. None of these variants were seen in the prolactinoma subsets of these studies. In addition, none of our cases fulfilled Knudson's two-hit model of tumour suppressor genes as no patients had germline variants in the 15 genes harbouring somatic variants of interest and the two patients with germline *CDH23* variants had no candidate somatic variants in *CDH23*. Copy number variation might have arguably been the second hit in some of these tumours as 11/15 (73%) of variants of interest were in

regions of copy number variation in a given tumour. Trisomy and tetrasomy may be especially relevant as increased mutant dosage could amplify a dominant negative effect by a sequence variant, thereby contributing to tumorigenesis. On the other hand, the maximum variant allele frequency (VAF) was 0.41 amongst the 15 variants of interest despite the frequent coexistence of copy number gain. Furthermore, most prolactinomas in our study harboured multiple CNVs and the CNVs were large; thus, it is unlikely that any single gene in these regions of CNV can explain the pathogenesis of prolactinomas.

Since our study was completed, Miao *et al.* (2019) published their findings of somatic biallelic variants in *POU6F2* in a giant prolactinoma resected from a 43-year-old man. The authors showed that the variants resulted in a decrease in *POU6F2* expression and that *POU6F2* inhibition increased cell proliferation and prolactin secretion in rat pituitary cells, whilst *POU6F2* overexpression had the opposite effects, consistent with the tumour suppressor gene model (Miao *et al.*, 2019). However, these changes have only been documented in a single tumour and we found no compelling *POU6F2* variants upon reanalysis of the tumour DNA results in our cohort, arguing against recurrent *POU6F2* driver mutations in the pathogenesis of prolactinomas.

A key limitation of our study is that WES does not detect intergenic variants, balanced translocations, fusion genes or epigenetic changes. As discussed in Chapter 9, WES will also typically miss deep intronic variants that may explain endocrine tumours when routine genetic testing has been uninformative. Integrative genomic analyses of both DNA and RNA (Branford *et al.*, 2018) as well as the emerging technology of long-read sequencing with real-time analysis of nucleotide binding (English *et al.*, 2012) may elucidate some of these possibilities. Furthermore, half of our tumour samples were FFPE, although we employed an optimised DNA extraction protocol to limit artefactual results because of this. Another

limitation of our study is that the identification of copy number variation and copy-neutral LOH was based on broad patterns in VAF and ploidy based on depth of coverage. We were thus only able to categorise copy number variation and copy-neutral LOH at the arm or chromosomal level. Smaller CNVs may have been missed, although other data support the predominance of large-scale CNVs, as observed in our tumours, over smaller CNVs (Bi *et al.*, 2017b). The relatively low allele frequencies of our variants of interest is also noteworthy. This may seemingly contradict the known monoclonal origin of PAs (Evans *et al.*, 2008). However, the <50% VAFs seen in our tumour DNA results may reflect CNVs, which was a common finding in our tumours, and/or normal tissue admixture, particularly as PAs are rarely resected *en bloc* and interspersed normal pituitary tissue is a common microscopic finding. VAFs <50% may alternatively represent the presence of multiple tumour clones. The possibility of this may be further assessed through spatial transcriptomics whereby sequencing results are overlaid with tissue sections to compare the transcriptomes of different tumour regions (Thrane *et al.*, 2018). Finally, the small size of this pilot study limited our ability to identify clinical predictors of the number of somatic variants or the number of chromosomes affected by copy number variation or copy-neutral LOH. An independent validation set of another group of prolactinomas using the same platforms employed in this study would have been ideal to further explore these putative clinicopathological correlations and our identified genes of interest; however, surgery is rarely performed for prolactinomas and thus further tumours were not available for investigation. With increasing recognition of DA-related toxicities such as the ICDs highlighted in Chapters 2 and 3, there may be greater demand for surgical resection of prolactinomas and hence improved tumour tissue availability in the future.

Larger studies involving sufficient numbers of different prolactinoma subsets (e.g., cystic prolactinomas or young-onset prolactinomas in males) with use of fresh frozen tumour

samples may better elucidate the genetic drivers of tumorigenesis. Our findings of suspicious albeit isolated somatic variants in strong candidate genes such as *DRD2* may be a function of the heterogenous patient and tumour case mix in the prolactinoma studies to date. We kept our inclusion criteria at a minimum in order to capture sufficient numbers of prolactinomas, which otherwise tend to be medically managed with DAs. Routine biobanking of PAs will help facilitate future studies, although resected tumour tissue is often piecemeal, in small quantity and potentially damaged by intraoperative cauterisation.

7.5 Conclusion

This systematic genomic study of all coding genes in a pure prolactinoma cohort demonstrated variants in genes of biologically plausible interest within individual tumours, without overlap between prolactinomas in this study or with the few other published pangenomic studies (Wang *et al.*, 2014; Gao *et al.*, 2015; Song *et al.*, 2016; Bi *et al.*, 2017b). We instead found a high degree of copy number variation, corroborating other preliminary studies of sporadic prolactinomas (Wierinckx *et al.*, 2018) and larger studies of mixed PA subtypes (Song *et al.*, 2016; Bi *et al.*, 2017a; Bi *et al.*, 2017b). Further research is required to determine how copy number variation may contribute to prolactinoma formation and ways in which this could be therapeutically targeted. Ongoing investigation into the molecular basis of prolactinomas and other causes of hyperprolactinaemia will be crucial in finding alternative medical agents for patients with DA resistance or dopamine receptor-mediated toxicities such as hypersexuality and the other ICDs described in Chapters 2 and 3.

Chapter 8: The aryl hydrocarbon receptor (*AHR*) gene in cyclical Cushing's disease: interaction with the cell cycle and clock genes

8.1 Introduction

Hyperprolactinaemia is frequently seen in the context of CD as highlighted in Chapter 5. The genetic basis of corticotrophinomas could thus inform the genetic basis of prolactinomas and *vice versa*. However, this has not been evident to date as prolactinomas lack mutations in the key corticotrophinoma gene, *USP8*, as shown in the last chapter. In the present chapter, we capitalised on our local experience in WES and tissue availability to perform the first molecular investigation of the pituitary form of cyclical CS.

Cyclical CS, characterised by intermittent biochemical hypercortisolism, accounts for approximately 20% of endogenous CS. Cycles may last days to years, often with a consistent intraindividual pattern (Meinardi *et al.*, 2007) suggesting an intrinsic fault in timekeeping. Competing theories for the pathogenesis of cyclical CS include: episodic haemorrhage; periodic growth/death of tumour cells; persistence of negative feedback; and, in cyclical CD only, altered hypothalamic control of the pituitary, via dopaminergic fluctuations for example (Meinardi *et al.*, 2007).

The only known genetic aetiology of cyclical CS is germline *PRKAR1A* mutations causing Carney's complex, including the common manifestation of ACTH-independent cyclical CS due to PPNAD (Powell *et al.*, 2008). However, PPNAD-associated CS may be either cyclical or non-cyclical (Powell *et al.*, 2008), and cyclicity is hence not necessarily explained by *PRKAR1A*. The molecular basis of cyclical CS has not otherwise been investigated. We performed WES in a man with cyclical CD, hypothesising that cyclical CS is due to perturbation in the clock genes responsible for circadian rhythms including the HPA axis.

8.2 Methods

A 47-year-old man was found to have left optic disc swelling, right partial oculomotor nerve palsy, and right proptosis and conjunctival injection suggesting ophthalmic vein compression. MRI revealed a 7.1 cm sellar mass invading the sphenoid sinus, bilateral cavernous sinuses and skull base, and impinging upon the third ventricle, right temporal lobe and midbrain (Figure 8.1). Transsphenoidal biopsy of the mass revealed an ACTH-positive PA. The patient had a history of obesity, hypertension, gout and renal calculi, but with no cyclical symptoms or blood pressure fluctuations. Body mass index was 52.1 kg/m² and he had a dorsocervical fat pad, though he had no supraclavicular fat pads, Cushingoid striae, facial plethora, ecchymoses or proximal weakness. He was eupituitary apart from fluctuating ACTH-dependent cortisol production ranging from normal to 35-fold normal (Figure 8.1). He was diagnosed with cyclical CD due to a giant corticotrophinoma with intermittent biochemical hypercortisolism; however, the precise temporal cyclicity could not be defined prior to transcranial surgery 1 week later. The tumour was partially resected, and histopathology confirmed a corticotrophinoma with a Ki-67 proliferation index of <1% and no significant mitotic activity. He was eucortisolaemic immediately pre- and postoperatively with ACTH lowering from 376 ng/L (upper limit of normal, 60) to 169 ng/L (Figure 8.1). Postoperative complications included acute kidney injury, transient hyperglycaemia, pneumonia, deep vein thrombosis and central hypothyroidism. He later noticed improved blood pressure control, reduced appetite, and improved satiety with early but transient weight loss. Serial MRI showed a stable 4.2 cm tumour remnant (Figure 8.1). Despite having typical CS comorbidities and postoperative complications, he has had no cyclical symptoms to guide the timing of biochemical investigations and no further episodes of overt hypercortisolism have been detected during intermittent testing. His family history is negative for endocrine tumours.

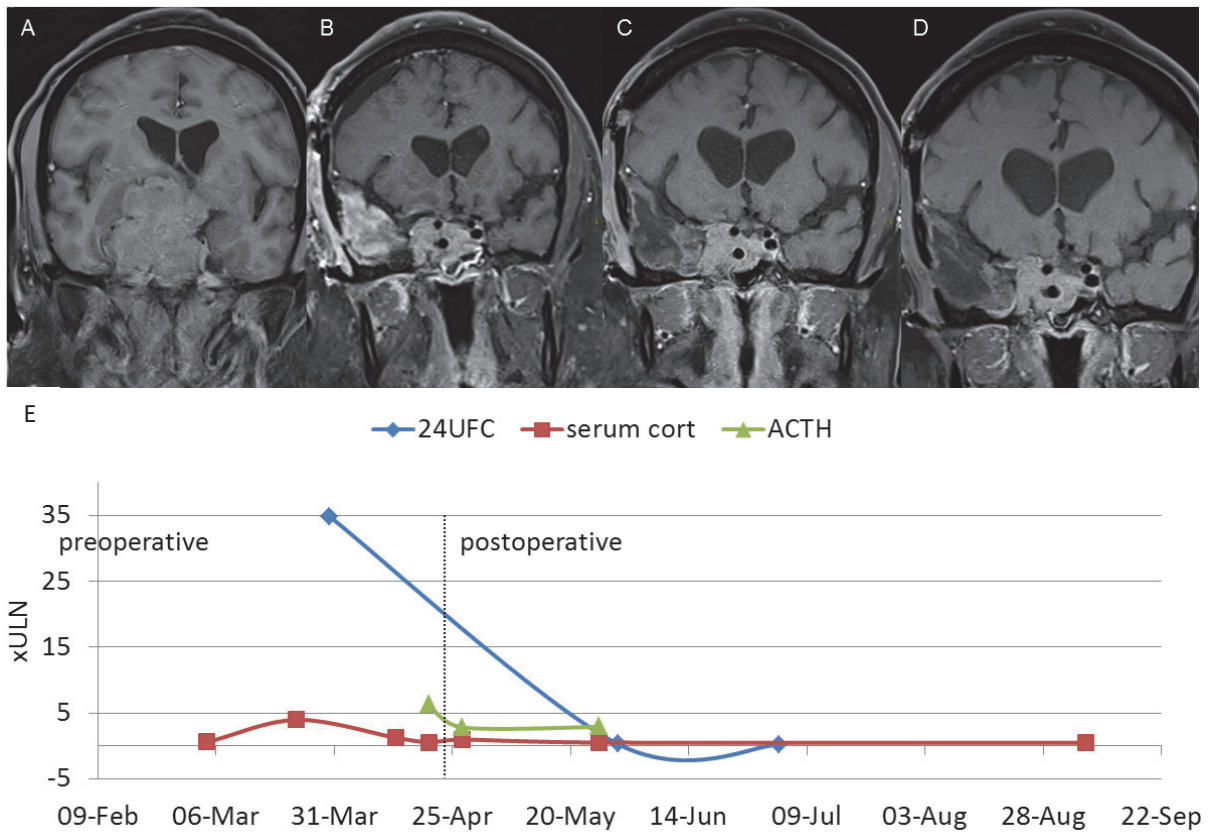


Figure 8.1. Radiological and biochemical features of the patient

A-D. MRI showing the corticotrophinoma at diagnosis (A) and 1 (B), 5 (C) and 9 (D) months following partial tumour resection by transcranial approach

E. Graphical representation of cyclical cortisol production relative to the upper limit of normal for each parameter pre- and postoperatively

24UFC, 24-hr urinary free cortisol; ACTH, adrenocorticotrophic hormone; serum cort, random serum cortisol

Molecular investigations were performed with institutional ethical approval (Melbourne Health: HREC/16/MH/132; Royal Adelaide Hospital: SSA/18/CALHN/445) and written informed consent. Tissue specimens from the corticotrophinoma were obtained at the time of surgery and stored in RNALater at -80°C . A fresh blood sample was obtained for extraction of germline DNA from peripheral blood leucocytes. Both tumour and germline DNA were extracted using commercially available kits (Qiagen) according to manufacturer protocols.

WES of tumour DNA and paired germline DNA was performed using Roche NimbleGen SeqCap EZ MedExome Target Enrichment Kit with mean depth of coverage of 100X and 95% of bases covered $\geq 20X$. Bioinformatic analysis was performed in ACRF (Adelaide, Australia) using GATK HaplotypeCaller (Van der Auwera *et al.*, 2013) to detect small variants (typically < 50 bp) and in-house scripts to identify CNVs.

Raw WES data were filtered for variants that were: rare ($< 1\%$ population prevalence); possibly damaging (by snpEFF impact, splicing/binding predictions, GERP or CADD); and of high quality (GATK GQ score > 50 and depth of coverage $> 30X$). All heterozygous and homozygous variants were then manually filtered for associations with the clock system, circadian rhythm, pituitary tumorigenesis or corticotroph function. Raw data were also compared against a list of candidate genes based on existing literature: *USP8*, *CABLES1*, *AIP*, *MEN1*, *CDKN1B*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *PRKAR1A*, *CDH23*, *NR3C1*, *EGFR*, *POMC*, *SMARCA4*, *HDAC2*, *GNAS*, *DICER1*, *CLOCK*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *TIMELESS*, *ARNTL*, *TIPIN*, *TBX19*, *HSP90AA1*, *NR2C2*, *AVPR1B*, *GPR101*, *PROP1*, *NPAS2*, *BHLHE40*, *BHLHE41*, *NFIL3*, *DBP*, *HLF*, *NR1D1*, *NR1D2*, *RORA*, *RORB*, *RORC*, *GSK3B*, *BTRC*, *FBXL3*, *FBXL21*, *FBXL15*, *RXRG* and *TH*. Both germline and tumour variants were considered. Whilst the slight overrepresentation of EAS in cyclical vs. non-cyclical CS (Meinardi *et al.*, 2007) suggests that the putative molecular cause is tumour-specific and therefore a somatic mutation, the ubiquity of cyclicity amongst the various CS aetiologies suggests that individuals could have a germline genetic predisposition that produces cyclicity if CS ever develops.

High confidence somatic variants were also used to generate a tumour mutational signature for comparison against the Sanger Institute's Signatures of Mutational Processes in Human Cancer (<https://cancer.sanger.ac.uk/cosmic/signatures>) (Alexandrov *et al.*, 2013).

A high-density single nucleotide polymorphism (SNP) array of tumour DNA was performed using the Illumina Infinium CytoSNP-850K BeadChip to validate CNV results from WES data analysis.

Tumour IHC was performed to evaluate the leading genetic variant of interest. We employed a rabbit polyclonal antibody raised against human aryl hydrocarbon receptor (AHR; Novus Biologicals, CO, USA, Cat # NBP1-89975) and a mouse monoclonal antibody raised against human AIP (clone 35-2, Novus Biologicals, CO, USA, Code # NB100-127) using a standard streptavidin-biotinylated immunoperoxidase technique. Tissue sections from the operative tumour specimen were dewaxed using xylene and then rehydrated through alcohols. Sections were then rinsed twice in phosphate-buffered saline (PBS; pH 7.4) for 5 min. Antigen retrieval was performed using Citrate buffer (pH 6). Slides were allowed to cool and washed twice in PBS (pH 7.4), then endogenous peroxidase activity was quenched. Non-specific proteins were blocked using normal horse serum for 30 min. The polyclonal AHR antibody was applied at a dilution of 1:100 and the monoclonal AIP antibody at a dilution of 1:1600 at room temperature overnight. The following day, the sections were washed twice in PBS, then either a biotinylated anti-rabbit secondary (Vector Laboratories, USA, Cat # BA-1000) or a biotinylated anti-mouse secondary (Vector Laboratories, USA, Cat # BA-2000) was applied for 60 min at room temperature. Following two further PBS washes, the slides were incubated for 1 hr at room temperature with a streptavidin-conjugated peroxidase tertiary (Pierce, USA, Cat # 21127). Sections were visualised using diaminobenzidinetetrahydrochloride, washed, counterstained with haematoxylin, dehydrated, cleared and mounted on glass slides.

8.3 Results

Germline DNA exhibited 14 rare, possibly damaging sequence variants in circadian/pituitary-associated genes (Table 8.1). Tumour DNA did not exhibit any additional variants. Amongst the 14 germline variants of interest, only one variant was considered to be reliable and relevant to both circadian rhythm and pituitary tumorigenesis. This germline exon 10 *AHR* variant (GRCh37/hg19, Chr 7:g.17379197C>T; ENST00000242057; c.1748C>T/p.Thr583Met) was present in the heterozygous state in both germline DNA (30/65 reads) and tumour DNA (86/177 reads). The variant is located at a site that is highly conserved and likely to be involved in phosphorylation (Figure 8.2) (Blom *et al.*, 1999). It is predicted to be damaging by five out of six *in silico* tools. ExAC allele frequency is 0.0001 with no homozygotes. It has been cited in Catalogue of Somatic Mutations in Cancer (COSMIC; cancer.sanger.ac.uk) in oesophageal adenocarcinoma (Dulak *et al.*, 2013).

Table 8.1. Germline variants in genes of interest

Genes of interest were those with a known relationship with the clock system and/or the pituitary

| Gene symbol | Reason for interest | Evidence against pathogenicity | Chr locus | Variant | EXAC (%) | CADD | GERP | Tumour DNA read count (WT,mutant) | Germline DNA read count (WT,mutant) |
|-------------|--|--|-------------|---------------------------------|----------|-------|------|-----------------------------------|-------------------------------------|
| AHR | Inhibits transcriptional activation of <i>PER1</i> (UniProtKB); binds known pituitary tumorigenesis gene, <i>A/P</i> | | 7:17379197 | c.1748C>T (p.Thr583Met) | 0.01 | 24.9 | 5.28 | 91,86 | 35,30 |
| AS1C3 | Strongly expressed in pituitary (UniProtKB) | Benign by 3 of 4 <i>in silico</i> tools | 7:150747594 | c.712C>G (p.Arg238Gly) | 0.042 | 25.1 | <2 | 72,82 | 33,34 |
| CDH23 | Known pituitary tumorigenesis gene | Synonymous | 10:73377084 | c.1083C>T (p.Ala361Ala) | 0.014 | 16.66 | 4.56 | 78,83 | 45,35 |
| CDH23 | Known pituitary tumorigenesis gene | 0.3% population prevalence | 10:73492073 | c.4060C>T (p.Arg1354Cys) | 0.259 | 31 | 4.02 | 73,67 | 41,28 |
| CLOCK | Canonical clock gene | 0.5% population prevalence; low mutant load in germline, mutant absent in tumour | 4:56304529 | c.2278_2280delCAG (p.Gln760del) | 0.479 | <10 | 4.47 | 36,0 | 46,5 |
| ERBB4 | Strongly expressed in pituitary (UniProtKB) | Intronic; low mutant load in germline, mutant absent in tumour | 2:212543728 | c.1622+49G>T | | <10 | 2.52 | 188,0 | 42,7 |
| GALR2 | Neuromodulator in hypothalamic-pituitary axis (RefSeq Gene) | 0.8% population prevalence | 17:74073386 | c.1038C>G (p.Ser346Arg) | 0.831 | 23.2 | 2.72 | 58,40 | 35,30 |
| PHLPP1 | Involved in circadian control by regulating circadian periodicity after resetting (UniProtKB) | Intronic | 18:60582301 | c.2804+60T>C | | <10 | 2.8 | 24,33 | 21,13 |

| | | | | | | | | | |
|----------------|--|--|--------------|-------------------------------|-------|-------|------|---------|-------|
| <i>PRLHR</i> | Encodes prolactin-releasing hormone receptor; only expressed in normal pituitary and PA (UniProtKB) | Synonymous | 10:120354258 | c.499C>T (p.Leu167Leu) | | <10 | 4.48 | 95,82 | 37,50 |
| <i>PTTG1IP</i> | Binds pituitary transcription factor, securin encoded by <i>PTTG1</i> (UniProtKB & RefSeq Gene) | Low mutant load in germline, mutant absent in tumour | 21:46276193 | c.361_363delTGC (p.Cys121del) | 0.196 | <10 | 4.85 | 37,0 | 53,6 |
| <i>RPTOR</i> | Strongly expressed in pituitary (UniProtKB) | Synonymous | 17:78921066 | c.3180C>T (p.Phe1060Phe) | 0.2 | 15.07 | 5.25 | 107,105 | 54,45 |
| <i>RXRG</i> | Suspected pituitary tumorigenesis gene | | 1:165379996 | c.856C>T (p.Arg286Cys) | | 34 | 5.1 | 37,52 | 19,21 |
| <i>SIX3</i> | Acts with HESX1 to control cell proliferation via Wnt/ β -catenin pathway in pituitary development (UniProtKB) | 0.5% population prevalence; low mutant load in germline, mutant absent in tumour | 2:45169429 | c.205_207delGGC (p.Gly69del) | 0.517 | <10 | 2.94 | 40,0 | 36,4 |
| <i>THRB</i> | Encodes thyroid hormone receptor | 0.5% population prevalence | 3:24206606 | c.240C>G (p.Ala80Ala) | 0.487 | 15.46 | 5.78 | 64,30 | 31,20 |

CADD, Combined Annotation Dependent Depletion score; Chr, chromosomal; ExAC, Exome Aggregation Consortium database; GERP, Genomic Evolutionary Rate Profiling score; HESX1, homeobox expressed in ES cells 1; PA, pituitary adenoma; WT, wild-type allele

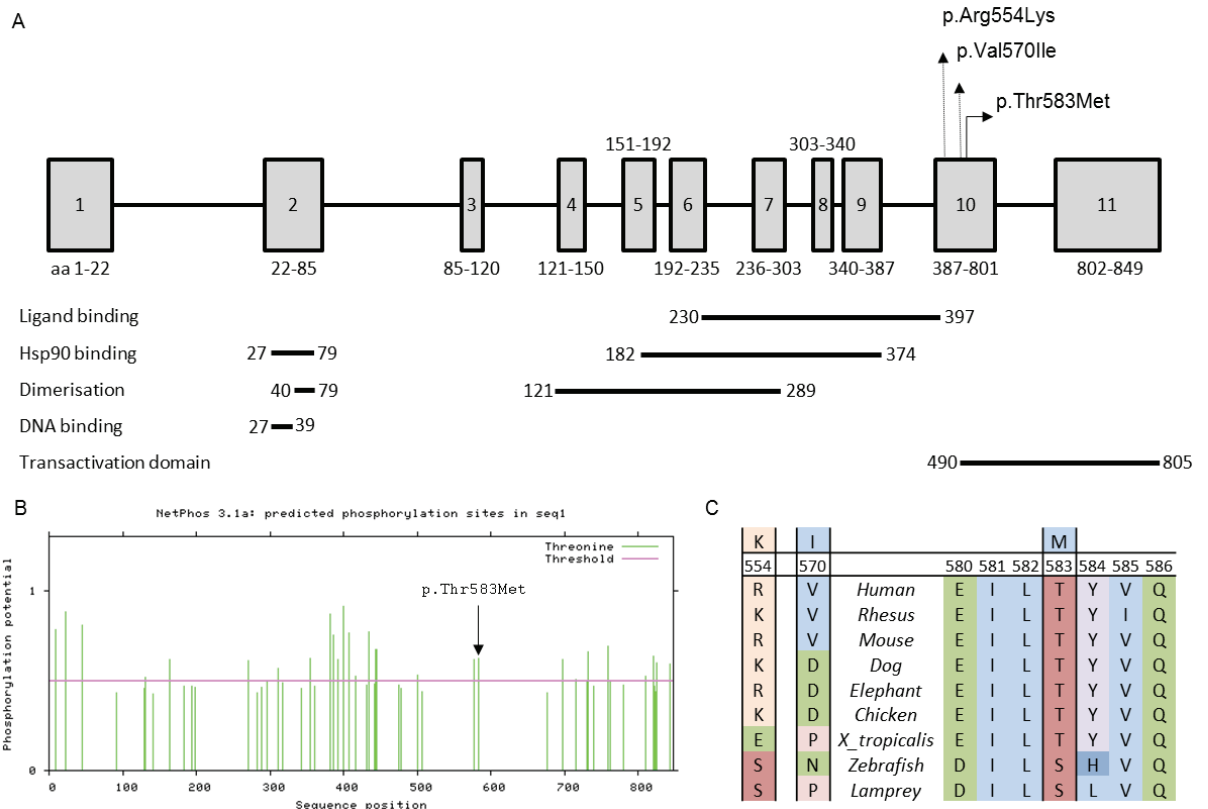


Figure 8.2 Position of AHR variant

A. Schematic diagram of the *AHR* gene showing the amino acid (aa) positions of all 11 exons (all coding), the location of the *AHR* variant in our patient (p.Thr583Met) and variants previously studied in the setting of acromegaly (p.Arg554Lys and p.Val570Ile), and functional domains based on extrapolations from mouse *Ahr* (Fukunaga *et al.*, 1995)

B. Threonine phosphorylation map of *AHR* by NetPhos 3.1 Server showing our patient's variant located at a predicted phosphorylation site (Blom *et al.*, 1999)

C. The alignment of protein sequences from different species showing our patient's variant at aa position 583 to be more conserved compared to the previously studied variants at aa positions 554 and 570

Tumour IHC showed restriction of AHR staining to the cytoplasm, whereas both cytoplasmic and nuclear AHR staining was seen in corticotrophinoma specimens from two male patients who had non-cyclical CD and no *AHR* variants on WES (Figure 8.3). By contrast, staining for the AHR chaperone, AIP, showed cytoplasmic and membranous staining in all three corticotrophinomas.

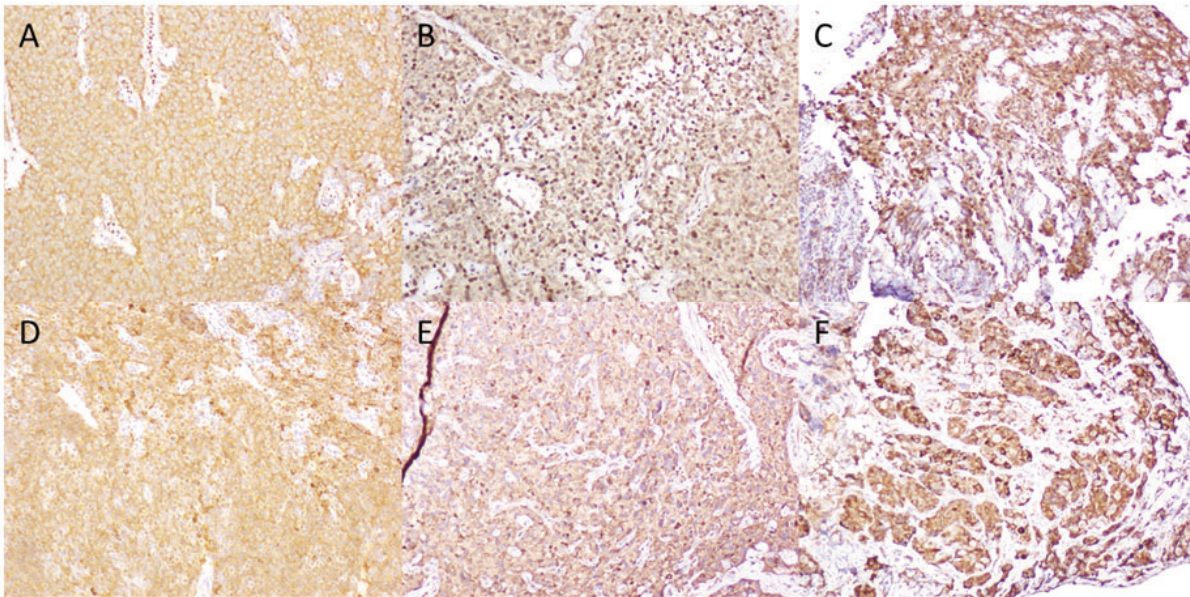


Figure 8.3 AHR and AIP tumour immunohistochemistry (100x magnification)

A. Only cytoplasmic AHR staining was observed in the patient's corticotrophinoma

B-C. Both cytoplasmic and nuclear AHR staining was observed in control corticotrophinoma specimens from two men with non-cyclical Cushing's disease and no *AHR* variants

D-F. Cytoplasmic and membranous staining for the AHR chaperone, AIP, was found in the corticotrophinomas from the patient (D) and the two controls (E, F)

WES also revealed a novel, damaging heterozygous germline *RXRG* variant (GRCh37/hg19, Chr 1:g. 165379996C>T; ENST00000359842; c.856C>T/p.Arg286Cys) situated in the ligand binding domain. Homology modelling of the human retinoid X receptor gamma (*RXRG*) protein using the mutant analysis server, HOPE (Venselaar *et al.*, 2010) (Figure 8.4), showed that the cysteine substitution at amino acid position 286 is smaller than the wild-type (WT) arginine, which may cause loss of external interactions. The charge of the WT residue is also lost by this substitution, which is predicted to disturb the ionic interaction made by the WT residue with nearby glutamic acid residues.

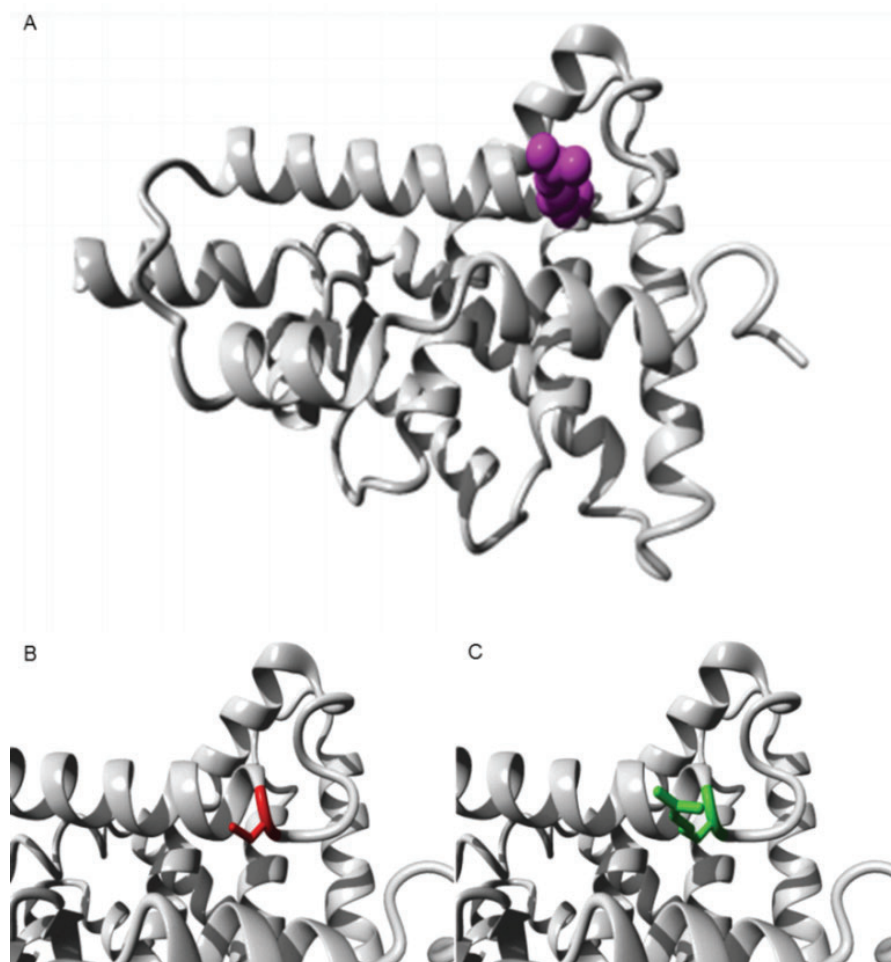


Figure 8.4 Structure of human wild-type and mutant RXRG protein

A. Arg286 (magenta) is part of the ligand binding domain and forms a salt bridge with glutamic acid at position 241 and glutamic acid at position 282

B-C. The substitution Arg286Cys (red) was shown through homology modelling to be smaller, and to lack charge, compared to the wild-type Arg286 (green)

CNV analysis of tumour WES data revealed multiple chromosomal gains involving Chr 5, 7, 8, 12-14, 16 and 18-22 (Figure 8.5). Orthogonal validation by SNP array showed $\text{arr}(3,5,7)\times 3,(8)\times 4,(12,13,14)\times 3,(16)\times 4,(18,19,20,21,22)\times 3$. Thus, SNP array confirmed tetrasomy of Chr 8 and 16 and trisomy of Chr 5, 7, 12-14 and 18-22 as shown by CNV analysis of the WES data, and also revealed Chr 3 trisomy. Involvement of Chr 7 by both WES and SNP array results indicated *AHR* copy number gain in the tumour, whilst the two different ploidy counts with Chr 8 and 16 tetrasomy and Chr 3, 5, 7, 12-14 and 18-22 trisomy by SNP array suggested the possibility of multiple tumour clones.

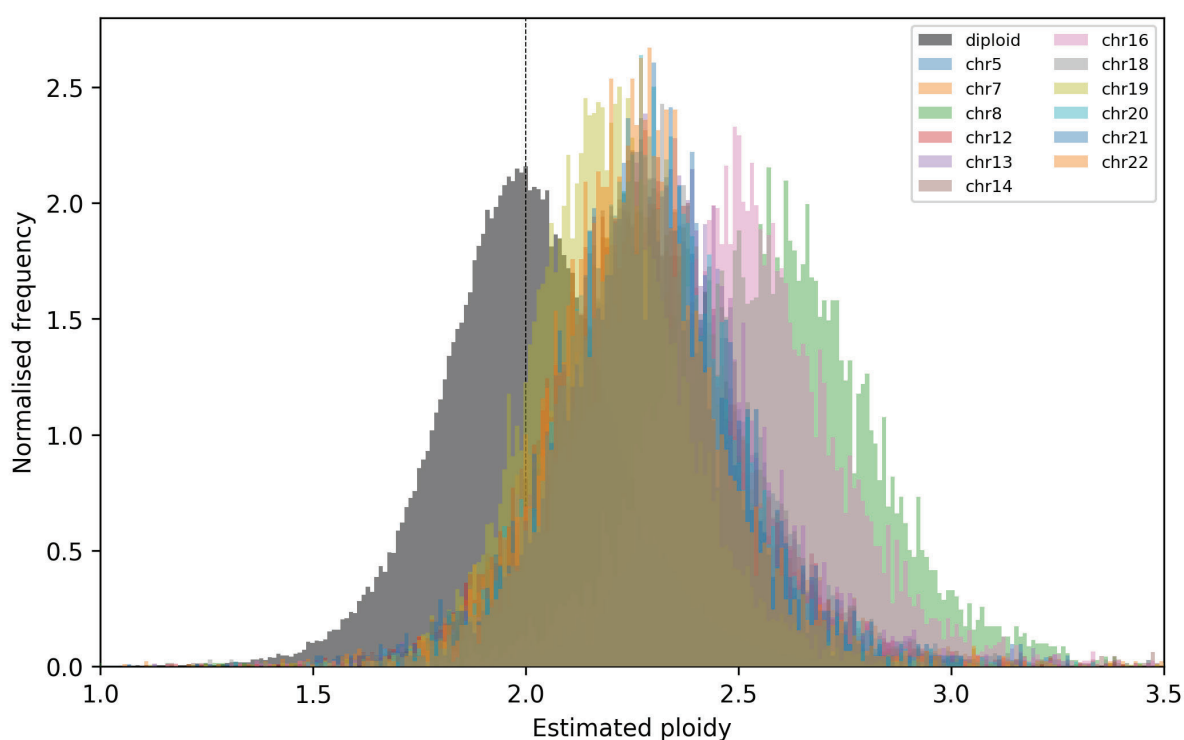


Figure 8.5. Tumour ploidy count

Plot of ploidy for each chromosome derived from WES copy number variation analysis, showing maximal gains in Chr 8 and 16 and intermediate gains in Chr 5, 7, 12-14 and 18-22

Tumour DNA disclosed 20 high confidence variants with a predominant mutational signature (Figure 8.6) matching that seen in most cancer types, though the aetiology of this signature is unknown (Alexandrov *et al.*, 2013).

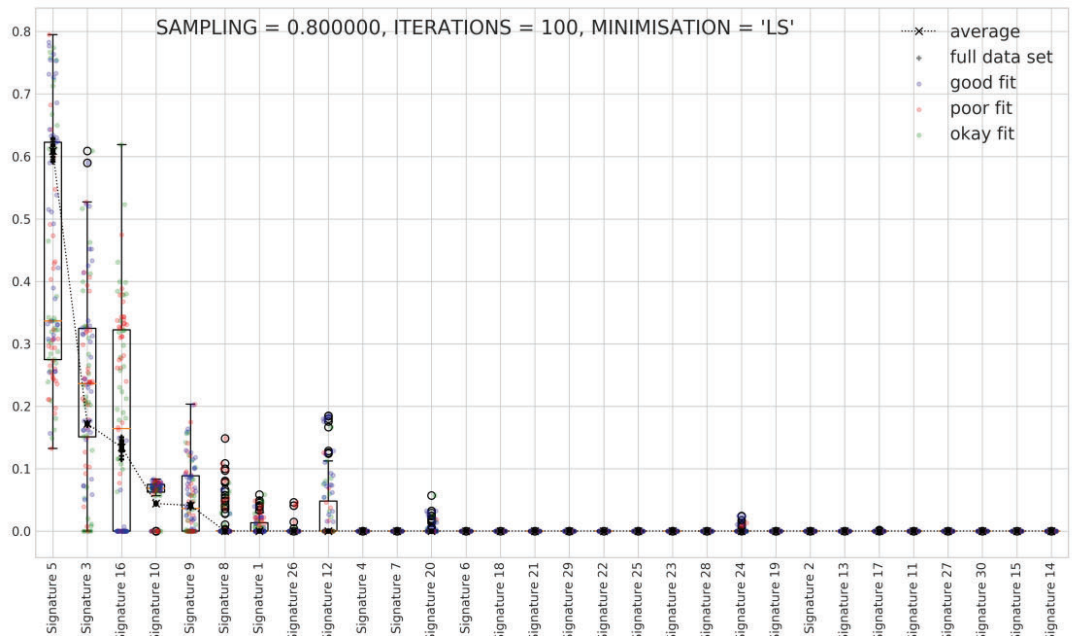


Figure 8.6. Mutational signature of tumour DNA

Plot of high confidence somatic variants according to Sanger Institute Mutational Signature types 1-30 showing the closest match to Signature 5, which has been found in all cancer types and most cancer samples (<https://cancer.sanger.ac.uk/cosmic/signatures>)

8.4 Discussion

The AHR protein exists in a cytoplasmic complex with AIP, heat shock protein 90 and p23 protein (Formosa *et al.*, 2017). Amongst many exogenous carcinogenic AHR ligands, the most potent is 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD; dioxin) (Cannavo *et al.*, 2016). Activation by such ligands causes dissociation and nuclear translocation of AHR, followed by heterodimerisation with Ah receptor nuclear translocator (ARNT) and transcription of target genes involved in the cell cycle and clock system (Jaffrain-Rea *et al.*, 2009; Jaffrain-Rea *et al.*, 2013; Cannavo *et al.*, 2016; Formosa *et al.*, 2017). In the present study, we identified a germline Thr583Met *AHR* variant in a man with cyclical CD. Our preliminary data suggest that this variant may have contributed to cyclical CD development via a loss of tumour suppressor function in the pituitary and disruption of circadian/infradian rhythms.

To the best of our knowledge, *AHR* mutations have not been reported in patients with pituitary adenomas, including recent WES cohort studies of patients with Cushing's disease (Reincke *et al.*, 2015; Chen *et al.*, 2018). However, other lines of evidence support a tumour suppressor role for *AHR* in the pituitary. *AHR* is the major binding partner of *AIP*, which is an established pituitary tumorigenesis gene (Jaffrain-Rea *et al.*, 2009; Jaffrain-Rea *et al.*, 2013). Loss of *AHR* stabilisation is postulated to be contributory to *AIP*-associated pituitary tumorigenesis, with somatotrophinomas from patients with germline *AIP* mutations typically showing decreased cytoplasmic and absent nuclear *AHR* staining (Jaffrain-Rea *et al.*, 2009). *AHR* may also have *AIP*-independent roles in pituitary tumorigenesis as *AHR* but not *AIP* expression is reduced in *GNAS*-mutated somatotrophinomas, and *AHR* activation increases the transcription of the pituitary tumorigenesis genes, *CDKN1B* (Jaffrain-Rea *et al.*, 2013). It is tempting to speculate that *AHR* may be a common step in tumorigenesis between *AIP*, *GNAS* and *CDKN1B*.

Whereas *AIP* germline mutations are most classically associated with somatotrophinomas, the *AHR* gene may be more relevant to corticotrophinomas. *AHR* immunostaining is found in the corticotroph-rich *pars intermedia* and normal corticotrophs demonstrate nuclear *AHR* immunostaining representative of activated *AHR* (Jaffrain-Rea *et al.*, 2009), although there have been no systematic *AHR* immunostaining studies of corticotrophinomas to date. Furthermore, *POMC* is overexpressed in mice and pituitary cell lines treated with the *AHR* ligand, dioxin (Huang *et al.*, 2002). The observed cross-talk between *AHR* and the oestrogen receptor (Cannavo *et al.*, 2016) may also be relevant to the predominance of CS in reproductive age women.

The location of our patient's *AHR* variant at a highly conserved phosphorylation site supports the pathogenicity of this variant. Loss of the normal nuclear pattern of *AHR* tumour staining

suggests that it is a loss-of-function variant causing failure of nuclear translocation. It is possible that the somatic Chr 7 trisomy amplified a dominant negative effect of this variant by increasing mutant dosage in the tumour. These features are consistent with *AHR* having a tumour suppressor function. However, other evidence suggests that *AHR* may have proto-oncogenic effects. Recapitulating animal models, an excess of NFPA and prolactinomas followed dioxin exposure from the 1976 Seveso accident in Italy (Jaffrain-Rea *et al.*, 2009), and acromegaly risk is eight-fold higher in Italian regions with high environmental exposure to other *AHR* ligands such as cadmium (Cannavo *et al.*, 2016). The *AHR* SNPs, rs2066853 (c.1661G>A, p.Arg554Lys) and rs4986826 (c.1708G>A, p.Val570Ile), are overrepresented in acromegalic patients in these regions with allele frequencies of 22.4% and 2.9%, respectively, compared to Caucasian ExAC allele frequencies of 9.9% and 0.3%, respectively (Cannavo *et al.*, 2016). Interestingly, these *AHR* SNPs and our patient's variant all reside in exon 10, encoding the transactivation domain (Figure 8.2) (Venselaar *et al.*, 2010). Exon 10 SNPs in *AHR* are associated with other neoplasms, including glioma, but pituitary studies have been limited to acromegaly (Cannavo *et al.*, 2016). The differential tumour suppressor and proto-oncogenic effects of *AHR* are yet to be fully elucidated but may depend on cell type.

AHR has an additional emerging role in the clock system, which entrains sleep, appetite, metabolism, locomotion and reproductive activity to 24-hr day-night cycles (Nader *et al.*, 2010; Jaeger *et al.*, 2017). The system's upstream mediators, circadian locomotor output cycle kaput (CLOCK) and brain-muscle-aryl hydrocarbon nuclear translocator-like protein 1 (BMAL1), heterodimerise and bind enhancer-box (E-box) regions in target genes, similarly to other members of the PER-ARNT-SIM (PAS) superfamily that includes *AHR* (Nader *et al.*, 2010). Downstream transcription of Periods (*PER1*, *PER2*, *PER3*) and Cytochromes (*CRY1*, *CRY2*) then creates diurnal patterns via various effector genes (Nader *et al.*, 2010; Jaeger *et*

al., 2017). Highly integrated with the clock system is the stress system, comprised of the HPA axis and autonomic nervous system (Nader *et al.*, 2010). In the hypothalamus, the light-activated central clock in the suprachiasmatic nucleus (SCN) projects to the paraventricular nuclei containing CRH- and arginine vasopressin-secreting neurons that regulate ACTH production (Nader *et al.*, 2010). Downstream, adrenocortical sensitivity to ACTH is modulated by SCN-mediated autonomic system activation and the peripheral clock in the adrenals (Nader *et al.*, 2010; Jaeger *et al.*, 2017), and the CLOCK/BMAL1 heterodimer can directly suppress glucocorticoid receptor-induced transcriptional activity (Nader *et al.*, 2010). Glucocorticoids in turn regulate *PER1* and *CRY1* expression, which might explain stress-induced circadian responses (Nader *et al.*, 2010). Finally, fluctuating clock gene expression allows the detection of day length, in turn imputing seasonal variation (Oster *et al.*, 2002), which may underlie observed infradian HPA rhythms (Meinardi *et al.*, 2007). Other clock and HPA interconnections include: cortisol lowering by both supraphysiological glucocorticoids and clock inputs (Nader *et al.*, 2010; Jaeger *et al.*, 2017); similar clinical manifestations due to sleep-wake disturbance vs. hypercortisolism (Nader *et al.*, 2010); and hypercortisolism with absent circadian rhythm in *Per1*-deficient mice (Nader *et al.*, 2010). Disruption of circadian/infradian HPA rhythms in cyclical CS and of circadian HPA rhythm in non-cyclical CS also indicates clock system and HPA axis interplay but, to the best of our knowledge, clock genes have not been previously assessed in CS.

Our patient's germline *AHR* variant was first of interest because it is a member of the PAS family (Cannavo *et al.*, 2016). Separate to the canonical pathways of AHR/ARNT heterodimerisation and BMAL1/CLOCK heterodimerisation described above, activated AHR can heterodimerise with BMAL1 via shared PAS domains and prevent BMAL1 from activating *PER1* transcription; thus, AHR indirectly inhibits *PER1* (Jaeger *et al.*, 2017). This is supported by greater amplitudes of downstream clock gene expression in *Ahr*-deficient vs. WT mice

(Jaeger *et al.*, 2017). On the other hand, pituitary adenomas are not reported in Ahr-deficient mice (Lund *et al.*, 2003; Vasquez *et al.*, 2003; Jaeger *et al.*, 2017). Our study findings may now provide a platform for further research to determine if inactivating *AHR* variants as found in our patient can account for the cyclicity of hypercortisolism in patients with cyclical CS.

Other variants might have acted synergistically with the *AHR* variant, particularly the novel germline *RXRG* variant which has a CADD score of 34.0 (Table 8.1). *RXRG* may act as a tumour suppressor gene in the pituitary as it is most highly expressed in the pituitary (GTEx; <https://www.gtexportal.org/home/>), and *RXRG* belongs to the retinoid X nuclear receptor family that mediates the antiproliferative effects of retinoic acid, which has shown some efficacy in the treatment of CD (Pecori Giraldi *et al.*, 2012). Another novel, likely damaging *RXRG* variant (p.R317H) in the ligand binding domain segregated upon WES of a familial prolactinoma kindred (Melo *et al.*, 2016). Although *RXRG* has not previously been studied in CD, the putative role of *RXRG* in this prolactinoma kindred is of interest given the associations between hyperprolactinaemia and CD as described in Chapter 5.

Our patient also had a somatic mutational signature typical of various cancer types (Alexandrov *et al.*, 2013). Though only 20 high confidence variants were available for the signature analysis, this is not uncommon in pituitary tumours (Song *et al.*, 2016; Bi *et al.*, 2017b), and the signature found raises the possibility of cooperation between the germline *AHR* and *RXRG* variants and somatic driver mutations.

A limitation of this pilot study is that the patient is currently in a prolonged state of eucortisolism, precluding additional investigations to demonstrate ongoing cyclicity. This is despite a significant tumour remnant, highlighting the discordance between structural and functional status in patients with cyclical CD. Given the rarity of CS in general and cyclical CD

in particular, collaborative research is required to further examine the potential relationship between *AHR* and cyclical CD.

In summary, our preliminary data suggest that the highly conserved *AHR* gene may represent a link between pituitary tumorigenesis, the HPA axis and the clock system, implicating it in the development of cyclical CD. With *AHR* known to be expressed in the pituitary, cyclical CD may occur because of the combination of *AHR*-mediated pituitary tumorigenesis and disordered clock control of the HPA axis. Our patient's somatic Chr 7 trisomy and germline *RXRG* variant might have been additive to his germline *AHR* variant, explaining how this variant can be seen in up to 1/10,000 individuals in population data despite the rarity of cyclical CD. Alternatively, *AHR* variants might lead to cyclicity in individuals who happen to develop CS. Further research is required to determine whether *AHR* is a true pituitary tumorigenesis gene or a phenotypic modifier gene accounting for cyclicity in CS of various aetiologies. If the former theory is supported, it may be informative to study the potential role of *AHR* in other unexplained pituitary tumours including prolactinomas, although reanalysis of the WES data from the prolactinoma cohort described in Chapter 7 did not reveal any *AHR* variants meeting our filtering criteria.

8.5 Conclusion

This is the first report of an *AHR* variant with predicted pathogenicity in the PA setting. Our preliminary data suggest that the highly conserved *AHR* gene may represent the missing link between pituitary tumorigenesis, the HPA axis and the clock system. Further research may indicate a role for the gene in the development of cyclical CD.

Chapter 9: Deep intronic *SDHC* mutation in familial paraganglioma with prolactinoma: a new mechanism of succinate dehydrogenase-related tumorigenesis

9.1 Introduction

As discussed in Chapter 7, prolactinomas may occur in a familial setting due to germline mutations in a number of PA predisposition genes. This includes the genes encoding the SDH complex whereby germline mutations predispose families to PPGL, GIST and RCC, in addition to PA. As in the last two chapters, we employed WES in the current chapter to investigate families with unexplained SDH-related tumours including a macroprolactinoma.

SDH is a heterotetramer protein complex comprised of four subunits: SDHA and SDHB comprise the catalytic domain; and SDHC and SDHD form a hydrophobic anchor in the inner mitochondrial membrane. The subunits are encoded by the *SDHA*, *SDHB*, *SDHC* and *SDHD* genes (Evenepoel *et al.*, 2015). Also critical to the function of SDH is SDH assembly factor 2 (SDHAF2), which is encoded by the *SDHAF2* gene and allows flavination of SDHA (Gill, 2018). The term '*SDHx*' is used to refer to *SDHA*, *SDHB*, *SDHC* and *SDHD* (Xekouki *et al.*, 2015), as well as *SDHAF2* by some authors (Papathomas *et al.*, 2015). SDH participates in both the tricarboxylic acid/Krebs cycle, where it oxidises succinate into fumarate, and the mitochondrial respiratory chain, where the donated electrons from succinate oxidation enable the reduction of coenzyme Q (Benn *et al.*, 2015; Evenepoel *et al.*, 2015; Belinsky *et al.*, 2017; Gill, 2018). *SDHx* mutations inactivate SDH, leading to reactive oxygen species and succinate accumulation (Benn *et al.*, 2015). The combined effect is inhibition of prolyl hydroxylases, resulting in decreased hydroxylation (inactivation) of hypoxia inducible factor α , angiogenesis, cellular proliferation and eventual tumorigenesis (Benn *et al.*, 2015; Evenepoel *et al.*, 2015).

In keeping with the tumour suppressor gene model, *SDHx* tumour syndromes result from germline heterozygous loss-of-function mutations distributed across all exons of the *SDHx* genes (Benn *et al.*, 2015). The somatic second hit is most commonly LOH, followed by somatic mutations, and, rarely, epigenetic inactivation (Evenepoel *et al.*, 2015). *SDHx* tumour syndromes are autosomal dominant with incomplete penetrance and variable expressivity (Toledo *et al.*, 2017), which is at least partly due to variability in the risk and timing of loss of the WT allele.

PPGL are the archetypal SDH-deficient tumour, but GIST, RCC and PA are now also established as classical SDH-related tumours (Benn *et al.*, 2015). Syndromes have been named according to the specific combination of SDH-related tumours: Carney triad for paraganglioma (PGL), GIST and pulmonary chondroma (Carney, 1999); Carney-Stratakis dyad for PGL and GIST (Carney & Stratakis, 2002); and 3PAs for PPGL and PA (Xekouki *et al.*, 2015). However, no individuals or families have been reported that exhibit all four SDH-related tumours. Distinguishing clinical features between the different SDH-related tumour syndromes include: a parent-of-origin effect with expression of the disorder primarily following paternal inheritance in *SDHD* and *SDHAF2*; PGL predilection for the head and neck region in *SDHD*; greater risks of malignant PPGL and RCC in *SDHB* and multifocal tumours in *SDHD*; and significantly lower prevalence and penetrance in *SDHA* and *SDHC* (Benn *et al.*, 2015; Evenepoel *et al.*, 2015).

Phaeochromocytomas and PGLs are neural crest derived tumours of the adrenal medulla and sympathetic/parasympathetic nervous system, respectively (Toledo *et al.*, 2017). PGLs may arise anywhere from the skull base to the pelvis (Benn *et al.*, 2015), making tumour surveillance onerous and emphasising the value of identifying causative mutations in PPGL kindreds to restrict follow-up to proven mutation carriers. PPGLs are regarded as the most

heritable tumours in humans (Toledo *et al.*, 2017), with germline mutations identifiable in >30% of all PPGL cases and 13-24% of sporadic cases (depending on age of onset, whether unifocal or multifocal, and whether the tumour is a pheochromocytoma or PGL) (Neumann *et al.*, 2002; Lenders *et al.*, 2014; Brito *et al.*, 2015; Curras-Freixes *et al.*, 2017; Sbardella *et al.*, 2018). Given the high mutation probability for most patients, and the clinical utility for patients and their families (Buffet *et al.*, 2019), genetic testing should be offered to all patients with PPGL (Lenders *et al.*, 2014). Knowledge of the genetic status of PPGL patients has recently been shown to improve patient follow-up with earlier recognition of recurrent disease and lower metastatic burden (Buffet *et al.*, 2019). The previous genetic investigation of choice was single or staged gene sequencing by direct sequencing with gene selection based on PGL location, hormone secretion and the presence or absence of metastasis and other tumours (Lenders *et al.*, 2014). This has been supplanted by comprehensive PPGL gene panels since the advent of accessible and affordable NGS technology allowing multiple genes to be tested simultaneously (Toledo *et al.*, 2017). Panel testing addresses the marked genetic heterogeneity in PPGL with >15 genes implicated to date (Toledo *et al.*, 2017). This encompasses cluster 1 pseudohypoxia inducing genes, including *SDHx*, and cluster 2 kinase signalling genes such as *RET*, *NF1*, *MAX* and *TMEM127* (Dahia, 2014). The major role of SDH deficiency in PPGL is underscored by *SDHx* mutations accounting for approximately half of all heritable PPGL syndromes (Lenders *et al.*, 2014; Gill, 2018).

GISTs are more commonly due to somatic gain-of-function receptor tyrosine kinase (RTK) mutations in *KIT* (70-80%) or *PDGFRA* (approximately 10%), which are collectively referred to as 'type 1' GIST (Belinsky *et al.*, 2017). SDH deficiency accounts for approximately half of the remaining 'type 2' RTK-WT GISTs (Belinsky *et al.*, 2017; Gill, 2018). The genetic drivers in SDH-deficient GISTs are germline *SDHA* mutations in 30%, germline mutations in *SDHB/SDHC/SDHD* in 20%, and postzygotic hypermethylation of the *SDHC* promoter in the

remaining 50% (Gill, 2018). Whereas RTK-mutated GISTs may arise anywhere along the gastrointestinal tract and show a predominant spindle cell morphology, SDH-deficient GISTs are almost exclusively gastric, commonly multifocal and typically epithelioid in cytomorphology (Benn *et al.*, 2015; Evenepoel *et al.*, 2015; Gill, 2018).

RCC is attributable to *SDHx* mutations in only 0.2% of cases (Benn *et al.*, 2015). However, RCC is evident in 14% of *SDHB* mutation carriers, 8% of *SDHD* mutation carriers, and occasional *SDHC* mutation carriers (Benn *et al.*, 2015). Features of SDH-deficient RCC include eosinophilic cytoplasm, intracytoplasmic vacuolations and sometimes cystic or sarcomatoid change (Papathomas *et al.*, 2014; Benn *et al.*, 2015; Gill, 2018).

PA is the least common manifestation of SDH deficiency, usually manifesting as prolactinomas or less often somatotrophinomas that are frequently macroadenomas with a more aggressive phenotype and a slight male predilection (Papathomas *et al.*, 2014; Evenepoel *et al.*, 2015; Xekouki *et al.*, 2015). Intracytoplasmic vacuoles have been reported as a defining feature (Dénes *et al.*, 2015), although the utility of this histopathological finding is limited by the predominantly medical management of prolactinomas with DAs.

SDHB and *SDHA* tumour IHC serves as an indicator of germline mutation status in all *SDHx*-related tumour types. Tumours stain negative (abnormal) for *SDHB* if there is biallelic inactivation of any SDH component (including *SDHAF2*), which almost always reflects the combination of a germline mutation and somatic loss of the WT allele in a given *SDHx* gene (Gill, 2018). Tumours may also stain negative for *SDHB* due to the more recently described mechanism of epigenetic silencing of *SDHC* by postzygotic *SDHC* promoter hypermethylation (Papathomas *et al.*, 2015). Loss of any SDH component destabilises the SDH complex or prevents it from forming with the subsequent release and degradation of the *SDHB* subunit, resulting in the loss of normal *SDHB* staining (Gill, 2018). Tumours stain negative (abnormal)

for SDHA only if there is biallelic inactivation of *SDHA* (Gill, 2018). SDH staining is reported as positive (normal) when there is granular cytoplasmic staining of comparable intensity to internal positive controls in the form of endothelial cells, sustentacular cells and lymphocytes, and negative when there is absent staining in the presence of internal positive controls (Papathomas *et al.*, 2015). SDHB/SDHA IHC was previously advocated to guide single/staged gene sequencing (Lenders *et al.*, 2014). With the advent of NGS, tumour IHC has become more useful in guiding the interpretation of *SDHx* variants as pathogenic vs. benign (Richter *et al.*, 2014; Evenepoel *et al.*, 2015; Papathomas *et al.*, 2015; Gill, 2018). However, 7-16% of patients with negative *SDHx* genetic testing have SDH-deficient tumours by IHC, and 18-19% of patients with SDH-deficient tumours by IHC lack identifiable *SDHx* mutations (Castelblanco *et al.*, 2013; Papathomas *et al.*, 2015). The significant correlation between malignancy and SDH deficiency even in the absence of *SDHx* mutations (Papathomas *et al.*, 2015) indicates that SDH-deficient tumours with negative genetic test results are an important clinical entity.

An adjunctive test in determining SDH deficiency is mass spectrometry with calculation of succinate:fumarate ratios, whereby a high ratio indicates a germline *SDHx* mutation causing loss of SDH function and succinate accumulation (Kim *et al.*, 2016; Gill, 2018). This may be particularly useful where IHC and genetic test results are discordant or if IHC is uninterpretable for histological reasons (Gill, 2018).

We report two families, with evidence of a common ancestor, with affected individuals collectively having all four SDH-related tumours. Comprehensive PPGL gene testing in standard commercial NGS facilities was negative. We hypothesised that their tumour predilection may be due to a mutation in a novel PPGL gene or in a location leading to altered gene transcription or expression that is not captured by standard genetic testing.

9.2 Methods

All genetic investigations were performed in a clinical setting by nationally accredited laboratory processes with informed patient consent. Written consent to publication was obtained from all living patients and from next of kin in the case of deceased relatives. The publication was ethically approved by the Royal Adelaide Hospital Human Research Ethics Committee in accordance with the National Health and Medical Research Council guidelines.

Case descriptions

Pedigrees and clinical features of the two families studied are shown in Figure 9.1 and Table 9.1, respectively.

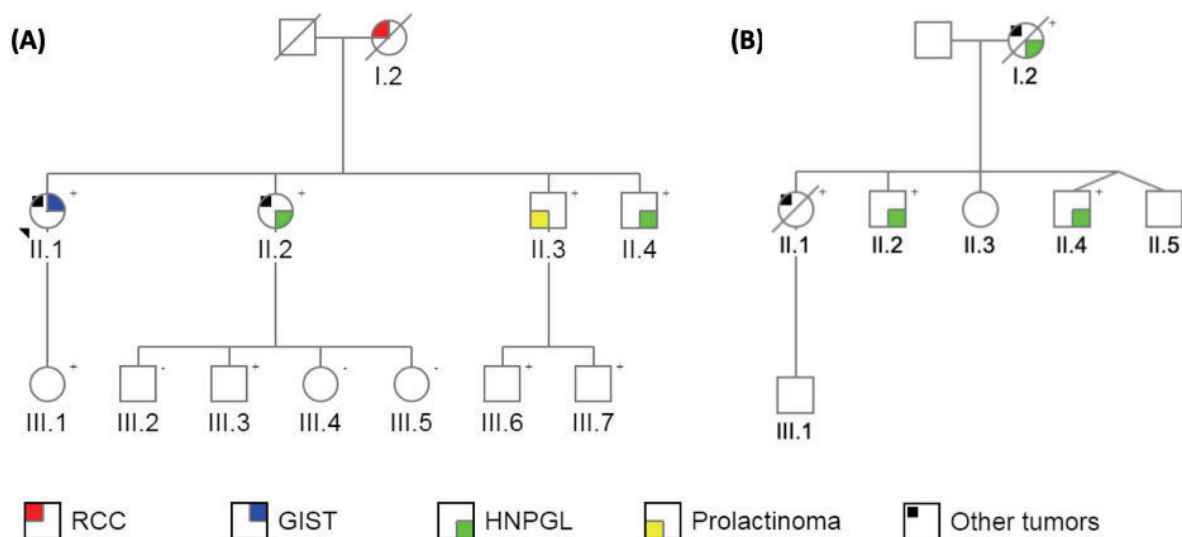


Figure 9.1. Pedigrees showing succinate dehydrogenase-related and other tumours

A. Family 1

B. Family 2

Genetic status in relation to the *SDHC* variant, c.20+74A>G, is indicated in the top-right-hand corner for all tested individuals: + variant present, - variant absent

Table 9.1. Clinical characteristics of affected relatives in Family 1 and 2

Findings consistent with SDH deficiency are indicated in bold

| Family | Individual (current age) | <i>SDHC</i> mutation status | Tumours (age at initial diagnosis) | <i>SDHB</i> tumour IHC | Succinate: fumarate ratio* |
|--------------|--------------------------|-----------------------------|---|------------------------|-------------------------------------|
| 1 | I.2 (died 61 yr) | N/T | RCC (60 yr) | Normal | N/T |
| | II.1 (59 yr) | + | Desmoid tumour (50 yr) | Normal | 10.600, 16.037, 11.681 ^a |
| | | | HCC (50 yr) | Normal | 29.458 |
| | | | Gastric GIST (51 yr) | Normal | 41.765 , 6.815 ^b |
| | | | Solitary fibrous tumour of lung (53 yr) | N/T | 13.895 |
| | | | Adrenocortical adenoma (53 yr) | N/T | N/T |
| | | | Meningioma (59 yr) | N/T | N/T |
| | II.2 (57 yr) | + | HNPGL (41 yr) | Abnormal | 89.490 |
| | | | Ovarian serous cystadenoma and cellular fibroma (53 yr) | N/T | 11.644 |
| | | | Meningioma (54 yr) | N/T | N/T |
| II.3 (54 yr) | + | Prolactinoma (41 yr) | N/T | N/T | |
| II.4 (52 yr) | + | HNPGL (34 yr) | Abnormal | 27.725 | |
| 2 | I.2 (died 61yr) | + | HNPGL (44 yr) | Abnormal | N/T |
| | | | Breast cancer (44 yr) | N/T | N/T |
| | | | Cholangiocarcinoma (61 yr) | N/T | N/T |
| | II.1 (died 38 yr) | + | Diffuse gastric carcinoma (38 yr) | Normal | N/T |
| | II.2 (38 yr) | + | Suspected HNPGL (37 yr) | N/T | N/T |
| II.4 (34 yr) | + | HNPGL (20 yr) | Abnormal | N/T | |

GIST, gastrointestinal stromal tumour; HCC, hepatocellular carcinoma; HNPGL, head and neck paraganglioma; IHC, immunohistochemistry; N/T, not tested; RCC, renal cell carcinoma; *SDHB*, succinate dehydrogenase subunit B; + mutation present; * ratios ≥ 23.48 consistent with SDH deficiency; ^a multiple ratios determined from serial resections of recurrent desmoid tumour; ^b multiple ratios determined from multifocal gastric GIST resected simultaneously

Family 1: The index family was of Italian ethnicity and consisted of four siblings with SDH-related tumours, with their mother having died from RCC (Figure 9.1A). Preliminary genetic testing in the proband, II.2, was negative for *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *VHL*, *RET* and *TMEM127* mutations by direct gene sequencing, and for *SDHB*, *SDHC*, *SDHD* and *VHL* CNVs by multiplex ligation-dependent probe amplification (MLPA).

I.2, a lifelong non-smoker, was diagnosed with RCC at age 60 and underwent left nephrectomy, demonstrating an 8.5 cm RCC with focal sarcomatoid appearance, microscopic invasion of perinephric fat and metastasis to peripelvic fat. Although *SDHB* tumour IHC was

historically reported as normal, the tissue slides were no longer available for review and other histological changes suggestive of SDH deficiency were noted in the original histopathological report, including vacuolated cytoplasm, cystic change and cells with eosinophilic cytoplasm.

II.1 first presented with a multifocal mesenteric desmoid tumour at age 50. She underwent right hemicolectomy at diagnosis, followed by small bowel and mesenteric resection and medical therapy with ibuprofen, tamoxifen, celecoxib and letrozole at age 54, and small bowel and sigmoid resection at age 55 for recurrent disease. Also at age 50, she was diagnosed with a well-differentiated hepatocellular carcinoma (HCC) for which she underwent resection at age 51 and radiofrequency ablation of a presumed second lesion at age 52. At age 51, she underwent resection of two gastric GISTs, measuring 6 mm at the lesser gastric curve and 20 mm at the body of the stomach. At age 53, she was found to have an 11 mm non-functioning adrenocortical adenoma which is being monitored, and a solitary fibrous tumour of lung which was resected. She was most recently diagnosed with a 4.2 cm left frontal meningioma at age 59 and is awaiting further management. SDHB IHC was performed on the desmoid tumour, HCC and GIST, with all samples showing normal staining. The two GIST specimens shared a similar appearance, with predominant spindle cell morphology and positive IHC for c-Kit/CD117 and Discovered on GIST-1 (DOG1), all of which suggested an RTK-mediated tumour.

II.2 presented with a non-secretory left cerebellopontine PGL at age 41 and underwent partial resection following embolisation. SDH tumour IHC was abnormal for SDHB and normal for SDHA. She recently completed radiotherapy at age 57 for the PGL remnant, which reached a diameter of 4.2 cm and was encasing the left ICA and impinging on the brainstem. She was also diagnosed with an ovarian serous cystadenoma and ovarian cellular

fibroma, both resected at age 53, and a parafalcine meningioma at age 54 which is under imaging surveillance.

II.3 was diagnosed with a 3.4 cm macroprolactinoma at age 41. He achieved a complete hormonal and tumour response with cabergoline.

II.4 was diagnosed with a non-secretory multifocal skull base PGL at age 34 and underwent partial resection following embolisation. The PGL remnant is stable on serial monitoring. SDH tumour IHC was abnormal for SDHB and normal for SDHA.

Because of the family history of PGL, all members of the second and third generations of Family 1 apart from III.5 have been screened for PPGL via MRI every 2-3 years and annual plasma metanephrines with no evidence of PPGL to date in any relatives other than II.2 and II.4.

Family 2: The second family was initially noteworthy for SDH-related and other neoplasia in two siblings and their mother (Figure 9.1B). Preliminary genetic testing in the proband with PGL, II.4, was negative for *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *VHL*, *RET* and *TMEM127* mutations by direct gene sequencing, and for *SDHB*, *SDHC*, *SDHD* and *VHL* CNVs by MLPA. All preliminary genetic tests in II.1, performed because of her history of gastric cancer, were negative, including: *CDH1*, *CTNNA1*, *MLH1*, *MSH2*, *MSH6*, *EPCAM*, *BRCA1* and *BRCA2* by NGS; *BRCA1*, *BRCA2* and *PMS2* by direct gene sequencing; and *CDH1*, *MLH1*, *MSH2*, *MSH6*, *EPCAM* and *PMS2* by MLPA. In view of the negative genetic test results and shared Italian ethnicity, Family 2 was selected for investigation for the mutation identified in Family 1 during the course of this study.

I.2 was diagnosed with a right jugular PGL at age 44 and underwent resection following embolisation. Histopathology confirmed PGL with lymph node metastases. SDHB IHC was

abnormal (SDHA IHC not performed). She was concurrently diagnosed with breast cancer, treated with mastectomy, axillary clearance and adjuvant chemoradiotherapy. She remained in remission of her PGL and breast cancer at age 61, when she died due to newly diagnosed metastatic cholangiocarcinoma.

II.1 presented with acute kidney injury and venous thromboembolism 2 months postpartum at age 38. She died from a presumed systemic inflammatory illness with multiple osteolytic lesions 3 weeks later. Postmortem examination revealed metastatic diffuse gastric carcinoma. Tumour IHC was normal for SDHB and SDHA.

II.4 underwent resection for a right jugulotympanic PGL at age 20. SDH tumour IHC was abnormal for SDHB and normal for SDHA.

The surviving second generation members of Family 2 recently underwent PPGL screening, revealing a likely head and neck PGL (HNPG) recurrence in II.4 and a new diagnosis of likely HNPG in II.2. PPGL screening was negative in II.3 and II.5.

DNA extraction

Fresh blood samples were obtained from II.1-4 of Family 1 and II.4 of Family 2 for extraction of germline DNA from peripheral blood leucocytes. Amongst the deceased individuals: no DNA was available from I.2 of Family 1; stored DNA was obtained from II.1 of Family 2; and only tumour DNA was available from I.2 of Family 2. Tumour DNA extraction was performed using FFPE tissue specimens of the 6 mm GIST in II.1 and the PGL in II.4 in Family 1, and the PGL and breast cancer in I.2 in Family 2. Other tumour specimens were not available for sequencing. Germline and tumour DNA were extracted using commercially available kits (Qiagen) according to manufacturer protocols. Additional DNA repair steps were performed

for the FFPE specimens to allow enzymatic removal of formalin-induced cytosine deamination artefacts.

Whole exome sequencing

WES was performed in Family 1 using the available germline and tumour DNA samples, the Roche NimbleGen SeqCap EZ MedExome Target Enrichment Kit and the Illumina NextSeq 500 sequencing platform. The average of mean depth of coverage amongst all samples was 97X, and 94% of target bases were covered $\geq 20X$. Bioinformatic analysis was performed in ACRF (Adelaide, Australia) using GATK HaplotypeCaller (Van der Auwera *et al.*, 2013) to detect small variants (typically < 50 bp) and in-house scripts and Sequenza to analyse CNVs. Raw WES data were filtered for variants that were: rare ($< 1\%$ population prevalence); possibly damaging (by snpEFF impact, splicing/binding predictions, GERP or CADD); and of high quality (by GATK internal filters). Germline variants were considered further if they were heterozygous in the germline DNA of all four siblings in Family 1 with GATK GQ score > 50 and depth of coverage $> 30X$. Variants in low complexity regions or duplicated segments were discarded. Candidate genes were prioritised based on existing literature. *In silico* splice site assessment was performed using Alamut Visual, incorporating SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer prediction models.

The Roche NimbleGen SeqCap EZ MedExome Target Enrichment Kit and the Illumina NextSeq 500 sequencing platform were also employed in the preliminary testing of gastric cancer predisposition genes in II.1 of Family 2.

Sanger sequencing

The leading germline variant of interest in Family 1 was assessed using germline DNA from II.1-4 of Family 1 and II.1 and II.4 of Family 2, and tumour DNA from I.2 from Family 2.

Bidirectional genomic DNA sequencing was performed using primers designed via Primer3Plus and raw data were visualised using MutationSurveyor version 2.51 (SoftGenetics LLC).

Sanger sequencing for the leading germline variant of interest was later performed to facilitate predictive cascade testing in other relatives of Family 1 and Family 2.

Haplotype analysis

Haplotype analysis was performed by considering rare variants (SNPs >20X coverage, ExAC and UK10K allele frequencies <0.01) in the WES data of II.2 of Family 1 and II.1 of Family 2 and mapping those loci which overlapped between the two individuals. For any rare variant identified in either individual, an unrelated individual would overlap at <1% of loci with random distribution throughout the genome. Conversely, relatedness due to identity by descent would be identified by a chain of shared rare variants that are non-randomly distributed throughout the genome.

Transcriptome analysis

Whole blood was obtained from II.2 of Family 1 for transcriptome analysis to further investigate the leading germline variant of interest in Family 1. RNA-Seq was performed via the Illumina TruSeq LT platform using 150 bp reads and poly(A) selected RNA to deplete ribosomal RNA.

Krebs cycle metabolomic studies

All available FFPE tumour specimens were tested in duplicate by mass spectrometry to measure succinate and fumarate levels and calculate succinate:fumarate ratios to aid the identification of SDH-deficient tumours as previously described (Kim *et al.*, 2016). A

threshold of 23.48, previously established in GIST FFPE specimens (Kim *et al.*, 2016), was used to define SDH deficiency with tumours exhibiting ratios above this considered to be SDH-deficient.

SDHC promoter methylation analysis

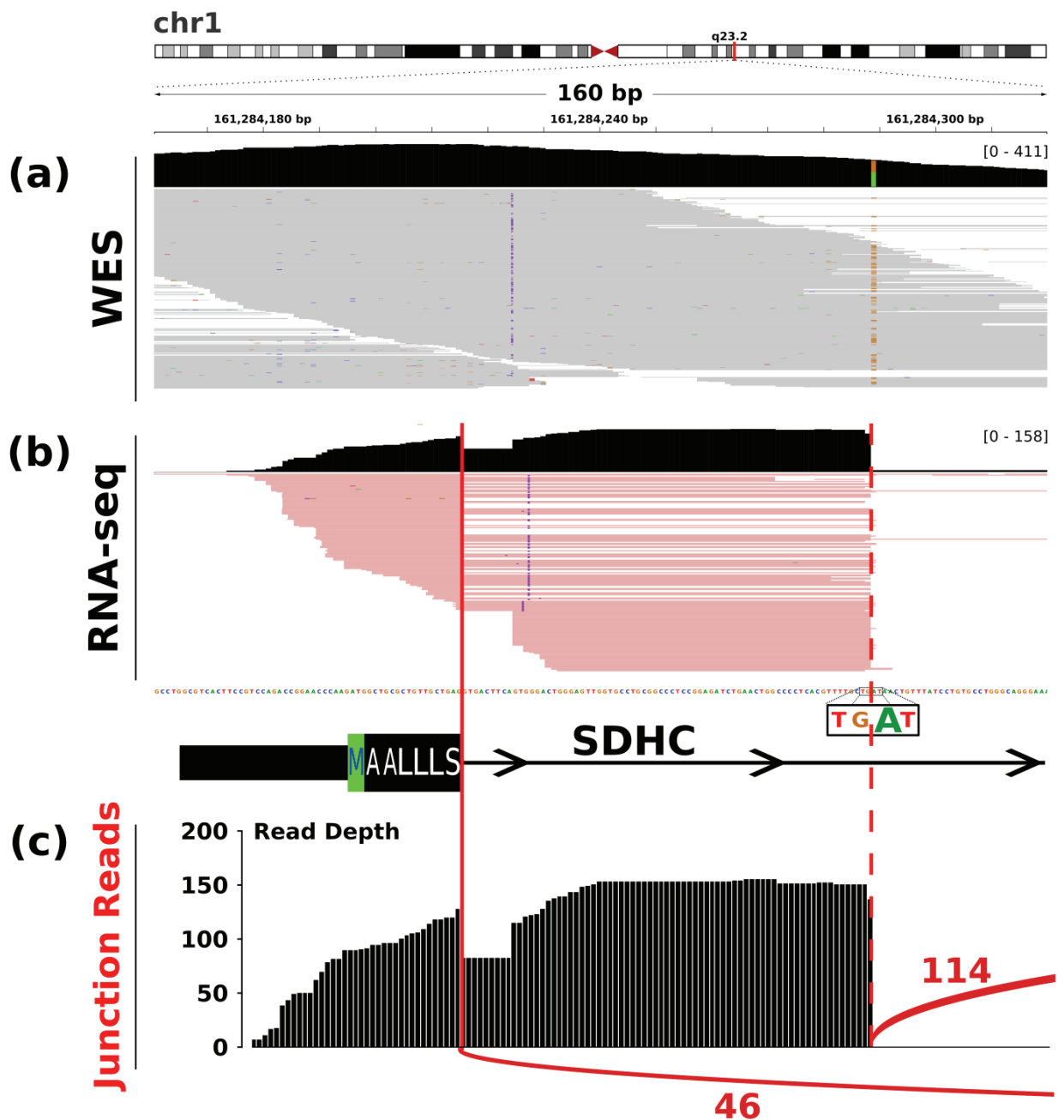
In view of the known mechanism of *SDHC* promoter hypermethylation in the pathogenesis of GISTs, methylation status of the *SDHC* promoter region was determined in both GIST specimens from II.1 of Family 1. Extracted tumour DNA was bisulfite treated using the Qiagen EpiTect Bisulfite Kit (EpiTect Bisulfite Kit, catalogue number 59104; Qiagen, Hilden, Germany) according to manufacturer instructions. As previously described (Flanagan *et al.*, 2012), a Qiagen PyroMark CpG assay was performed and pyrograms were analysed using PyroMark Q24 software (Qiagen, Hilden, Germany) to calculate percentage methylation at four CpG sites (Chr 1: 161,313,986; Chr 1: 161,313,998; Chr 1: 161,314,011 and Chr 1: 161,314,022) and mean methylation across these four CpGs for each sample.

9.3 Results

Germline genetic analysis in Family 1

After filtration of raw data, WES of germline DNA revealed 19,581 rare variants with at least some evidence of pathogenicity, including 130 high-quality heterozygous variants in all four siblings of Family 1. One variant (GRCh37/hg19, Chr 1:g.161284289A>G; NM_003001; c.20+74A>G) was found in an intronic region of the known candidate gene, *SDHC* (Figure 9.2A), at >20X coverage (21 WT reads, 32 mutant allele reads in II.1; 93,80 in II.2; 25,24 in II.3; and 35,30 in II.4). Sanger sequencing confirmed the variant in all four siblings. This deep intronic *SDHC* variant is situated in a conserved region (GERP 2.38) of intron 1 and has not been previously reported. It is absent in public genomic datasets, including: 1KGP; UK10K;

ExAC; and gnomAD, containing 31,378 control alleles in the vicinity of this variant, including 106 alleles from patients of Southern European ethnicity. All four component splicing models of Alamut Visual predicted introduction of an alternate 5' (donor) splice site at the location of the variant.



(d) Nucleotide sequence (exon1):

atg gctg cgc t g t t g c t g a g g t g a c t t c a g t g t g g g a c t g g g a g t t g g t g c c t g c g g c c c
t c c g g a g a t c t g a a c t g g c c c t c a c g t t t t g c t g

Amino Acid sequence (whole gene):

MAALLLR*LQ CGTGSWCLRP SGLDNWPLTF C*HVGRHCLR AHFSPQLCIR NAVPLGTTAK
EEMERFWNKN IGSNRPLSPH ITIYSWSLPM AMSICHRGTG IALSAGVSLF GMSALLLPGN
FESYLELVKS LCLGPALIHT AKFALVFPLM YHTWNGIRHL MWDLGKGLKI PQLYQSGVVV
LVLTVLSSMG LAAM

Final SDHC protein product (whole gene)

MAALLLR*

Figure 9.2. DNA and RNA representations of the *SDHC* variant, c.20+74A>G

A. WES result of germline DNA as depicted in Integrative Genomics Viewer showing the heterozygous substitution of guanine (brown) for adenosine (green) at genomic DNA position 161,284,289 of Chr 1

B. RNA-Seq result as depicted in Integrative Genomics Viewer showing alternative splicing of exon 1 (the canonical splice site is indicated by the solid red line and the novel splice site by the dotted red line, coinciding with the A>G substitution)

C. Junction counts of individual mRNA reads showing preferential expression of the aberrantly spliced transcript ($n=114$) vs. normal transcript ($n=46$)

D. Nucleotide, amino acid and final protein product sequences produced by the 75 bp inclusion observed on RNA-Seq (the start codon is indicated in blue, the intronic inclusion in exon 1 created by the *SDHC* c.20+74A>G variant is indicated in red and premature stop codons are indicated by the red asterisks)

Subsequent RNA-Seq of whole blood from II.2 of Family 1 showed aberrant splicing with *SDHC* mRNA reads extending into intron 1 (Figure 9.2B), due to conversion of a non-splicing region (TG|AT) into a canonical splice site (TG|GT) because of the familial *SDHC* variant. There was evidence of preferential expression of the alternatively spliced transcript ($n=114$) compared to the normal transcript ($n=46$) (Figure 9.2C). The alternatively spliced transcript was absent in publicly accessible databases (University of California, Santa Cruz (UCSC) Genome Browser, Ensembl, GTEx, National Center for Biotechnology Information) as well as in-house RNA-Seq results from >700 samples.

The retained segment size is 75 bp due to the upstream inclusion of a common 2 bp *SDHC* insertion listed as benign by ClinVar (*SDHC*, NM_003001.3, c.20+11_20+12dupTG). Thus, frameshift does not occur. However, the retained intronic segment produces a premature stop codon immediately after exon 1. The final *SDHC* protein product is significantly shortened (Figure 9.2D) and predicted to result in nonsense-mediated decay.

Overall, the familial *SDHC* variant fulfilled the American College of Medical Genetics and Genomics (ACMG) criteria for a pathogenic (class 5) variant (PVS1: null variant; PS3: functional evidence; PM2: absent from controls; PP1: cosegregation) (Richards *et al.*, 2015).

Given the history of additional tumours in II.1 and II.2 of Family 1, germline data from these individuals was independently interrogated for variants in genes predisposing to GIST (*KIT*, *PDGFRA*), meningioma (*NF2*, *SMARB1*, *SMARCE1*, *SUFU*, *LZTR1*) and desmoid tumours (*APC*). Applying our basic filters, we found no germline variants in these genes.

Tumour genetic analysis in Family 1

WES and copy number analysis of the PGL from II.4 of Family 1 revealed 0.4-0.5X ploidy loss of Chr 1 (Figure 9.3). This could represent either loss of one copy of Chr 1 in 40-50% of

tumour cells or loss of both copies of Chr 1 in 20-25% of tumour cells. Mutant allele frequency would be expected to be unchanged if both chromosomes were lost in a subset of cells or if there was unbiased loss of Chr 1 between different cells. By contrast, the deep intronic *SDHC* mutation load on WES rose from 47% in the germline DNA of II.4 to 60% in the tumour DNA of II.4. It was thus deduced that 40% of cells lost the *SDHC* WT allele (producing a 3:5 WT:mutant ratio in tumour DNA vs. a 1:1 ratio in heterozygous germline DNA). This chromosomal loss was considered to be the second hit in the tumour suppressor gene two-hit model, thus supporting the germline deep intronic *SDHC* variant as the causative mutation. Whole chromosome loss of Chr 11 was also detected, as is commonly observed in PGL specimens (Dannenber *et al.*, 2001).

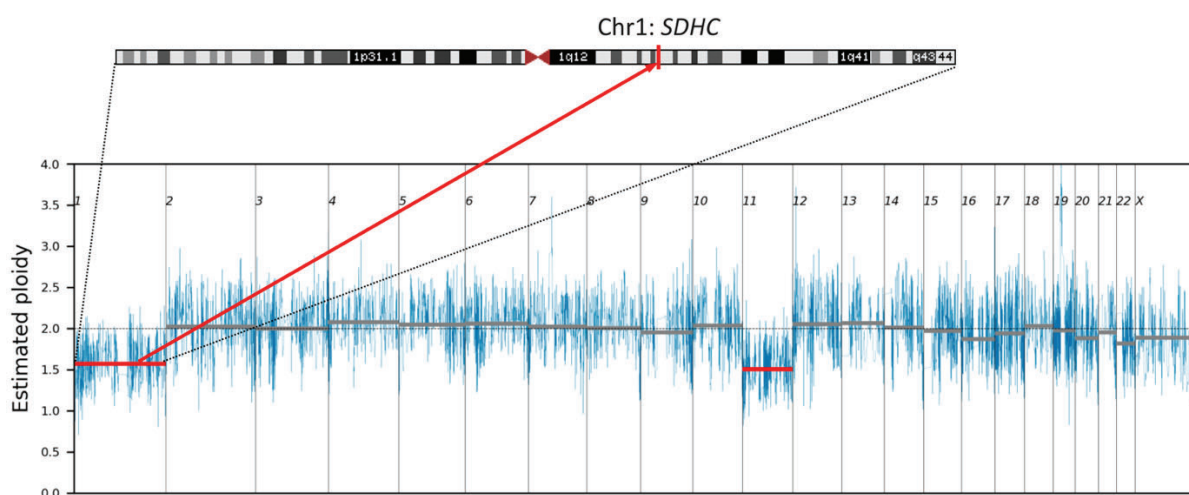


Figure 9.3. Chr 1 and 11 loss in the paraganglioma of II.4 from Family 1

Chromosomal loss as demonstrated by whole exome sequencing of tumour DNA with the position of the *SDHC* gene on Chr 1 indicated by the red vertical line

WES of the 6 mm GIST from II.1 of Family 1 demonstrated a previously described (Joensuu *et al.*, 2015) gain-of-function *KIT* mutation (GRCh37/hg19, Chr 2:g.55593610T>G; ENST00000288135; p.Val559Gly/c.1676T>G). Other than the germline c.20+74A>G variant, no point mutations or CNVs involving *SDHC* were found in this specimen. Each of the non-

contiguous GIST specimens from II.1 of Family 1 showed low *SDHC* promoter methylation: range 7-24%, average 15.3% in the 6 mm GIST; range 9-22%, average 14.8% in the 20 mm GIST. The methylation rate in these tumours fell in the bottom 10% of internal FFPE GIST control specimens ($n=15$) (Kim *et al.*, 2016), excluding *SDHC* promoter hypermethylation in the pathogenesis of this patient's GISTs.

Genetic linkage between Family 1 and Family 2

Sanger sequencing in Family 2 using germline DNA from II.1 and II.4 and tumour DNA from the PGL and breast cancer of I.2 revealed the same deep intronic *SDHC* variant in these three family members with neoplasia.

As hotspot mutations have not been described in *SDHC* and because of the current geographical proximity and shared Italian ancestry of Family 1 and Family 2, haplotype analysis was performed to investigate possible cryptic relatedness. This revealed multiple regions of identity by descent, including the deep intronic *SDHC* mutation, consistent with a shared common ancestor (Figure 9.4). Deeper family history taking revealed that the two families originated from the same small region in Italy.

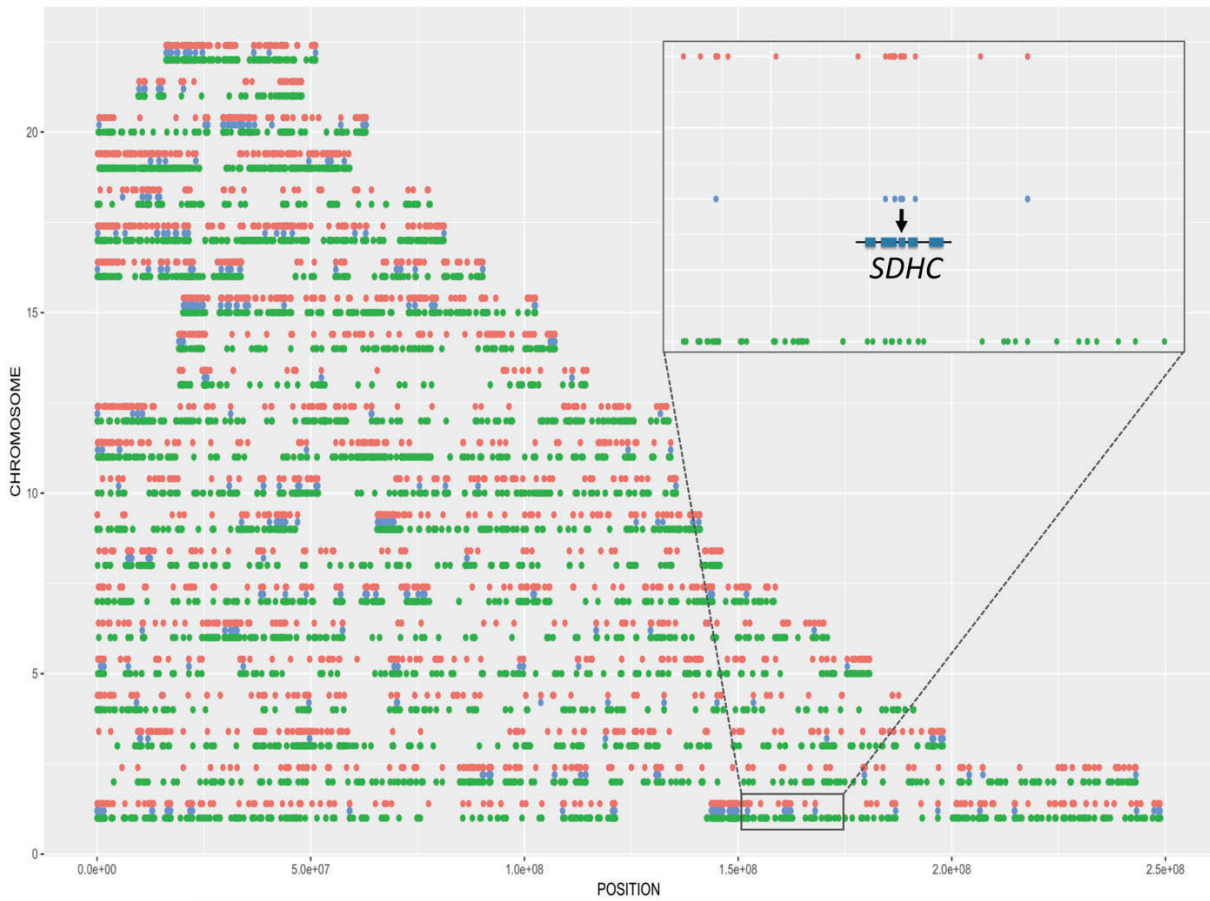


Figure 9.4. Haplotype analysis using exome data from the 22 autosomes

Regions unique to Family 1 are shown in red, regions unique to Family 2 in green, and regions shared between the two families (representing identity by descent) in blue; the inset shows the shared region on Chr 1 that includes *SDHC*

Metabolomic profiles

Succinate:fumarate ratios (Table 9.1) indicated SDH deficiency in both PGLs in Family 1, consistent with the SDHB IHC results for these tumours. The HCC and 6 mm GIST specimens from II.1 of Family 1 were also classified as SDH-deficient with ratios of 29.458 and 41.768, respectively. This was despite normal SDHB IHC results for both tumours and the WES finding of a known *KIT* mutation in the 6 mm GIST. The 20 mm GIST specimen from the same patient, as well as all other examined tumours, were classified as SDH-sufficient.

Cascade testing

The c.20+74A>G *SDHC* mutation was subsequently detected in III.1, III.3, III.6 and III.7 of Family 1, and in II.2 of Family 2. Apart from II.2 of Family 2 who was recently diagnosed with a likely HNPGL, all of these mutation carriers appear to be unaffected to date.

The mutation was absent in III.2, III.4 and III.5 of Family 1, and in II.5 of Family 2.

Cascade testing has not yet been performed in II.3 and III.1 of Family 2.

9.4 Discussion

This study revealed a novel genetic mechanism for the development of SDH-related tumours: the presence of deep intronic mutations that lead to incomplete translation of the *SDHC* gene. This was identified in two families currently living in the same city, with historical links to the same small region in Italy, and shown to be distantly related by haplotyping. Contrary to the fortuitous findings in this WES study, deep intronic mutations will usually be missed by WES and NGS gene panels performed in the clinical testing of individuals and families with PPGL. Such intronic mutations may comprise a significant proportion of individuals and families with SDH-related tumours and negative genetic

testing, especially those with abnormal SDH IHC. This has implications for testing in PPGL. WGS, though more costly, may be required in cases with negative routine genetic testing despite a characteristic phenotype. The extent to which this is necessary will depend on the frequency of deep intronic *SDHx* mutations in unexplained SDH-deficient tumour syndromes. A recent international consensus statement on the use of NGS in PPGL genetic testing recognised the possibility but cited the lack of evidence for deep intronic mutations playing a part in PPGL (Toledo *et al.*, 2017). Intron analysis has also been limited by sheer size, with intronic regions being approximately 20-fold longer than exonic regions (Vaz-Drago *et al.*, 2017). Others have hypothesised that SDH-deficient tumours lacking *SDHx* mutations despite exhaustive genetic testing might relate to large-scale chromosomal abnormalities or epigenetic changes (Richter *et al.*, 2014). We have now described the first deep intronic mutation in an *SDHx* gene and propose deep intronic *SDHx* mutations as a novel mechanism in previously unexplained cases of SDH deficiency. To the best of our knowledge, Family 1 also represents the first family to manifest all four SDH-related tumours, albeit with insufficient tissue data to confirm a causative role for the *SDHC* mutation in all tumours. In addition, the cryptic relatedness revealed by haplotype analysis indicates that the presented two families form one of the largest *SDHC* kindreds to date.

SDH-related tumours have been described in the setting of a range of germline *SDHx* variant types, including start codon, missense, nonsense and frameshift variants, and whole exon and gene deletions (Evenepoel *et al.*, 2015; Andrews *et al.*, 2018). Epigenetic variation has also been described with *SDHC* promoter hypermethylation now known to account for Carney triad (Haller *et al.*, 2014) and the half of SDH-deficient GISTs that were previously considered unexplained due to a lack of germline *SDHx* mutations (Benn *et al.*, 2015; Gill, 2018). Splice site mutations are another well-described mechanism of tumorigenesis with

germline *SDHC* splicing variants accounting for 15% of PGL and 30% of GIST (Evenepoel *et al.*, 2015). However, such splicing variants are typically only 1-2 bp away from the intron-exon boundary. Of 557 publicly available variants in *SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2* reported in the Leiden Open Variant Database (<http://www.lovd.nl/3.0/home>) as anything but benign or likely benign, 126 are in intronic regions including 38 variants suspected or proven to cause aberrant splicing according to the submitted classification. The deepest of such variants are only +/-7 bp away from the exon-intron boundary. Deep intronic variants have previously referred to variants >100 bp away from exon-intron boundaries (Vaz-Drago *et al.*, 2017). However, the c.20+74A>G *SDHC* mutation is considered to be a deep intronic variant as it falls outside the usual 10-20 bp region that is typically assessed in clinical genetic testing, explaining why the mutation was undetected in the preceding sequential genetic testing which spanned 12 years in Family 1.

Though deep intronic mutations are a novel finding amongst the *SDHx* genes, this variant type has been described in >75 other disease-associated genes (Vaz-Drago *et al.*, 2017), including *NF1* which has a small contribution to familial PPGL syndromes (Lenders *et al.*, 2014; Vaz-Drago *et al.*, 2017). The precise location of deep intronic variants predicts functional consequence. Deep intronic variants <150 bp away from the exon-intron boundary typically cause weakening of the canonical splice site (Vaz-Drago *et al.*, 2017), as shown through RNA-Seq in this study. By contrast, the most common mechanism of deep intronic variants overall is an intronic point mutation or small deletion creating a novel donor splice site at/near the variant and activating an upstream pre-existing non-canonical acceptor splice site. This creates a pseudo-exon, typically disrupting the reading frame and introducing a premature stop codon that directs the mutant mRNA towards nonsense-mediated decay (Vaz-Drago *et al.*, 2017).

The *SDHC*-related familial PGL syndrome (also referred to as hereditary PGL syndrome type 3; PGL3) is widely considered to be a less severe disorder than the more common familial syndromes associated with *SDHD* (PGL1) and *SDHB* (PGL4) mutations (Else *et al.*, 2014; Benn *et al.*, 2015). PGL3 typically manifests as unifocal non-secretory HNPGL with low malignant potential, reduced risk of RCC and PA, and overall low penetrance (Benn *et al.*, 2015). Consistent with this classical phenotype, our families developed non-secretory HNPGLs with metastasis in only one case. All PGLs stained negative for SDHB and positive for SDHA, as expected. Succinate:fumarate ratios also classified the two available PGLs as SDH-deficient, noting that relatively lower ratios may be observed in: SDH-deficient PGLs in the head and neck region presumably due to differences in tumour cellularity (Richter *et al.*, 2014); in *SDHC*-mutated tumours compared to *SDHB* (Richter *et al.*, 2014); and in FFPE compared to fresh frozen specimens (Richter *et al.*, 2014; Gill, 2018). Furthermore, the one PGL available for WES exhibited Chr 1 loss, consistent with loss of the WT *SDHC* allele.

Unusually for the *SDHC* gene in particular, Family 1 also exhibited the other three classical SDH-related tumours. The RCC was deemed sufficient by SDHB IHC performed soon after surgery around the advent of SDH IHC, but contemporary IHC studies, metabolomic profiling and WES were not possible in this specimen which was later destroyed, or in the prolactinoma which was successfully treated with a DA. The development of an RCC in a non-smoker with SDH-related histological findings and a large prolactin-secreting macroadenoma in a 41-year-old male remain suspicious for SDH deficiency in the absence of more detailed tissue data.

The GIST in Family 1 stained positive for c-Kit/CD117, showed normal SDHB IHC, and had predominant spindle cell morphology, all consistent with the somatic gain-of-function *KIT* mutation found on WES. However, metabolomic analysis showed one GIST focus to have a

significantly raised succinate:fumarate ratio, suggestive of SDH deficiency. We did not identify any *SDHC* somatic second hits on WES of the GIST and *SDHC* promoter hypermethylation studies were negative. However, a lack of *SDHD* somatic second hits has been similarly reported in some pheochromocytomas in *SDHD* mutation carriers and the possibility of haploinsufficiency has been raised (Papathomas *et al.*, 2014).

Though speculative, it is possible that the gain-of-function *KIT* mutation detected in our patient's GIST may have served as the second hit in a model of digenic tumorigenesis. RTK and *SDHx* mutations in GIST have traditionally been considered mutually exclusive (Gill, 2018), but there is emerging evidence for possible cooperation between the RTK and pseudohypoxia pathways. In a case similar to ours, a 38-year-old woman with Carney-Stratakis dyad (PGL and GIST) and a germline frameshift *SDHD* mutation was found to have a gain-of-function *KIT* mutation in her GIST (Gasparotto *et al.*, 2016). Although, the role of *SDHD* in her GIST has been challenged because of the rectal location, spindle cell morphology, lack of a somatic second hit and weak focal SDHB staining (Belinsky *et al.*, 2017). Cases involving gastric GISTs have been reported, including: a man with Carney-Stratakis dyad (PPGL and GIST), a germline truncating *SDHD* mutation and a somatic gain-of-function *KIT* mutation in his GIST (Ayala-Ramirez *et al.*, 2010); a man with Carney-Stratakis dyad (PGL and GIST), a germline *SDHB* 5 bp deletion and a somatic gain-of-function *KIT* mutation in his GIST that also demonstrated negative SDHB IHC and *SDHB* LOH (Jove *et al.*, 2014); and a patient with SDHB IHC-negative multifocal GIST with dual somatic mutations in *PDGFRA* and *SDHB* which were gain- and loss-of-function, respectively (Belinsky *et al.*, 2017). The importance of determining the relative contributions of the different genes in digenic models is underscored by the treatment of RTK-mutated tumours with imatinib, with genotype-dependent tumour responses, and the emergence of novel RTK inhibitors

(Belinsky *et al.*, 2017), as well as preliminary data supporting a role for poly(ADP)-ribose polymerase (PARP) inhibitors in SDH-deficient tumours (Sulkowski *et al.*, 2018).

In addition to the four classical SDH-related tumours, our patients demonstrated a range of other tumours. The HCC was most suggestive of a possible relationship with the germline *SDHC* mutation as, despite normal SDHB IHC, it showed a high succinate:fumarate ratio consistent with SDH deficiency. *SDHB* expression is frequently downregulated in human HCC and *SDHB* knockdown in murine models increases HCC proliferation and metastasis (Tseng *et al.*, 2018), but we are unaware of other cases of HCC in *SDHx* carriers.

We observed other tumours that have been previously reported in patients with *SDHx* abnormalities, namely: adrenocortical adenoma (Carney, 1999; Else *et al.*, 2014; Richter *et al.*, 2016); meningioma (Niemeijer *et al.*, 2015); breast cancer (Evenepoel *et al.*, 2015; Niemeijer *et al.*, 2015); and diffuse gastric cancer (Habano *et al.*, 2003; Hansford *et al.*, 2015). Whether any of these tumours relate to the germline *SDHC* mutation remains to be elucidated in the absence of informative tumour data. This includes the adrenocortical adenoma and meningiomas that have not required surgery, the breast cancer which was unavailable for investigation, and the gastric cancer which showed normal SDHB IHC but was unavailable for further study.

The desmoid tumour, solitary fibrous tumour of lung, ovarian serous cystadenoma, ovarian cellular fibroma and cholangiocarcinoma observed in our families have not been previously linked with *SDHx* mutations. Arguing against a role for *SDHC* in tumorigenesis, the desmoid tumour showed normal SDHB IHC and succinate:fumarate ratio and the solitary fibrous tumour of lung and ovarian tumours showed normal succinate:fumarate ratios. The cholangiocarcinoma was unavailable for investigation.

Although most of the tumours in these families are individually rare and the combination of tumours would be exceedingly rare, we cannot exclude the incidental co-occurrence of sporadic tumours. This is particularly true for the tumours that were only partly studied or not studied at all due to specimen availability. Moreover, doubt remains regarding the general association of *SDHx* mutations and PA as the germline *SDHx* variants in patients with PA have frequently been classified as likely benign variants or variants of unknown significance (Xekouki *et al.*, 2015; De Sousa *et al.*, 2017b), and tumour studies have been limited, partly due to the predominance of prolactinomas in these patients which are usually medically treated (Papathomas *et al.*, 2014; Evenepoel *et al.*, 2015; Xekouki *et al.*, 2015). Another possibility in the families reported here is multiple inherited neoplasia allele syndrome (known as MINAS) (Whitworth *et al.*, 2016); however, WES did not demonstrate suspicious germline variants in other relevant tumour predisposition genes. The apparently high burden of tumours in the *SDHC* mutation carriers described here may also relate to their close surveillance, especially given that several of the tumours were classified as SDH-sufficient by SDHB IHC and metabolomic studies.

Considering the wide expression and critical role of the *SDHx* genes (Papathomas *et al.*, 2014), the *SDHC* mutation in these families may have still played a role in the tumours that appeared to be SDH-sufficient. The sensitivity of SDHB IHC in identifying patients with *SDHx* mutations ranges from 84% to 99% amongst expert observers, giving a false normal rate for SDHB IHC of up to 16% (Papathomas *et al.*, 2015). Others have described what appears to be falsely normal SDHB tumour IHC despite *SDHx* variants that were truncating (Evenepoel *et al.*, 2015) or frameshift (Papathomas *et al.*, 2015), located at the start codon with demonstrated LOH (Papathomas *et al.*, 2015), predicted to be pathogenic by *in silico* analysis (Evenepoel *et al.*, 2015), and/or shown to cause SDH deficiency in other tumours in the same or other individuals (Evenepoel *et al.*, 2015). Pulmonary chondromas associated with the

Carney triad have exhibited normal SDHB staining contrary to the now known mechanism of *SDHC* promoter hypermethylation (Gill, 2018). Explanations for falsely normal SDH IHC results include staining artefacts, incorrect interpretation, technical differences in fixation time and/or formalin concentrations, age of FFPE specimens, and mechanisms of inactivation that do not impact upon the staining antibody epitope (Evenepoel *et al.*, 2015; Papathomas *et al.*, 2015). Succinate:fumarate ratios in *SDHx*-mutated PPGLs can also be falsely normal, possibly due to SDH dysfunction in electron transport rather than the Krebs cycle (Kim *et al.*, 2016). Intratumour heterogeneity in operative specimens may impact on both IHC and metabolomic results (Papathomas *et al.*, 2014; Kim *et al.*, 2016). Our conclusions regarding the SDH status of tumours in these families is also limited by the lack of normative SDH IHC and metabolomic data in tumours other than PPGL, GIST, RCC and PA.

The high prevalence of SDH-related tumours in these families contrasts against previously reported *SDHC* penetrance rates of 20-25% based on case series of *SDHC*-mutated patients (Else *et al.*, 2014; Andrews *et al.*, 2018). With *SDHC* mutations evenly distributed amongst the six coding exons, genotype-phenotype correlations are unclear (Benn *et al.*, 2015). Studies of large kindreds, such as that reported here, have been promoted as a more accurate method of determining penetrance due to the ascertainment bias associated with only studying probands (Andrews *et al.*, 2018). It is tempting to speculate that the c.20+74A>G *SDHC* mutation is associated with a more aggressive phenotype as 8/12 confirmed mutation carriers in our families developed SDH-related and/or other neoplasms and three patients succumbed from malignancy. Although, the average age of first neoplasia diagnosis in these families was relatively late at 41.6 yr for classical SDH-related tumours and 47.4 yr for all tumours, compared to 29.3 yr (range 15-40 yr) in probands with *SDHC* mutations (Else *et al.*, 2014). Splicing variants have not been definitively associated with more aggressive disease, but a higher RCC risk has been suggested for the *SDHB* splice site

mutation, c.423+1G>A (Benn *et al.*, 2015), and *SDHx* splice site mutations are slightly overrepresented in malignant PPGL (17% vs. 11% of all PPGL) (Evenepoel *et al.*, 2015).

Finally, this study highlights the utility of: metabolomic studies in determining SDH deficiency; FFPE specimens for tumour DNA studies; RNA-Seq to evaluate deep intronic variants; and NGS data in CNV and haplotype analysis. The cryptic relatedness suspected in the families because of their shared ethnicity was confirmed by a contemporary form of haplotype analysis whereby identity by descent was determined by comparing rare variants deduced from NGS and ExAC and UK10K reference data. This methodology may be used to evaluate other suspected *SDHx* founder mutations. A partial *SDHC* gene deletion in apparently unrelated patients of Yemenite ethnicity has been suggested but unproven to be due to a founder mutation (Else *et al.*, 2014). Previous genealogy work using demographic data traced a large cohort of French-Canadian patients with *SDHC*-related PGLs due to a truncating founder mutation (Bourdeau *et al.*, 2016). Confirming cryptic relatedness is not only of biological interest, but also clinically significant as it guides cascade testing.

In summary, we report a novel *SDHC* pathogenic variant, c.20+74A>G, which represents the first deep intronic mutation in an *SDHx* gene. Whilst we showed the PGLs in these families to be SDH-deficient, conclusive results were not reached in the other tumours that either showed normal SDHB IHC or were unable to be studied because of a lack of tumour specimens. The association between *SDHx* mutations and prolactinomas has been particularly problematic throughout the literature because of the efficacy of DA treatment that diminishes the availability of operative tumour specimens. Further research is required to assess the causative role of the *SDHC* mutation in the wide tumour spectrum described here, noting that previous *SDHC* descriptions have been limited by the underrepresentation of *SDHC* amongst the familial *SDHx* tumour syndromes. For now, deep intronic *SDHx*

mutations should be considered in patients with classical SDH-related tumours, especially in the roughly 20% of SDH-deficient tumours with no identifiable mutation on routine genetic testing (Castelblanco *et al.*, 2013; Papathomas *et al.*, 2015). Large validation studies are required to determine the cost-benefit analysis of testing for deep intronic mutations – for example, through RNA-Seq – in patients with PPGL and other seemingly inherited disorders (Bagnall *et al.*, 2018).

9.5 Conclusion

This is the first report of a deep intronic *SDHx* mutation, highlighting a unique mechanism of SDH-related tumorigenesis and explaining at least some previously unsolved cases of SDH-deficient tumours. Our results also highlight the possibilities of digenic tumorigenesis in GIST and a role for *SDHx* mutations in HCC.

Chapter 10: Conclusions

This thesis was undertaken with the broad aims of optimising the assessment and management of patients with hyperprolactinaemia and to better understand the mechanisms underlying prolactin excess due to prolactinomas and related disorders. The most significant finding of the clinical studies was that the current first-line treatment of prolactinomas with DAs produces significant neuropsychological changes in a subset of patients. Given that prolactinomas are rarely fatal and may necessitate medical treatment for several years, these neuropsychological changes may be considered unacceptable by patients and clinicians alike. This highlights the need for an improved molecular understanding of prolactinomas in order to identify alternative medical treatments. The major molecular study of this thesis failed to identify druggable driver mutations in prolactinomas and instead showed various CNVs across chromosomes. Further research is required to determine whether this high burden of copy number variation signifies a common underlying genetic event in mitotic pathways. If so, this may represent a future target of medical treatment analogous to the use of PARP inhibitors that prevent cell rescue from double-strand DNA breaks in tumours with failure of homologous recombination such as in *BRCA1/2* mutation carriers.

Other key findings in this thesis include:

- Overestimation of serum prolactin by the Roche assay compared to the Siemens assay, with the potential for misdiagnosing either hyperprolactinaemia in normoprolactinaemic patients or prolactinomas in patients with mild and otherwise explained hyperprolactinaemia;
- Consistent petrosal sinus co-lateralisation of prolactin with ACTH in CD, invalidating the use of prolactin-corrected ACTH calculations in the interpretation of IPSS results;

- Marked hyperprolactinaemia in patients with ICA aneurysms of the cavernous sinus, which may otherwise be misdiagnosed as a prolactinoma;
- A rare germline missense variant in the *AHR* gene in the setting of cyclical CD, which may represent the genetic link between pituitary tumorigenesis and the clock system in cyclical Cushing's; and
- The first reported deep intronic mutations in the *SDHx* genes, highlighting a novel mechanism of SDH-related tumorigenesis.

The findings of this thesis are clinically significant. In general terms, the diagnostic pitfalls and the risk of DA-induced ICDs revealed by the clinical studies argue for hyperprolactinaemic patients to be managed in expert centres, as is routine for patients with other secretory pituitary tumours. The molecular studies show the value of NGS, RNA analysis and clinicopathological correlation in elucidating the mechanisms of endocrine tumorigenesis. The failure of our RT-PCR study in identifying the prolactin secretagogue responsible for vasculogenic hyperprolactinaemia highlights the limitations of targeted genetic studies compared to the pangenomic approaches used in the other molecular studies of this thesis.

Practice changes supported by the findings of this thesis include:

- Repeating serum prolactin on a different platform in patients where serum prolactin is incongruent with the clinical scenario;
- Avoidance of prolactin measurement during IPSS in the evaluation of ACTH-dependent CS;
- Consideration of carotid aneurysms in the differential diagnosis of patients with marked hyperprolactinaemia and sellar/parasellar masses;

- Educating and monitoring all DA-treated hyperprolactinaemic patients – especially men who become eugonadal – regarding the risk of ICDs and considering surgery if an ICD develops;
- Use of RNA studies and possibly WGS in patients with unexplained SDH-deficient tumours to look for *SDHx* deep intronic mutations that may be missed on routine genetic testing that only assesses coding DNA regions; and
- Development of clinical databases and tissue biobanks to support translational research with the aim of improving the future care of patients with hyperprolactinaemia.

To raise awareness amongst clinicians and to promote research into some of the issues evaluated in this thesis, we introduced new terms that characterise particular associations. The term ‘dopa-testotoxicosis’ was introduced to highlight the male predilection and predominance of hypersexuality in the DA-induced ICDs. Our findings that male gender and eugonadism at the time of neuropsychological assessment are predictive of ICD risk indicate a likely synergy between relative increases in testosterone into the normal range and off-target D3 dopamine receptor stimulation in the development of these ICDs. The term ‘vasculogenic hyperprolactinaemia’ was proposed to encompass the marked hyperprolactinaemia that may occur in association with carotid aneurysms. Although we were unable to identify a vascular-derived prolactin secretagogue, this term may help clinicians be mindful that prolactinomas and pregnancy are not the only causes of prolactin levels >10-fold normal and that further imaging should be considered to identify a carotid aneurysm.

Many of the findings reported here are not addressed in current guidelines. Both the 2006 Pituitary Society guidelines on prolactinomas (Casanueva *et al.*, 2006) and the 2011

Endocrine Society guidelines on hyperprolactinaemia (Melmed *et al.*, 2011) advise that macroprolactinomas are typically associated with serum prolactin levels >250-500 mcg/L. These thresholds of hyperprolactinaemia are given in absolute terms rather than in reference to the upper limit of normal which is advocated in this thesis based on our findings of significant interassay discordance. Although drugs and pregnancy are cited as differential diagnoses for marked hyperprolactinaemia, there is no mention of the possibility of a carotid aneurysm. This is despite carotid aneurysms being the only other cause of both marked hyperprolactinaemia and an isolated sellar/parasellar mass, with catastrophic consequences if transsphenoidal resection is attempted because of an apparent lack of tumour shrinkage with DA therapy. There is also no mention of the risk of DA-induced ICDs in either guideline, which is especially concerning given the multiple successful class actions that have been filed against pharmaceutical companies for failing to warn patients of ICD risks in the DA treatment of Parkinson's disease and restless legs syndrome. On the topic of prolactin measurement during IPSS, the 2015 Endocrine Society guidelines on the management of CS (Nieman *et al.*, 2015) outline indications for performing IPSS, but they do not discuss whether or not prolactin should be measured in addition to ACTH. By contrast, some local guidelines (Machado *et al.*, 2016) support the use of prolactin-corrected ACTH ratios, which is of concern given the prolactin intersinus gradient demonstrated by our study. Regarding our novel finding of *SDHC* deep intronic mutations, a 2017 international consensus statement on genetic testing in patients with PPGL syndromes (Toledo *et al.*, 2017) advised that there has been no evidence to date of deep intronic mutations playing a part in these tumours. Particularly in the case of the older guidelines on prolactin excess, our findings support updating of these guidelines to reflect the contemporary clinical pitfalls discussed herein.

Most significantly, this thesis challenges the overall treatment paradigm in the management of patients with prolactinomas. The high risk of DA-induced ICDs reported in Chapters 3 and 4 argues for a lower threshold for considering surgery, either upfront in patients with tumours that appear to be resectable or early in the course of treatment if ICD symptoms emerge. On the other hand, our tumour findings reported in Chapter 7 illustrate the high risk of postoperative recurrence in patients with prolactinomas. This is especially true for patients with macroadenomas or giant prolactinomas, which tend to be overrepresented in dedicated pituitary centres such as ours. The high demand for treatment alternatives combined with the suboptimal operative outcomes in patients with prolactinomas strongly support ongoing molecular studies to identify novel drug targets in patients with prolactinomas.

The chief limitation of this thesis is the small sample sizes of some of the studies, reflecting the time constraints of doctoral studies and the rarity of the disorders under investigation. This was somewhat mitigated in our study on vasculogenic hyperprolactinaemia and our initial study on dopa-testotoxicosis by collation of all published cases along with our cases to draw meaningful clinical conclusions. In multiple studies, we collaborated with other tertiary referral centres interstate and searched local clinical databases to identify eligible patients. This was particularly fruitful in our cross-sectional analysis of the risk of ICDs in hyperprolactinaemic patients vs. controls, which is the largest such study on this topic internationally. Our use of retrospective cases was, however, challenging in the molecular studies as tumour specimens in our institutions have not been routinely stored in conditions that allow DNA/RNA analysis and operative specimens are routinely destroyed after a certain period of time lapses according to local laboratory protocols. This limitation in tissue availability was partly overcome by employing data from published papers and publicly accessible genetic databases to better interpret our results.

Further investigation is required to evaluate the various molecular findings of this thesis, including: the high burden of copy number variation in prolactinomas; the putative role of the *AHR* gene in cyclical CD; and deep intronic mutations in the *SDHx* genes. This will require routine biobanking of operative specimens under suitable conditions (e.g., fresh frozen tissue) to allow future DNA/RNA studies, and larger collaborations extending beyond Australia, especially for the rarer disorders (i.e., vasculogenic hyperprolactinaemia and cyclical CD). Greater patient numbers may also allow for better subcategorisation of patients, which may then lead to the finding of recurrent genetic variants that may be missed in small heterogenous cohorts. It is plausible that different genetic events may be responsible for aggressive giant prolactinomas in male patients compared to microprolactinomas in female patients undergoing surgery because of DA intolerance rather than resistance. As highlighted by the novel *SDHC* mutation reported in Chapter 9, WGS studies may also be required to solve the unanswered question of driver mutations in prolactinomas if the causative variants are restricted to deep intronic regions that are not captured by WES.

The preliminary findings of this thesis will be investigated during the course of my postdoctoral studies. Thus far, we have expanded our interstate collaborations and found another three patients with cyclical CS to further investigate the role of *AHR* and other clock genes in the pathogenesis of this disorder. We have also identified another twelve patients with SDH-deficient tumours but negative routine genetic testing; WGS and RNA-Seq will be performed in these patients to look for deep intronic mutations across the *SDHx* genes. WGS will also allow us to evaluate other possible explanations for such cryptic SDH-deficient tumours based on emerging findings from other centres. For example, one group has recently demonstrated an *SDHB* variant with only 15% mutant load in blood DNA as causative of recurrent PGL (Cardot-Bauters *et al.*, 2019). Low-level mosaicism of this degree

will usually be missed on Sanger sequencing, whereas NGS can demonstrate low mutant loads provided there is sufficient depth of coverage. Another group has recently shown evidence that *DLST* is a new PPGL-predisposition gene belonging to the pseudohypoxia cluster of causative genes (Remacha *et al.*, 2019). WGS will allow interrogation of all such novel candidate genes as they transpire. Finally, we are exploring the possibility of screening for DA-induced ICDs in patients with NFPAs using the same neuropsychological tool utilised in this thesis. The proposed study would be a national collaboration investigating multiple aspects of the DA treatment of NFPAs, with our centre responsible for the neuropsychological assessment of patients. This study would be highly valuable in delineating the differential contributions of disease, treatment and patient characteristics in the development of ICDs, anxiety and depression.

Studying hyperprolactinaemic patients at the genomic level through WES and dissecting the risk factors contributing to DA side effects are examples of the increasing effort towards personalised care in pituitary medicine. Other growing areas of personalised pituitary research include: ¹¹C-methionine positron emission tomography to visualise functional PAs and thereby guide the targeted treatment of tumours that are not evident by MRI alone (Koulouri *et al.*, 2015; Koulouri *et al.*, 2016); somatostatin receptor tumour IHC to predict somatostatin analogue responses in acromegaly (Chiloiro *et al.*, 2019); and proton beam therapy in patients with craniopharyngiomas to deliver high-precision radiotherapy that maximises radiation dose at the tumour site whilst sparing adjacent structures (Toussaint *et al.*, 2020). The latter is of particular relevance locally as Adelaide prepares to be the first proton therapy unit in the southern hemisphere as of 2021. Frequently, these studies, as presented here, are performed in isolation based on the investigating centre's local expertise, thus limiting their external validity. A future task will comprise the integration of these disparate datasets into a comprehensive personalised schema that can be applied to

individual patients. The Pituitary Society recently outlined criteria for Pituitary Tumor Centers of Excellence (PTCOE), including neuroradiology, neuropathology, radiation oncology and neuro-ophthalmology expertise in addition to endocrinologists and neurosurgeons with dedicated pituitary training (Casanueva *et al.*, 2017). Such centres should help consolidate different disciplines of pituitary research, allowing direct comparison of the various pituitary tumour treatment modalities and ultimately improved care of individual patients.

Appendices

Appendix 1: Aggressive pituitary tumours and pituitary carcinomas

(De Sousa & McCormack, 2019)

LIBRARY NOTE:

is i (pages 196-228) s
i

It is also available online to authorised users at:
s i i

Appendix 2: Germline variants in familial pituitary tumour syndrome genes are common in young patients and families with additional endocrine tumours

(De Sousa *et al.*, 2017b)

LIBRARY NOTE:

is i (pages 230-239) s
i

It is also available online to authorised users at:
s i

Appendix 3: Genetic testing in endocrinology

(De Sousa *et al.*, 2018)

LIBRARY NOTE:

is i (pages 241-252) s
i

It is also available online to authorised users at:
s i i i s

Appendix 4: Case report of whole genome sequencing in the XY female: identification of a novel *SRY* mutation and revision of a misdiagnosis of androgen insensitivity syndrome

(De Sousa *et al.*, 2016)

LIBRARY NOTE:

is i (pages 254-260) s
i

It is also available online to authorised users at:

s i s

Appendix 5: Familial *GATA6* mutation causing variably expressed diabetes mellitus and cardiac and renal abnormalities

(Du *et al.*, 2019)

LIBRARY NOTE:

is i (pages 262-267) s
i

It is also available online to authorised users at:

s i

Appendix 6: *ARMC5* is not implicated in familial hyperaldosteronism type II (FH-II)

(De Sousa *et al.*, 2017e)

LIBRARY NOTE:

is i (pages 269-271) s
i

It is also available online to authorised users at:
<https://doi.org/10.1038/jhh.2017.71>

Bibliography

Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjord JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Illicic T, Imbeaud S, Imielinski M, Jager N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, Lopez-Otin C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN, Span PN, Teague JW, Totoki Y, Tutt AN, Valdes-Mas R, van Buuren MM, van 't Veer L, Vincent-Salomon A, Waddell N, Yates LR, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ & Stratton MR. Signatures of mutational processes in human cancer. *Nature*. 2013;500:415-421.

American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders* (4th edn, text revision). Washington, DC: American Psychiatric Association. 2000.

American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders* (5th edn). Arlington, VA: American Psychiatric Association. 2013.

Andela CD, Scharloo M, Pereira AM, Kaptein AA & Biermasz NR. Quality of life (QoL) impairments in patients with a pituitary adenoma: a systematic review of QoL studies. *Pituitary*. 2015;18:752-776.

Andrews KA, Ascher DB, Pires DEV, Barnes DR, Vialard L, Casey RT, Bradshaw N, Adlard J, Aylwin S, Brennan P, Brewer C, Cole T, Cook JA, Davidson R, Donaldson A, Fryer A, Greenhalgh L, Hodgson SV, Irving R, Laloo F, McConachie M, McConnell VPM, Morrison PJ, Murday V, Park SM, Simpson HL, Snape K, Stewart S, Tomkins SE, Wallis Y, Izatt L, Goudie D, Lindsay RS, Perry CG, Woodward ER, Antoniou AC & Maher ER. Tumour risks and genotype-phenotype correlations associated with germline variants in succinate dehydrogenase subunit genes SDHB, SDHC and SDHD. *J Med Genet*. 2018;55:384-394.

Antony MM, Bieling PJ, Cox BJ, Enns MW & Swinson RP. Psychometric properties of the 42-item and 21-item versions of the Depression Anxiety Stress Scales in clinical groups and a community sample. *Psychol Assess*. 1998;10:176-181.

Asa SL, Kelly MA, Grandy DK & Low MJ. Pituitary lactotroph adenomas develop after prolonged lactotroph hyperplasia in dopamine D2 receptor-deficient mice. *Endocrinology*. 1999;140:5348-5355.

Asa SL, Penz G, Kovacs K & Ezrin C. Prolactin cells in the human pituitary. A quantitative immunocytochemical analysis. *Arch Pathol Lab Med*. 1982;106:360-363.

Assié G, Libé R, Espiard S, Rizk-Rabin M, Guimier A, Luscap W, Barreau O, Lefèvre L, Sibony M, Guignat L, Rodriguez S, Perlemoine K, René-Corail F, Letourneur F, Trabulsi B, Poussier A, Chabbert-Buffet N, Borson-Chazot F, Groussin L, Bertagna X, Stratakis C, Ragazzon B & Bertherat J. ARMC5 mutations in macronodular adrenal hyperplasia with Cushing's syndrome. *N Engl J Med*. 2013;369:2105-2114.

- Athanasoulia AP, Ising M, Pfister H, Mantzoros CS, Stalla GK & Sievers C. Distinct dopaminergic personality patterns in patients with prolactinomas: a comparison with nonfunctioning pituitary adenoma patients and age- and gender-matched controls. *Neuroendocrinology*. 2012a;96:204-211.
- Athanasoulia AP, Sievers C, Ising M, Brockhaus AC, Yassouridis A, Stalla GK & Uhr M. Polymorphisms of the drug transporter gene ABCB1 predict side effects of treatment with cabergoline in patients with PRL adenomas. *Eur J Endocrinol*. 2012b;167:327-335.
- Athanasoulia-Kaspar AP, Popp KH & Stalla GK. Neuropsychiatric and metabolic aspects of dopaminergic therapy: perspectives from an endocrinologist and a psychiatrist. *Endocrine Connect*. 2018;7:R88-94.
- Ayala-Ramirez M, Callender GG, Kupferman ME, Rich TA, Chuang HH, Trent J, Perrier ND, Goodarzi M & Jimenez C. Paraganglioma syndrome type 1 in a patient with Carney-Stratakis syndrome. *Nat Rev Endocrinol*. 2010;6:110-115.
- Aylwin SJ, Bodi I & Beaney R. Pronounced response of papillary craniopharyngioma to treatment with vemurafenib, a BRAF inhibitor. *Pituitary*. 2016;19:544-546.
- Bagnall RD, Ingles J, Dinger ME, Cowley MJ, Ross SB, Minoche AE, Lal S, Turner C, Colley A, Rajagopalan S, Berman Y, Ronan A, Fatkin D & Semsarian C. Whole Genome Sequencing Improves Outcomes of Genetic Testing in Patients With Hypertrophic Cardiomyopathy. *J Am Coll Cardiol*. 2018;72:419-429.
- Bancos I, Nannenga MR, Bostwick JM, Silber MH, Erickson D & Nippoldt TB. Impulse control disorders in patients with dopamine agonist-treated prolactinomas and nonfunctioning pituitary adenomas: a case-control study. *Clin Endocrinol (Oxf)*. 2014;80:863-868.
- Bancos I, Nippoldt TB & Erickson D. Hypersexuality in men with prolactinomas treated with dopamine agonists. *Endocrine*. 2017;56:456-457.
- Barake M, Evins AE, Stoeckel L, Pachas GN, Nachtigall LB, Miller KK, Biller BM, Tritos NA & Klibanski A. Investigation of impulsivity in patients on dopamine agonist therapy for hyperprolactinemia: a pilot study. *Pituitary*. 2014;17:150-156.
- Barake M, Klibanski A & Tritos NA. MANAGEMENT OF ENDOCRINE DISEASE: Impulse control disorders in patients with hyperprolactinemia treated with dopamine agonists: how much should we worry? *Eur J Endocrinol*. 2018;179:R287-296.
- Barbieri A, Giuliani E, Genazzani A, Baraldi E, Ferrari A, D'Amico R & Coppi G. Analgesia and endocrine surgical stress: effect of two analgesia protocols on cortisol and prolactin levels during abdominal aortic aneurysm endovascular repair. *Neuro Endocrinol Lett*. 2011;32:526-529.
- Bartke A & Kopchick JJ. The forgotten lactogenic activity of growth hormone: important implications for rodent studies. *Endocrinology*. 2015;156:1620-1622.
- Bastiaens J, Dorfman BJ, Christos PJ & Nirenberg MJ. Prospective cohort study of impulse control disorders in Parkinson's disease. *Mov Disord*. 2013;28:327-333.

Beckers A, Aaltonen LA, Daly AF & Karhu A. Familial isolated pituitary adenomas (FIPA) and the pituitary adenoma predisposition due to mutations in the aryl hydrocarbon receptor interacting protein (AIP) gene. *Endocr Rev.* 2013;34:239-277.

Belinsky MG, Cai KQ, Zhou Y, Luo B, Pei J, Rink L & von Mehren M. Succinate dehydrogenase deficiency in a PDGFRA mutated GIST. *BMC Cancer.* 2017;17:512.

Ben-Jonathan N, Neill MA, Arbogast LA, Peters LL & Hoefler MT. Dopamine in hypophysial portal blood: relationship to circulating prolactin in pregnant and lactating rats. *Endocrinology.* 1980;106:690-696.

Bengtsson D, Joost P, Aravidis C, Askmalm Stenmark M, Backman AS, Melin B, von Salome J, Zagoras T, Gebre-Medhin S & Burman P. Corticotroph Pituitary Carcinoma in a Patient With Lynch Syndrome (LS) and Pituitary Tumors in a Nationwide LS Cohort. *J Clin Endocrinol Metab.* 2017;102:3928-3932.

Benn DE, Robinson BG & Clifton-Bligh RJ. 15 YEARS OF PARAGANGLIOMA: Clinical manifestations of paraganglioma syndromes types 1-5. *Endocr Relat Cancer.* 2015;22:T91-103.

Beumont P, Bruwer J, Pimstone B, Vinik A & Utian W. Brom-ergocryptine in the treatment of phenothiazine-induced galactorrhoea. *Br J Psychiatry.* 1975;126:285-288.

Bi WL, Greenwald NF, Ramkissoon SH, Abedalthagafi M, Coy SM, Ligon KL, Mei Y, MacConaill L, Ducar M, Min L, Santagata S, Kaiser UB, Beroukhim R, Laws ER Jr. & Dunn IF. Clinical Identification of Oncogenic Drivers and Copy-Number Alterations in Pituitary Tumors. *Endocrinology.* 2017a;158:2284-2291.

Bi WL, Horowitz P, Greenwald NF, Abedalthagafi M, Agarwalla PK, Gibson WJ, Mei Y, Schumacher SE, Ben-David U, Chevalier A, Carter S, Tiao G, Brastianos PK, Ligon AH, Ducar M, MacConaill L, Laws ER Jr., Santagata S, Beroukhim R & Dunn IF. Landscape of Genomic Alterations in Pituitary Adenomas. *Clin Cancer Res.* 2017b;23:1841-1851.

Biller BM, Luciano A, Crosignani PG, Molitch M, Olive D, Rebar R, Sanfilippo J, Webster J & Zacur H. Guidelines for the diagnosis and treatment of hyperprolactinemia. *J Reprod Med.* 1999;44:1075-1084.

Bird PH, Hendry KA, Shaw DC, Wilde CJ & Nicholas KR. Progressive changes in milk protein gene expression and prolactin binding during lactation in the tammar wallaby (*Macropus eugenii*). *J Mol Endocrinol.* 1994;13:117-125.

Blom N, Gammeltoft S & Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol.* 1999;294:1351-1362.

Bourdeau I, Grunenwald S, Burnichon N, Khalifa E, Dumas N, Binet MC, Nolet S & Gimenez-Roqueplo AP. A SDHC Founder Mutation Causes Paragangliomas (PGLs) in the French Canadians: New Insights on the SDHC-Related PGL. *J Clin Endocrinol Metab.* 2016;101:4710-4718.

Bouthillier A, van Loveren HR & Keller JT. Segments of the internal carotid artery: a new classification. *Neurosurgery.* 1996;38:425-432.

- Boyd A. Bromocriptine and psychosis: a literature review. *Psychiatr Q.* 1995;66:87-95.
- Brancati F, Camerota L, Colao E, Vega-Warner V, Zhao X, Zhang R & Bottillo I. Biallelic variants in the ciliary gene TMEM67 cause RHYNS syndrome. *Eur J Hum Genet.* 2018;26:1266-1271.
- Branford S, Wang P, Yeung DT, Thomson D, Purins A, Wadham C, Shahrin NH, Marum JE, Nataren N, Parker WT, Geoghegan J, Feng J, Shanmuganathan N, Mueller MC, Dietz C, Stangl D, Donaldson Z, Altamura H, Georgievski J, Braley J, Brown A, Hahn C, Walker I, Kim SH, Choi SY, Park SH, Kim DW, White DL, Yong ASM, Ross DM, Scott HS, Schreiber AW & Hughes TP. Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. *Blood.* 2018;132:948-961.
- Brastianos PK, Taylor-Weiner A, Manley PE, Jones RT, Dias-Santagata D, Thorner AR, Lawrence MS, Rodriguez FJ, Bernardo LA, Schubert L, Sunkavalli A, Shillingford N, Calicchio ML, Lidov HG, Taha H, Martinez-Lage M, Santi M, Storm PB, Lee JY, Palmer JN, Adappa ND, Scott RM, Dunn IF, Laws ER Jr., Stewart C, Ligon KL, Hoang MP, Van Hummelen P, Hahn WC, Louis DN, Resnick AC, Kieran MW, Getz G & Santagata S. Exome sequencing identifies BRAF mutations in papillary craniopharyngiomas. *Nat Genet.* 2014;46:161-165.
- Breves JP, Serizier SB, Goffin V, McCormick SD & Karlstrom RO. Prolactin regulates transcription of the ion uptake Na⁺/Cl⁻ cotransporter (ncc) gene in zebrafish gill. *Mol Cell Endocrinol.* 2013;369:98-106.
- Brito JP, Asi N, Bancos I, Gionfriddo MR, Zeballos-Palacios CL, Leppin AL, Undavalli C, Wang Z, Domecq JP, Prustsky G, Elraiyah TA, Prokop LJ, Montori VM & Murad MH. Testing for germline mutations in sporadic pheochromocytoma/paraganglioma: a systematic review. *Clin Endocrinol (Oxf).* 2015;82:338-345.
- Buffet A, Ben Aim L, Leboulleux S, Drui D, Vezzosi D, Libe R, Ajzenberg C, Bernardeschi D, Cariou B, Chabolle F, Chabre O, Darrouzet V, Delemer B, Desailoud R, Goichot B, Esvant A, Offredo L, Herman P, Laboureau S, Lefebvre H, Pierre P, Raingeard I, Reznik Y, Sadoul JL, Hadoux J, Tabarin A, Tauveron I, Zenaty D, Favier J, Bertherat J, Baudin E, Amar L & Gimenez-Roqueplo AP. Positive impact of genetic test on the management and outcome of patients with paraganglioma and/or pheochromocytoma. *J Clin Endocrinol Metab.* 2019;104:1109-1118.
- Bulwer C, Conn R, Shankar A, Ferrau F, Kapur S, Ederies A, Korbonits M & Spodeas HA. Cabergoline-related impulse control disorder in an adolescent with a giant prolactinoma. *Clin Endocrinol (Oxf).* 2017;86:862-864.
- Burnstock G. Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat.* 1999;194:335-342.
- Cai WY, Alexander JM, Hedley-Whyte ET, Scheithauer BW, Jameson JL, Zervas NT & Klibanski A. ras mutations in human prolactinomas and pituitary carcinomas. *J Clin Endocrinol Metab.* 1994;78:89-93.
- Calarge CA, Ellingrod VL, Acion L, Miller DD, Moline J, Tansey MJ & Schlechte JA. Variants of the dopamine D2 receptor gene and risperidone-induced hyperprolactinemia in children and adolescents. *Pharmacogenet Genomics.* 2009;19:373-382.

Caldas JG, Cavalheiro S, Nalli DR, Frudit ME & Puglia P Jr. Endovascular treatment of intracavernous carotid aneurysm with hyperprolactinaemia. *Interv Neuroradiol*. 1998;4:253-256.

Cannavo S, Ragonese M, Puglisi S, Romeo PD, Torre ML, Alibrandi A, Scaroni C, Occhi G, Ceccato F, Regazzo D, De Menis E, Sartorato P, Arnaldi G, Trementino L, Trimarchi F & Ferrau F. Acromegaly Is More Severe in Patients With AHR or AIP Gene Variants Living in Highly Polluted Areas. *J Clin Endocrinol Metab*. 2016;101:1872-1879.

Caputo C, Prior D & Inder WJ. The need for annual echocardiography to detect cabergoline-associated valvulopathy in patients with prolactinoma: a systematic review and additional clinical data. *Lancet Diabetes Endocrinol*. 2015;3:906-913.

Cardot-Bauters C, Carnaille B, Aubert S, Crepin M, Boury S, Burnichon N & Pigny P. A Full Phenotype of Paraganglioma Linked to a Germline SDHB Mosaic Mutation. *J Clin Endocrinol Metab*. 2019;104:3362-3366.

Carney JA. Gastric stromal sarcoma, pulmonary chondroma, and extra-adrenal paraganglioma (Carney Triad): natural history, adrenocortical component, and possible familial occurrence. *Mayo Clin Proc*. 1999;74:543-552.

Carney JA, Gordon H, Carpenter PC, Shenoy BV & Go VL. The complex of myxomas, spotty pigmentation, and endocrine overactivity. *Medicine (Baltimore)*. 1985;64:270-283.

Carney JA & Stratakis CA. Familial paraganglioma and gastric stromal sarcoma: a new syndrome distinct from the Carney triad. *Am J Med Genet*. 2002;108:132-139.

Casanueva FF, Barkan AL, Buchfelder M, Klibanski A, Laws ER, Loeffler JS, Melmed S, Mortini P, Wass J, Giustina A & Pituitary Society Expert Group on Pituitary Tumors. Criteria for the definition of Pituitary Tumor Centers of Excellence (PTCOE): A Pituitary Society Statement. *Pituitary*. 2017;20:489-498.

Casanueva FF, Molitch ME, Schlechte JA, Abs R, Bonert V, Bronstein MD, Brue T, Cappabianca P, Colao A, Fahlbusch R, Fideleff H, Hadani M, Kelly P, Kleinberg D, Laws E, Marek J, Scanlon M, Sobrinho LG, Wass JA & Giustina A. Guidelines of the Pituitary Society for the diagnosis and management of prolactinomas. *Clin Endocrinol (Oxf)*. 2006;65:265-273.

Castelblanco E, Santacana M, Valls J, de Cubas A, Cascon A, Robledo M & Matias-Guiu X. Usefulness of negative and weak-diffuse pattern of SDHB immunostaining in assessment of SDH mutations in paragangliomas and pheochromocytomas. *Endocr Pathol*. 2013;24:199-205.

Cavallaro R, Cocchi F, Angelone SM, Lattuada E & Smeraldi E. Cabergoline treatment of risperidone-induced hyperprolactinemia: a pilot study. *J Clin Psychiatry*. 2004;65:187-190.

Cebrián A, Ruiz-Llorente S, Cascon A, Pollan M, Diez J, Pico A, Telleria D, Benitez J & Robledo M. Mutational and gross deletion study of the MEN1 gene and correlation with clinical features in Spanish patients. *J Med Genet*. 2003;40:e72.

- Celik E, Ozkaya HM, Poyraz BC, Saglam T & Kadioglu P. Impulse control disorders in patients with prolactinoma receiving dopamine agonist therapy: a prospective study with 1 year follow-up. *Endocrine*. 2018;62:692-700.
- Chen J, Jian X, Deng S, Ma Z, Shou X, Shen Y, Zhang Q, Song Z, Li Z, Peng H, Peng C, Chen M, Luo C, Zhao D, Ye Z, Shen M, Zhang Y, Zhou J, Fahira A, Wang Y, Li S, Zhang Z, Ye H, Li Y, Shen J, Chen H, Tang F, Yao Z, Shi Z, Chen C, Xie L, Wang Y, Fu C, Mao Y, Zhou L, Gao D, Yan H, Zhao Y, Huang C & Shi Y. Identification of recurrent USP48 and BRAF mutations in Cushing's disease. *Nat Commun*. 2018;9:3171.
- Chiloiro S, Bima C, Tartaglione T, Giampietro A, Gessi M, Lauretti L, Anile C, Colosimo C, Rindi G, Pontecorvi A, De Marinis L & Bianchi A. Pasireotide and Pegvisomant Combination Treatment in Acromegaly Resistant to Second-Line Therapies: A Longitudinal Study. *J Clin Endocrinol Metab*. 2019;104:5478-5482.
- Clapp C, Lopez-Gomez FJ, Nava G, Corbacho A, Torner L, Macotela Y, Duenas Z, Ochoa A, Noris G, Acosta E, Garay E & Martinez de la Escalera G. Expression of prolactin mRNA and of prolactin-like proteins in endothelial cells: evidence for autocrine effects. *J Endocrinol*. 1998;158:137-144.
- Cohen M, Persky R, Stegemann R, Hernandez-Ramirez LC, Zeltser D, Lodish MB, Chen A, Keil MF, Tatsi C, Faucz F, Buchner D, Stratakis CA & Tiosano D. Germline USP8 mutation associated with pediatric Cushing disease and other clinical features: a new syndrome. *J Clin Endocrinol Metab*. 2019;104:4676-4682.
- Colao A, Di Sarno A, Landi ML, Scavuzzo F, Cappabianca P, Pivonello R, Volpe R, Di Salle F, Cirillo S, Annunziato L & Lombardi G. Macroprolactinoma shrinkage during cabergoline treatment is greater in naive patients than in patients pretreated with other dopamine agonists: a prospective study in 110 patients. *J Clin Endocrinol Metab*. 2000;85:2247-2252.
- Colao A, Vitale G, Cappabianca P, Briganti F, Ciccarelli A, De Rosa M, Zarrilli S & Lombardi G. Outcome of cabergoline treatment in men with prolactinoma: effects of a 24-month treatment on prolactin levels, tumor mass, recovery of pituitary function, and semen analysis. *J Clin Endocrinol Metab*. 2004;89:1704-1711.
- Collins MT, Singer FR & Eugster E. McCune-Albright syndrome and the extraskeletal manifestations of fibrous dysplasia. *Orphanet J Rare Dis*. 2012;7:S4.
- Cooke NE, Coit D, Shine J, Baxter JD & Martial JA. Human prolactin. cDNA structural analysis and evolutionary comparisons. *J Biol Chem*. 1981;256:4007-4016.
- Corenblum B & Donovan L. The safety of physiological estrogen plus progestin replacement therapy and with oral contraceptive therapy in women with pathological hyperprolactinemia. *Fertil Steril*. 1993;59:671-673.
- Cotton E & Ray D. DICER1 mutation and pituitary prolactinoma. *Endocrinol Diabetes Metab Case Rep*. 2018;2018:18-0087.
- Crock PA, Pestell RG, Calenti AJ, Gilford EJ, Henderson JK, Best JD & Alford FP. Multiple pituitary hormone gradients from inferior petrosal sinus sampling in Cushing's disease. *Acta Endocrinol (Copenh)*. 1988;119:75-80.

Curras-Freixes M, Pineiro-Yanez E, Montero-Conde C, Apellaniz-Ruiz M, Calsina B, Mancikova V, Remacha L, Richter S, Ercolino T, Rogowski-Lehmann N, Deutschbein T, Calatayud M, Guadalix S, Alvarez-Escola C, Lamas C, Aller J, Sastre-Marcos J, Lazaro C, Galofre JC, Patino-Garcia A, Meoro-Aviles A, Balmana-Gelpi J, De Miguel-Novoa P, Balbin M, Matias-Guiu X, Leton R, Inglada-Perez L, Torres-Perez R, Roldan-Romero JM, Rodriguez-Antona C, Fliedner SMJ, Opocher G, Pacak K, Korpershoek E, de Krijger RR, Vroonen L, Mannelli M, Fassnacht M, Beuschlein F, Eisenhofer G, Cascon A, Al-Shahrour F & Robledo M. PheoSeq: A Targeted Next-Generation Sequencing Assay for Pheochromocytoma and Paraganglioma Diagnostics. *J Mol Diagn.* 2017;19:575-588.

Dahia PL. Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. *Nat Rev Cancer.* 2014;14:108-119.

Daly AF, Rixhon M, Adam C, Dempegioti A, Tichomirowa MA & Beckers A. High prevalence of pituitary adenomas: a cross-sectional study in the province of Liege, Belgium. *J Clin Endocrinol Metab.* 2006;91:4769-4775.

Dannenbergh H, de Krijger RR, Zhao J, Speel EJ, Saremaslani P, Dinjens WN, Mooi WJ, Roth J, Heitz PU & Komminoth P. Differential loss of chromosome 11q in familial and sporadic parasympathetic paragangliomas detected by comparative genomic hybridization. *Am J Pathol.* 2001;158:1937-1942.

Daousi C, Nixon T, Javadpour M, Hayden K & MacFarlane IA. Inferior petrosal sinus ACTH and prolactin responses to CRH in ACTH-dependent Cushing's syndrome: a single centre experience from the United Kingdom. *Pituitary.* 2010;13:95-104.

De Bellis A, Colao A, Pivonello R, Savoia A, Battaglia M, Ruocco G, Tirelli G, Lombardi G, Bellastella A & Bizzarro A. Antipituitary antibodies in idiopathic hyperprolactinemic patients. *Ann N Y Acad Sci.* 2007;1107:129-135.

De Coopman J. Breastfeeding after pituitary resection: support for a theory of autocrine control of milk supply? *J Hum Lact.* 1993;9:35-40.

de Jong OG, Verhaar MC, Chen Y, Vader P, Gremmels H, Posthuma G, Schiffelers RM, Gucek M & van Balkom BW. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. *J Extracell Vesicles.* 2012;1.

de Kock L, Sabbaghian N, Plourde F, Srivastava A, Weber E, Bouron-Dal Soglio D, Hamel N, Choi JH, Park SH, Deal CL, Kelsey MM, Dishop MK, Esbenshade A, Kuttesch JF, Jacques TS, Perry A, Leichter H, Maeder P, Brundler MA, Warner J, Neal J, Zacharin M, Korbonits M, Cole T, Traunecker H, McLean TW, Rotondo F, Lepage P, Albrecht S, Horvath E, Kovacs K, Priest JR & Foulkes WD. Pituitary blastoma: a pathognomonic feature of germ-line DICER1 mutations. *Acta Neuropathol.* 2014;128:111-122.

De Rosa M, Zarrilli S, Vitale G, Di Somma C, Orio F, Tauchmanova' L, Lombardi G & Colao A. Six months of treatment with cabergoline restores sexual potency in hyperprolactinemic males: an open longitudinal study monitoring nocturnal penile tumescence. *J Clin Endocrinol Metab.* 2004;89:621-625.

De Sousa SM, Chapman IM, Falhammar H & Torpy DJ. Dopa-testotoxicosis: disruptive hypersexuality in hypogonadal men with prolactinomas treated with dopamine agonists. *Endocrine*. 2017a;55:618-624.

De Sousa SM, Hardy TS, Scott HS & Torpy DJ. Genetic Testing in Endocrinology. *Clin Biochem Rev*. 2018;39:17-28.

De Sousa SM, Kassahn KS, McIntyre LC, Chong CE, Scott HS & Torpy DJ. Case report of whole genome sequencing in the XY female: identification of a novel SRY mutation and revision of a misdiagnosis of androgen insensitivity syndrome. *BMC Endocr Disord*. 2016;16:58.

De Sousa SM, McCabe MJ, Wu K, Roscioli T, Gayevskiy V, Brook K, Rawlings L, Scott HS, Thompson TJ, Earls P, Gill AJ, Cowley MJ, Dinger ME & McCormack AI. Germline variants in familial pituitary tumour syndrome genes are common in young patients and families with additional endocrine tumours. *Eur J Endocrinol*. 2017b;176:635-644.

De Sousa SMC, Baranoff J, Rushworth RL, Butler J, Sorbello J, Vorster J, Thompson T, McCormack AI, Inder WJ & Torpy DJ. Impulse control disorders in dopamine agonist-treated hyperprolactinemia: prevalence and risk factors. *J Clin Endocrinol Metab*. 2020a;105:e108-e118.

De Sousa SMC, Manavis J, Feng J, Wang P, Schreiber AW, Scott HS & Torpy DJ. A putative role for the aryl hydrocarbon receptor (AHR) gene in a patient with cyclical Cushing's disease. *BMC Endocr Disord*. 2020b;20:18.

De Sousa SMC & McCormack AI. Aggressive Pituitary Tumors and Pituitary Carcinomas. In KR Feingold, B Anawalt, A Boyce, G Chrousos, K Dungan, A Grossman, JM Hershman, G Kaltsas, C Koch, P Kopp, M Korbonits, R McLachlan, JE Morley, M New, L Perreault, J Purnell, R Rebar, F Singer, DL Trencce, A Vinik & DP Wilson (Eds.), *Endotext*. South Dartmouth, MA: MDText.com Inc. 2019.

De Sousa SMC, McCormack AI, McGrath S & Torpy DJ. Prolactin correction for adequacy of petrosal sinus cannulation may diminish diagnostic accuracy in Cushing's disease. *Clin Endocrinol (Oxf)*. 2017c;87:515-522.

De Sousa SMC, Meyer EJ, Rankin W, Brautigan PJ, Burt MG & Torpy DJ. Vasculogenic hyperprolactinemia: severe prolactin excess in association with internal carotid artery aneurysms. *Pituitary*. 2017d;20:676-682.

De Sousa SMC, Saleem M, Rankin W & Torpy DJ. Serum prolactin overestimation and risk of misdiagnosis. *Endocrinol Diabetes Metab*. 2019a;2:e00065.

De Sousa SMC, Stowasser M, Feng J, Schreiber AW, Wang P, Hahn CN, Gordon RD, Torpy DJ, Scott HS & Gagliardi L. ARMC5 is not implicated in familial hyperaldosteronism type II (FH-II). *J Hum Hypertens*. 2017e;31:857-859.

De Sousa SMC, Wang PPS, Santoreneos S, Shen A, Yates CJ, Babic M, Eshraghi L, Feng J, Koszyca B, Roberts-Thomson S, Schreiber AW, Torpy DJ & Scott HS. The Genomic Landscape of Sporadic Prolactinomas. *Endocr Pathol*. 2019b;30:318-328.

Delcour C, Robin G, Young J & Dewailly D. PCOS and Hyperprolactinemia: what do we know in 2019? *Clin Med Insights Reprod Health*. 2019;13:1-7.

Dénes J, Swords F, Rattenberry E, Stals K, Owens M, Cranston T, Xekouki P, Moran L, Kumar A, Wassif C, Fersht N, Baldeweg S, Morris D, Lightman S, Agha A, Rees A, Grieve J, Powell M, Boguszewski C, Dutta P, Thakker R, Srirangalingam U, Thompson C, Druce M, Higham C, Davis J, Eeles R, Stevenson M, O'Sullivan B, Tanriere P, Skordilis K, Gabrovska P, Barlier A, Webb S, Aulinas A, Drake W, Bevan J, Preda C, Dalantaeva N, Ribeiro-Oliveira AJ, Garcia I, Yordanova G, Iotova V, Evanson J, Grossman A, Trouillas J, Ellard S, Stratakis C, Maher E, Roncaroli F & Korbonits M. Heterogeneous genetic background of the association of pheochromocytoma/paraganglioma and pituitary adenoma—results from a large patient cohort. *J Clin Endocrinol Metab*. 2015;100:E531-541.

Dhuffar MK & Griffiths MD. Understanding the role of shame and its consequences in female hypersexual behaviours: A pilot study. *J Behav Addict*. 2014;3:231-237.

Dogansen SC, Cikrikcili U, Oruk G, Kutbay NO, Tanrikulu S, Hekimsoy Z, Hadzalic A, Gorar S, Omma T, Mert M, Akbaba G, Yalin GY, Bayram F, Ozkan M & Yarman S. Dopamine Agonist-Induced Impulse Control Disorders in Patients With Prolactinoma: A Cross-Sectional Multicenter Study. *J Clin Endocrinol Metab*. 2019;104:2527-2534.

Doppman JL, Chang R, Oldfield EH, Chrousos G, Stratakis CA & Nieman LK. The hypoplastic inferior petrosal sinus: a potential source of false-negative results in petrosal sampling for Cushing's disease. *J Clin Endocrinol Metab*. 1999;84:533-540.

Du YT, Moore L, Poplawski NK & De Sousa SMC. Familial GATA6 mutation causing variably expressed diabetes mellitus and cardiac and renal abnormalities. *Endocrinol Diabetes Metab Case Rep*. 2019;19-0022.

Duarte FH, Machado MC, Lima JR & Salgado LR. Severe hyperprolactinemia associated with internal carotid artery aneurysm: differential diagnosis between prolactinoma and hypothalamic-pituitary disconnection. *Arq Bras Endocrinol Metabol*. 2008;52:1189-1193.

Dulak AM, Stojanov P, Peng S, Lawrence MS, Fox C, Stewart C, Bandla S, Imamura Y, Schumacher SE, Shefler E, McKenna A, Carter SL, Cibulskis K, Sivachenko A, Saksena G, Voet D, Ramos AH, Auclair D, Thompson K, Sougnez C, Onofrio RC, Guiducci C, Beroukheim R, Zhou Z, Lin L, Lin J, Reddy R, Chang A, Landrenau R, Pennathur A, Ogino S, Luketich JD, Golub TR, Gabriel SB, Lander ES, Beer DG, Godfrey TE, Getz G & Bass AJ. Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. *Nat Genet*. 2013;45:478-486.

Dwight T, Mann K, Benn DE, Robinson BG, McKelvie P, Gill AJ, Winship I & Clifton-Bligh RJ. Familial SDHA mutation associated with pituitary adenoma and pheochromocytoma/paraganglioma. *J Clin Endocrinol Metab*. 2013;98:E1103-1108.

Ehara Y, Siler T, VandenBerg G, Sinha YN & Yen SS. Circulating prolactin levels during the menstrual cycle: episodic release and diurnal variation. *Am J Obstet Gynecol*. 1973;117:962-970.

- Elenkova A, Shabani R, Kalinov K & Zacharieva S. Increased prevalence of subclinical cardiac valve fibrosis in patients with prolactinomas on long-term bromocriptine and cabergoline treatment. *Eur J Endocrinol*. 2012;167:17-25.
- Else T, Marvin ML, Everett JN, Gruber SB, Arts HA, Stoffel EM, Auchus RJ & Raymond VM. The clinical phenotype of SDHC-associated hereditary paraganglioma syndrome (PGL3). *J Clin Endocrinol Metab*. 2014;99:E1482-1486.
- Elston MS, McDonald KL, Clifton-Bligh RJ & Robinson BG. Familial pituitary tumor syndromes. *Nat Rev Endocrinol*. 2009;5:453-461.
- English AC, Richards S, Han Y, Wang M, Vee V, Qu J, Qin X, Muzny DM, Reid JG, Worley KC & Gibbs RA. Mind the gap: upgrading genomes with Pacific Biosciences RS long-read sequencing technology. *PLoS One*. 2012;7:e47768.
- Evans CO, Moreno CS, Zhan X, McCabe MT, Vertino PM, Desiderio DM & Oyesiku NM. Molecular pathogenesis of human prolactinomas identified by gene expression profiling, RT-qPCR, and proteomic analyses. *Pituitary*. 2008;11:231-245.
- Evenepoel L, Papathomas TG, Krol N, Korpershoek E, de Krijger RR, Persu A & Dinjens WN. Toward an improved definition of the genetic and tumor spectrum associated with SDH germ-line mutations. *Genet Med*. 2015;17:610-620.
- Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML & McCutcheon IE. The prevalence of pituitary adenomas: a systematic review. *Cancer*. 2004;101:613-619.
- Fahy UM, Foster PA, Torode HW, Hartog M & Hull MG. The effect of combined estrogen/progestogen treatment in women with hyperprolactinemic amenorrhea. *Gynecol Endocrinol*. 1992;6:183-188.
- Falhammar H & Yarker JY. Pathological gambling and hypersexuality in cabergoline-treated prolactinoma. *Med J Aust*. 2009;190:97.
- Fernandez A, Karavitaki N & Wass JA. Prevalence of pituitary adenomas: a community-based, cross-sectional study in Banbury (Oxfordshire, UK). *Clin Endocrinol (Oxf)*. 2010;72:377-382.
- Fernandez-Real JM, Fernandez-Castaner M, Villabona C, Sagarra E, Gomez-Saez JM & Soler J. Giant intrasellar aneurysm presenting with panhypopituitarism and subarachnoid hemorrhage: case report and literature review. *Clin Investig*. 1994;72:302-306.
- Findling JW, Kehoe ME & Raff H. Identification of patients with Cushing's disease with negative pituitary adrenocorticotropin gradients during inferior petrosal sinus sampling: prolactin as an index of pituitary venous effluent. *J Clin Endocrinol Metab*. 2004;89:6005-6009.
- Flanagan S, Lee M, Li CC, Suter CM & Buckland ME. Promoter Methylation Analysis of IDH Genes in Human Gliomas. *Front Oncol*. 2012;2:193.
- Formosa R, Borg J & Vassallo J. Aryl hydrocarbon receptor (AHR) is a potential tumour suppressor in pituitary adenomas. *Endocr Relat Cancer*. 2017;24:445-457.

Frederiksen A, Rossing M, Hermann P, Ejersted C, Thakker RV & Frost M. Clinical Features of Multiple Endocrine Neoplasia Type 4: Novel Pathogenic Variant and Review of Published Cases. *J Clin Endocrinol Metab.* 2019;104:3637-3646.

Freeman ME, Kanyicska B, Lerant A & Nagy G. Prolactin: structure, function, and regulation of secretion. *Physiol Rev.* 2000;80:1523-1631.

Friesen H, Guyda H & Hardy J. The biosynthesis of human growth hormone and prolactin. *J Clin Endocrinol Metab.* 1970;31:611-624.

Friesen HG. The discovery of human prolactin: a very personal account. *Clin Invest Med.* 1995;18:66-72.

Fujii R, Fukusumi S, Hosoya M, Kawamata Y, Habata Y, Hinuma S, Sekiguchi M, Kitada C, Kurokawa T, Nishimura O, Onda H, Sumino Y & Fujino M. Tissue distribution of prolactin-releasing peptide (PrRP) and its receptor. *Regul Pept.* 1999;83:1-10.

Fukunaga BN, Probst MR, Reisz-Porszasz S & Hankinson O. Identification of functional domains of the aryl hydrocarbon receptor. *J Biol Chem.* 1995;270:29270-29278.

Gahr M, Connemann BJ & Schonfeldt-Lecuona CJ. [Pathological gambling induced by dopamine agonists]. *Fortschr Neurol Psychiatr.* 2011;79:470-474.

Gao H, Wang F, Lan X, Li C, Feng J, Bai J, Cao L, Gui S, Hong L & Zhang Y. Lower PRDM2 expression is associated with dopamine-agonist resistance and tumor recurrence in prolactinomas. *BMC Cancer.* 2015;15:272.

Garg SK & Dash RJ. Galactorrhoea amenorrhoea syndrome due to internal carotid artery aneurysm. *Postgrad Med J.* 1985;61:611-613.

Gasparotto D, Rossi S, Campagna D, Scavina P, Tiziano FD, Marzotto A, Toffolatti L, Vitelli CE, Amini M, Dei Tos AP & Maestro R. Imatinib-Sensitizing KIT Mutation in a Carney-Stratakis-Associated GI Stromal Tumor. *J Clin Oncol.* 2016;34:e99-103.

Gellersen B, Bonhoff A, Hunt N & Bohnet HG. Decidual-type prolactin expression by the human myometrium. *Endocrinology.* 1991;129:158-168.

Gibney J, Smith TP & McKenna TJ. The impact on clinical practice of routine screening for macroprolactin. *J Clin Endocrinol Metab.* 2005;90:3927-3932.

Gill AJ. Succinate dehydrogenase (SDH) and mitochondrial driven neoplasia. *Pathology.* 2012;44:285-292.

Gill AJ. Succinate dehydrogenase (SDH)-deficient neoplasia. *Histopathology.* 2018;72:106-116.

Gillam MP, Molitch ME, Lombardi G & Colao A. Advances in the treatment of prolactinomas. *Endocr Rev.* 2006;27:485-534.

Goldman MH, Gruber AT & Herman MA. Concurrent Panhypopituitarism and Hyperprolactinemia Due to a Giant Internal Carotid Aneurysm Revealed by Thyroid Hormone

- Withdrawal During Follow-Up Management of Thyroid Cancer. *AACE Clin Case Rep.* 2016;2:e167-171.
- Gonzales GF, Velasquez G & Garcia-Hjarles M. Hypoprolactinemia as related to seminal quality and serum testosterone. *Arch Androl.* 1989;23:259-265.
- Gorvin CM, Newey PJ, Rogers A, Stokes V, Neville MJ, Lines KE, Ntali G, Lees P, Morrison PJ, Singhellakis PN, Malandrinou FC, Karavitaki N, Grossman AB, Karpe F & Thakker RV. Association of prolactin receptor (PRLR) variants with prolactinomas. *Hum Mol Genet.* 2019;28:1023–1037.
- Grall-Bronnec M, Victorri-Vigneau C, Donnio Y, Leboucher J, Rousselet M, Thiabaud E, Zreika N, Derkinderen P & Challet-Bouju G. Dopamine Agonists and Impulse Control Disorders: A Complex Association. *Drug Saf.* 2018;41:19-75.
- Grant P, Dworakowska D & Carroll P. Maximizing the accuracy of Inferior petrosal sinus sampling: validation of the use of Prolactin as a marker of pituitary venous effluent in the diagnosis of Cushing's disease. *Clin Endocrinol (Oxf).* 2012;76:555-559.
- Grattan DR. 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-prolactin axis. *J Endocrinol.* 2015;226:T101-122.
- Grattan DR & Averill RL. Absence of short-loop autoregulation of prolactin during late pregnancy in the rat. *Brain Res Bull.* 1995;36:413-416.
- Grossmann M. Mutant prolactin receptor and familial hyperprolactinemia. *N Engl J Med.* 2014;370:976-977.
- Gungor A, Gokkaya N, Bilen A, Bilen H, Akbas EM, Karadeniz Y & Eren S. Pituitary Insufficiency and Hyperprolactinemia Associated with Giant Intra- and Suprasellar Carotid Artery Aneurysm. *Case Rep Med.* 2015;2015:536191.
- Gupta A & Zimmerman RS. Hypersexuality in cabergoline-treated prolactinoma. Paper presented at the 93rd Annual Meeting of the Endocrine Society, Boston, MA. 2011.
- Guyda HJ & Friesen HG. Serum prolactin levels in humans from birth to adult life. *Pediatr Res.* 1973;7:534-540.
- Habano W, Sugai T, Nakamura S, Uesugi N, Higuchi T, Terashima M & Horiuchi S. Reduced expression and loss of heterozygosity of the SDHD gene in colorectal and gastric cancer. *Oncol Rep.* 2003;10:1375-1380.
- Hall WA, Luciano MG, Doppman JL, Patronas NJ & Oldfield EH. Pituitary magnetic resonance imaging in normal human volunteers: occult adenomas in the general population. *Ann Intern Med.* 1994;120:817-820.
- Haller F, Moskalev EA, Faucz FR, Barthelmess S, Wiemann S, Bieg M, Assie G, Bertherat J, Schaefer IM, Otto C, Rattenberry E, Maher ER, Strobel P, Werner M, Carney JA, Hartmann A, Stratakis CA & Agaimy A. Aberrant DNA hypermethylation of SDHC: a novel mechanism of tumor development in Carney triad. *Endocr Relat Cancer.* 2014;21:567-577.

Hansford S, Kaurah P, Li-Chang H, Woo M, Senz J, Pinheiro H, Schrader KA, Schaeffer DF, Shumansky K, Zogopoulos G, Santos TA, Claro I, Carvalho J, Nielsen C, Padilla S, Lum A, Talhouk A, Baker-Lange K, Richardson S, Lewis I, Lindor NM, Pennell E, MacMillan A, Fernandez B, Keller G, Lynch H, Shah SP, Guilford P, Gallinger S, Corso G, Roviello F, Caldas C, Oliveira C, Pharoah PD & Huntsman DG. Hereditary Diffuse Gastric Cancer Syndrome: CDH1 Mutations and Beyond. *JAMA Oncol.* 2015;1:23-32.

Harris C. Mutant prolactin receptor and familial hyperprolactinemia. *N Engl J Med.* 2014;370:976.

Hays RD, Hayashi T & Stewart AL. A five-item measure of socially desirable response set. *Educ Psychol Measur.* 1989;49:629-636.

Henry JD & Crawford JR. The short-form version of the Depression Anxiety Stress Scales (DASS-21): construct validity and normative data in a large non-clinical sample. *Br J Clin Psychol.* 2005;44:227-239.

Hernandez-Ramirez LC, Gam R, Valdes N, Lodish MB, Pankratz N, Balsalobre A, Gauthier Y, Faucz FR, Trivellin G, Chittiboina P, Lane J, Kay DM, Dimopoulos A, Gaillard S, Neou M, Bertherat J, Assie G, Villa C, Mills JL, Drouin J & Stratakis CA. Loss-of-function mutations in the CABLES1 gene are a novel cause of Cushing's disease. *Endocr Relat Cancer.* 2017;24:379-392.

Heshmati HM, Fatourechi V, Dagam SA & Piepgras DG. Hypopituitarism caused by intrasellar aneurysms. *Mayo Clin Proc.* 2001;76:789-793.

Horvath E, Kovacs K & Scheithauer BW. Pituitary hyperplasia. *Pituitary.* 1999;1:169-179.

Howie PW, McNeilly AS, McArdle T, Smart L & Houston M. The relationship between suckling-induced prolactin response and lactogenesis. *J Clin Endocrinol Metab.* 1980;50:670-673.

Hu Y, Ding Y, Yang M & Xiang Z. Serum prolactin levels across pregnancy and the establishment of reference intervals. *Clin Chem Lab Med.* 2018;56:803-807.

Huang P, Ceccatelli S, Hakansson H, Grandison L & Rannug A. Constitutive and TCDD-induced expression of Ah receptor-responsive genes in the pituitary. *Neurotoxicology.* 2002;23:783-793.

Iacovazzo D, Caswell R, Bunce B, Jose S, Yuan B, Hernandez-Ramirez LC, Kapur S, Caimari F, Evanson J, Ferrau F, Dang MN, Gabrovskaja P, Larkin SJ, Ansorge O, Rodd C, Vance ML, Ramirez-Renteria C, Mercado M, Goldstone AP, Buchfelder M, Burren CP, Gurlek A, Dutta P, Choong CS, Cheetham T, Trivellin G, Stratakis CA, Lopes MB, Grossman AB, Trouillas J, Lupski JR, Ellard S, Sampson JR, Roncaroli F & Korbonits M. Germline or somatic GPR101 duplication leads to X-linked acrogigantism: a clinico-pathological and genetic study. *Acta Neuropathol Commun.* 2016;4:56.

Ing R, Petrakis NL & Ho JH. Unilateral breast-feeding and breast cancer. *Lancet.* 1977;2:124-127.

- Invitti C, Pecori Giraldi F, Tagliaferri A, Scacchi M, Dubini A & Cavagnini F. Enhanced prolactin responsiveness to galanin in patients with Cushing's disease. *Clin Endocrinol (Oxf)*. 1993;39:213-216.
- Ioachimescu AG, Fleseriu M, Hoffman AR, Vaughan TB III & Katznelson L. Psychological effects of dopamine agonist treatment in patients with hyperprolactinemia and prolactin-secreting adenomas. *Eur J Endocrinol*. 2019;180:31-40.
- Jaeger C, Khazaal AQ, Xu C, Sun M, Krager SL & Tischkau SA. Aryl Hydrocarbon Receptor Deficiency Alters Circadian and Metabolic Rhythmicity. *J Biol Rhythms*. 2017;32:109-120.
- Jaffrain-Rea ML, Angelini M, Gargano D, Tichomirowa MA, Daly AF, Vanbellinhen JF, D'Innocenzo E, Barlier A, Giangaspero F, Esposito V, Ventura L, Arcella A, Theodoropoulou M, Naves L, Fajardo C, Zacharieva S, Rohmer V, Brue T, Gulino A, Cantore G, Alesse E & Beckers A. Expression of aryl hydrocarbon receptor (AHR) and AHR-interacting protein in pituitary adenomas: pathological and clinical implications. *Endocr Relat Canc*. 2009;16:1029-1043.
- Jaffrain-Rea ML, Rotondi S, Turchi A, Occhi G, Barlier A, Peverelli E, Rostomyan L, Defilles C, Angelini M, Oliva MA, Ceccato F, Maiorani O, Daly AF, Esposito V, Buttarelli F, Figarella-Branger D, Giangaspero F, Spada A, Scaroni C, Alesse E & Beckers A. Somatostatin analogues increase AIP expression in somatotropinomas, irrespective of Gsp mutations. *Endocr Relat Cancer*. 2013;20:753-766.
- Joensuu H, Rutkowski P, Nishida T, Steigen SE, Brabec P, Plank L, Nilsson B, Braconi C, Bordoni A, Magnusson MK, Sufliarsky J, Federico M, Jonasson JG, Hostein I, Bringuier PP & Emile JF. KIT and PDGFRA mutations and the risk of GI stromal tumor recurrence. *J Clin Oncol*. 2015;33:634-642.
- Jove M, Mora J, Sanjuan X, Rodriguez E, Robledo M, Farran L & Garcia del Muro X. Simultaneous KIT mutation and succinate dehydrogenase (SDH) deficiency in a patient with a gastrointestinal stromal tumour and Carney-Stratakis syndrome: a case report. *Histopathology*. 2014;65:712-717.
- Kafka MP. Hypersexual disorder: a proposed diagnosis for DSM-V. *Arch Sex Behav*. 2010;39:377-400.
- Kahn SR, Leblanc R, Sadikot AF & Fantus IG. Marked hyperprolactinemia caused by carotid aneurysm. *Can J Neurol Sci*. 1997;24:64-66.
- Kalenga MK, Thomas K, de Gasparo M & De Hertogh R. Determination of renin, angiotensin converting enzyme and angiotensin II levels in human placenta, chorion and amnion from women with pregnancy induced hypertension. *Clin Endocrinol (Oxf)*. 1996;44:429-433.
- Kalogeras KT, Nieman LK, Friedman TC, Doppman JL, Cutler GB Jr., Chrousos GP, Wilder RL, Gold PW & Yanovski JA. Inferior petrosal sinus sampling in healthy subjects reveals a unilateral corticotropin-releasing hormone-induced arginine vasopressin release associated with ipsilateral adrenocorticotropin secretion. *J Clin Invest*. 1996;97:2045-2050.
- Karavitaki N, Thanabalasingham G, Shore HC, Trifanescu R, Ansorge O, Meston N, Turner HE & Wass JA. Do the limits of serum prolactin in disconnection hyperprolactinaemia need re-

definition? A study of 226 patients with histologically verified non-functioning pituitary macroadenoma. *Clin Endocrinol (Oxf)*. 2006;65:524-529.

Kars M, Roelfsema F, Romijn JA & Pereira AM. Malignant prolactinoma: case report and review of the literature. *Eur J Endocrinol*. 2006;155:523-534.

Karunakaran S, Page RC & Wass JA. The effect of the menopause on prolactin levels in patients with hyperprolactinaemia. *Clin Endocrinol (Oxf)*. 2001;54:295-300.

Kauppila A, Martikainen H, Puistola U, Reinila M & Ronnberg L. Hypoprolactinemia and ovarian function. *Fertil Steril*. 1988;49:437-441.

Kelly MA, Rubinstein M, Asa SL, Zhang G, Saez C, Bunzow JR, Allen RG, Hnasko R, Ben-Jonathan N, Grandy DK & Low MJ. Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron*. 1997;19:103-113.

Kim E, Wright MJ, Sioson L, Novos T, Gill AJ, Benn DE, White C, Dwight T & Clifton-Bligh RJ. Utility of the succinate: Fumarate ratio for assessing SDH dysfunction in different tumor types. *Mol Genet Metab Rep*. 2016;10:45-49.

Klein V, Rettenberger M & Briken P. Self-reported indicators of hypersexuality and its correlates in a female online sample. *J Sex Med*. 2014;11:1974-1981.

Kobza AO, Dizon S & Arnaout A. Case report of bilateral pheochromocytomas due to a novel MAX mutation in a patient known to have a pituitary prolactinoma. *AACE Clinical Case Rep*. 2018;4:e453-456.

Korali Z, Wittchen HU, Pfister H, Hofler M, Oefelein W & Stalla GK. Are patients with pituitary adenomas at an increased risk of mental disorders? *Acta Psychiatr Scand*. 2003;107:60-68.

Korytnaya E, Liu J, Camelo-Piragua S, Sullivan S, Auchus RJ & Barkan A. Ectopic prolactin secretion from a perivascular epithelioid cell tumor (PEComa). *J Clin Endocrinol Metab*. 2014;99:3960-3964.

Koulouri O, Kandasamy N, Hoole AC, Gillett D, Heard S, Powlson AS, O'Donovan DG, Annamalai AK, Simpson HL, Akker SA, Aylwin SJ, Brooke A, Buch H, Levy MJ, Martin N, Morris D, Parkinson C, Tysome JR, Santarius T, Donnelly N, Buscombe J, Boros I, Smith R, Aigbirhio F, Antoun NM, Burnet NG, Cheow H, Mannion RJ, Pickard JD & Gurnell M. Successful treatment of residual pituitary adenoma in persistent acromegaly following localisation by 11C-methionine PET co-registered with MRI. *Eur J Endocrinol*. 2016;175:485-498.

Koulouri O, Steuwe A, Gillett D, Hoole AC, Powlson AS, Donnelly NA, Burnet NG, Antoun NM, Cheow H, Mannion RJ, Pickard JD & Gurnell M. A role for 11C-methionine PET imaging in ACTH-dependent Cushing's syndrome. *Eur J Endocrinol*. 2015;173:M107-120.

Krishnamoorthy S, Rajan R, Banerjee M, Kumar H, Sarma G, Krishnan S, Sarma S & Kishore A. Dopamine D3 receptor Ser9Gly variant is associated with impulse control disorders in Parkinson's disease patients. *Parkinsonism Relat Disord*. 2016;30:13-17.

Lecoq AL, Kamenický P, Guiochon-Mantel A & Chanson P. Genetic mutations in sporadic pituitary adenomas - what to screen for? *Nat Rev Endocrinol*. 2015;11:43-54.

- Lee MS, Song HC, An H, Yang J, Ko YH, Jung IK & Joe SH. Effect of bromocriptine on antipsychotic drug-induced hyperprolactinemia: eight-week randomized, single-blind, placebo-controlled, multicenter study. *Psychiatry Clin Neurosci*. 2010;64:19-27.
- Lenders JW, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SK, Murad MH, Naruse M, Pacak K & Young WF Jr. Pheochromocytoma and Paraganglioma: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2014;99:1915-1942.
- Loli P, Boccardi E, Branca V, Bramerio M, Barberis M, Losa M, Terreni MT, Lodrini S, Pollo B & Vignati F. Growth hormone and prolactin responses to corticotrophin-releasing-hormone in patients with Cushing's disease: a paracrine action of the adenomatous corticotrophic cells? *Clin Endocrinol (Oxf)*. 1998;49:433-439.
- Lovibond SH & Lovibond PF. *Manual for the Depression Anxiety Stress Scales* (2nd edn). Sydney, Australia: Psychology Foundation. 1995.
- Lund AK, Goens MB, Kanagy NL & Walker MK. Cardiac hypertrophy in aryl hydrocarbon receptor null mice is correlated with elevated angiotensin II, endothelin-1, and mean arterial blood pressure. *Toxicol Appl Pharmacol*. 2003;193:177-187.
- Ma ZY, Song ZJ, Chen JH, Wang YF, Li SQ, Zhou LF, Mao Y, Li YM, Hu RG, Zhang ZY, Ye HY, Shen M, Shou XF, Li ZQ, Peng H, Wang QZ, Zhou DZ, Qin XL, Ji J, Zheng J, Chen H, Wang Y, Geng DY, Tang WJ, Fu CW, Shi ZF, Zhang YC, Ye Z, He WQ, Zhang QL, Tang QS, Xie R, Shen JW, Wen ZJ, Zhou J, Wang T, Huang S, Qiu HJ, Qiao ND, Zhang Y, Pan L, Bao WM, Liu YC, Huang CX, Shi YY & Zhao Y. Recurrent gain-of-function USP8 mutations in Cushing's disease. *Cell Res*. 2015;25:306-317.
- Machado MC, Fragoso MC, Moreira AC, Boguszewski CL, Vieira LN, Naves LA, Vilar L, Araujo LA, Czepielewski MA, Gadelha MR, Musolino NR, Miranda PA, Bronstein MD & Ribeiro-Oliveira A Jr. Recommendations of the Neuroendocrinology Department of the Brazilian Society of Endocrinology and Metabolism for the diagnosis of Cushing's disease in Brazil. *Arch Endocrinol Metab* 2016;60:267-286.
- Martinkova J, Trejbalova L, Sasikova M, Benetin J & Valkovic P. Impulse control disorders associated with dopaminergic medication in patients with pituitary adenomas. *Clin Neuropharmacol*. 2011;34:179-181.
- Marzioni D, Fiore G, Giordano A, Nabissi M, Florio P, Verdenelli F, Petraglia F & Castellucci M. Placental expression of substance P and vasoactive intestinal peptide: evidence for a local effect on hormone release. *J Clin Endocrinol Metab*. 2005;90:2378-2383.
- Maslar IA, Powers-Craddock P & Ansbacher R. Decidual prolactin production by organ cultures of human endometrium: effects of continuous and intermittent progesterone treatment. *Biol Reprod*. 1986;34:741-750.
- McCormack A, Dekkers OM, Petersenn S, Popovic V, Trouillas J, Raverot G, Burman P & ESE survey collaborators. Treatment of aggressive pituitary tumours and carcinomas: results of a European Society of Endocrinology (ESE) survey 2016. *Eur J Endocrinol*. 2018;178:265-276.

McLennan K, Jeske Y, Cotterill A, Cowley D, Penfold J, Jones T, Howard N, Thomsett M & Choong C. Combined pituitary hormone deficiency in Australian children: clinical and genetic correlates. *Clin Endocrinol (Oxf)*. 2003;58:785-794.

McNally PG, Bolia A, Absalom SR, Falconer-Smith J & Howlett TA. Preliminary observations using endocrine markers of pituitary venous dilution during bilateral simultaneous inferior petrosal sinus catheterization in Cushing's syndrome: is combined CRF and TRH stimulation of value? *Clin Endocrinol (Oxf)*. 1993;39:681-686.

Meinardi JR, Wolffenbuttel BH & Dullaart RP. Cyclic Cushing's syndrome: a clinical challenge. *Eur J Endocrinol*. 2007;157:245-254.

Melmed S, Casanueva FF, Hoffman AR, Kleinberg DL, Montori VM, Schlechte JA & Wass JA. Diagnosis and treatment of hyperprolactinemia: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2011;96:273-288.

Melmed S, Kleinberg D & Ho K. Pituitary Physiology and Diagnostic Evaluation. In S Melmed, KS Polonsky, PR Larsen & HM Kronenberg (Eds.), *Williams Textbook of Endocrinology* (13th edn). Philadelphia, PA: Elsevier. 2016.

Melo FM, Couto PP, Bale AE, Bastos-Rodrigues L, Passos FM, Lisboa RG, Ng JM, Curran T, Dias EP, Friedman E & De Marco L. Whole-exome identifies RXRG and TH germline variants in familial isolated prolactinoma. *Cancer Genet*. 2016;209:251-257.

Merchenthaler I. Induction of enkephalin in tuberoinfundibular dopaminergic neurons of pregnant, pseudopregnant, lactating and aged female rats. *Neuroendocrinology*. 1994;60:185-193.

Miao Y, Li C, Guo J, Wang H, Gong L, Xie W & Zhang Y. Identification of a novel somatic mutation of POU6F2 by whole-genome sequencing in prolactinoma. *Mol Genet Genomic Med*. 2019;e1022.

Missale C, Nash SR, Robinson SW, Jaber M & Caron MG. Dopamine receptors: from structure to function. *Physiol Rev*. 1998;78:189-225.

Mohammad HP, Abbud RA, Parlow AF, Lewin JS & Nilson JH. Targeted overexpression of luteinizing hormone causes ovary-dependent functional adenomas restricted to cells of the Pit-1 lineage. *Endocrinology*. 2003;144:4626-4636.

Molitch ME. Medication-induced hyperprolactinemia. *Mayo Clin Proc*. 2005a;80:1050-1057.

Molitch ME. Pharmacologic resistance in prolactinoma patients. *Pituitary*. 2005b;8:43-52.

Molitch ME. Mutant prolactin receptor and familial hyperprolactinemia. *N Engl J Med*. 2014;370:977.

Molitch ME. Endocrinology in pregnancy: management of the pregnant patient with a prolactinoma. *Eur J Endocrinol*. 2015;172:R205-213.

Moore TJ, Glenmullen J & Mattison DR. Reports of pathological gambling, hypersexuality, and compulsive shopping associated with dopamine receptor agonist drugs. *JAMA Intern Med*. 2014;174:1930-1933.

- Moreira AC, Antonini SR & de Castro M. MECHANISMS IN ENDOCRINOLOGY: A sense of time of the glucocorticoid circadian clock: from the ontogeny to the diagnosis of Cushing's syndrome. *Eur J Endocrinol*. 2018;179:R1-18.
- Mulligan GB, Eray E, Faiman C, Gupta M, Pineyro MM, Makdissi A, Suh JH, Masaryk TJ, Prayson R, Weil RJ & Hamrahian AH. Reduction of false-negative results in inferior petrosal sinus sampling with simultaneous prolactin and corticotropin measurement. *Endocr Pract*. 2011;17:33-40.
- Mulligan GB, Faiman C, Gupta M, Kennedy L, Hatipoglu B, Hui F, Weil RJ & Hamrahian AH. Prolactin measurement during inferior petrosal sinus sampling improves the localization of pituitary adenomas in Cushing's disease. *Clin Endocrinol (Oxf)*. 2012;77:268-274.
- Nader N, Chrousos GP & Kino T. Interactions of the circadian CLOCK system and the HPA axis. *Trends Endocrinol Metab*. 2010;21:277-286.
- Nannenga MR, Tebben PJ & Nippoldt TB. Development of impulse control disorders in patients with dopamine agonist treated prolactinomas: a case series. Paper presented at the 93rd Annual Meeting of the Endocrine Society, Boston, MA. 2011.
- Napier TC & Persons AL. Pharmacological insights into impulsive-compulsive spectrum disorders associated with dopaminergic therapy. *Eur J Neurosci*. 2019;50:2492-2502.
- Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Althoefer C, Zerres K, Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peczkowska M, Szmigielski C & Eng C. Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med*. 2002;346:1459-1466.
- Newell-Price J, Bertagna X, Grossman AB & Nieman LK. Cushing's syndrome. *Lancet*. 2006;367:1605-1617.
- Newey PJ, Gorvin CM, Cleland SJ, Willberg CB, Bridge M, Azharuddin M, Drummond RS, van der Merwe PA, Klenerman P, Bountra C & Thakker RV. Mutant prolactin receptor and familial hyperprolactinemia. *N Engl J Med*. 2013a;369:2012-2020.
- Newey PJ, Nesbit MA, Rimmer AJ, Head RA, Gorvin CM, Attar M, Gregory L, Wass JA, Buck D, Karavitaki N, Grossman AB, McVean G, Ansorge O & Thakker RV. Whole-exome sequencing studies of nonfunctioning pituitary adenomas. *J Clin Endocrinol Metab*. 2013b;98:E796-800.
- Niall HD, Hogan ML, Sauer R, Rosenblum IY & Greenwood FC. Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene reduplication. *Proc Natl Acad Sci U S A*. 1971;68:866-870.
- Nieman LK, Biller BM, Findling JW, Murad MH, Newell-Price J, Savage MO & Tabarin A. Treatment of Cushing's Syndrome: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2015;100:2807-2831.
- Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, Stewart PM & Montori VM. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2008;93:1526-1540.

Niemeijer ND, Papathomas TG, Korpershoek E, de Krijger RR, Oudijk L, Morreau H, Bayley JP, Hes FJ, Jansen JC, Dinjens WN & Corssmit EP. Succinate Dehydrogenase (SDH)-Deficient Pancreatic Neuroendocrine Tumor Expands the SDH-Related Tumor Spectrum. *J Clin Endocrinol Metab.* 2015;100:E1386-1393.

Oldfield EH, Doppman JL, Nieman LK, Chrousos GP, Miller DL, Katz DA, Cutler GB Jr. & Loriaux DL. Petrosal sinus sampling with and without corticotropin-releasing hormone for the differential diagnosis of Cushing's syndrome. *N Engl J Med.* 1991;325:897-905.

Ooi TC & Russell NA. Hypopituitarism resulting from an intrasellar carotid aneurysm. *Can J Neurol Sci.* 1986;13:70-71.

Oster H, Maronde E & Albrecht U. The circadian clock as a molecular calendar. *Chronobiol Int.* 2002;19:507-516.

Owerbach D, Rutter WJ, Cooke NE, Martial JA & Shows TB. The prolactin gene is located on chromosome 6 in humans. *Science.* 1981;212:815-816.

Pack SD, Kirschner LS, Pak E, Zhuang Z, Carney JA & Stratakis CA. Genetic and histologic studies of somatomammotropic pituitary tumors in patients with the "complex of spotty skin pigmentation, myxomas, endocrine overactivity and schwannomas" (Carney complex). *J Clin Endocrinol Metab.* 2000;85:3860-3865.

Pap E, Falus A, Mihalyi D, Borck H, Diel F & Pallinger E. Histamine regulates placental cytokine expression - in vivo study on HDC knockout mice. *Placenta.* 2007;28:239-244.

Papathomas TG, Gaal J, Corssmit EP, Oudijk L, Korpershoek E, Heimdal K, Bayley JP, Morreau H, van Dooren M, Papaspyrou K, Schreiner T, Hansen T, Andresen PA, Restuccia DF, van Kessel I, van Leenders GJ, Kros JM, Looijenga LH, Hofland LJ, Mann W, van Nederveen FH, Mete O, Asa SL, de Krijger RR & Dinjens WN. Non-pheochromocytoma (PCC)/paraganglioma (PGL) tumors in patients with succinate dehydrogenase-related PCC-PGL syndromes: a clinicopathological and molecular analysis. *Eur J Endocrinol.* 2014;170:1-12.

Papathomas TG, Oudijk L, Persu A, Gill AJ, van Nederveen F, Tischler AS, Tissier F, Volante M, Matias-Guiu X, Smid M, Favier J, Rapizzi E, Libe R, Curras-Freixes M, Aydin S, Huynh T, Lichtenauer U, van Berkel A, Canu L, Domingues R, Clifton-Bligh RJ, Bialas M, Vikkula M, Baretton G, Papotti M, Nesi G, Badoual C, Pacak K, Eisenhofer G, Timmers HJ & Beuschlein F. SDHB/SDHA immunohistochemistry in pheochromocytomas and paragangliomas: a multicenter interobserver variation analysis using virtual microscopy: a Multinational Study of the European Network for the Study of Adrenal Tumors (ENS@T). *Mod Pathol.* 2015;28:807-821.

Pecori Giraldi F, Ambrogio AG, Andrioli M, Sanguin F, Karamouzis I, Corsello SM, Scaroni C, Arvat E, Pontecorvi A & Cavagnini F. Potential role for retinoic acid in patients with Cushing's disease. *J Clin Endocrinol Metab.* 2012;97:3577-3583.

Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Hofler H, Fend F, Graw J & Atkinson MJ. Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. *Proc Natl Acad Sci U S A.* 2006;103:15558-15563.

- Pepe S, Korbonits M & Iacovazzo D. Germline and mosaic mutations causing pituitary tumours: genetic and molecular aspects. *J Endocrinol*. 2019;240:R21-45.
- Perovich RM, Lieberman JA, Fleischhacker WW & Alvir J. The behavioral toxicity of bromocriptine in patients with psychiatric illness. *J Clin Psychopharmacol*. 1989;9:417-422.
- Petakov MS, Damjanovic SS, Nikolic-Durovic MM, Dragojlovic ZL, Obradovic S, Gligorovic MS, Simic MZ & Popovic VP. Pituitary adenomas secreting large amounts of prolactin may give false low values in immunoradiometric assays. The hook effect. *J Endocrinol Invest*. 1998;21:184-188.
- Pinzone JJ, Katznelson L, Danila DC, Pauler DK, Miller CS & Klibanski A. Primary medical therapy of micro-and macroprolactinomas in men. *J Clin Endocrinol Metab*. 2000;85:3053-3057.
- Poncin J, Stevenaert A & Beckers A. Somatic MEN1 gene mutation does not contribute significantly to sporadic pituitary tumorigenesis. *Eur J Endocrinol*. 1999;140:573-576.
- Powell AC, Stratakis CA, Patronas NJ, Steinberg SM, Batista D, Alexander HR, Pingpank JF, Keil M, Bartlett DL & Libutti SK. Operative management of Cushing syndrome secondary to micronodular adrenal hyperplasia. *Surgery*. 2008;143:750-758.
- Premaratne VS, Saeger I & Macdonald BK. Lesson of the month (1): Cabergoline - 'I eat funny on that'. *Clin Med (Lond)*. 2014;14:205-207.
- Primeau V, Raftopoulos C & Maiter D. Outcomes of transsphenoidal surgery in prolactinomas: improvement of hormonal control in dopamine agonist-resistant patients. *Eur J Endocrinol*. 2012;166:779-786.
- Qiao X, Ye H, Zhang X, Zhao W, Zhang S, Lu B, Wang X, Zhang Z, Wu X, He M, Zhao X, Li S, Zhou L, Yang Y, Hu R & Li Y. The value of prolactin in inferior petrosal sinus sampling with desmopressin stimulation in Cushing's disease. *Endocrine*. 2015;48:644-652.
- Raitila A, Georgitsi M, Karhu A, Tuppurainen K, Makinen MJ, Birkenkamp-Demtroder K, Salmenkivi K, Orntoft TF, Arola J, Launonen V, Vahteristo P & Aaltonen LA. No evidence of somatic aryl hydrocarbon receptor interacting protein mutations in sporadic endocrine neoplasia. *Endocr Relat Cancer*. 2007;14:901-906.
- Ratliff JK & Oldfield EH. Multiple pituitary adenomas in Cushing's disease. *J Neurosurg*. 2000;93:753-761.
- Reid RC, Garos S & Carpenter BN. Reliability, validity, and psychometric development of the Hypersexual Behavior Inventory in an outpatient sample of men. *Sex Addict Compulsivity*. 2011;18:30-51.
- Reid RC, Garos S & Fong T. Psychometric development of the hypersexual behavior consequences scale. *J Behav Addict*. 2012;1:115-122.
- Reid RC & Kafka MP. Controversies About Hypersexual Disorder and the DSM-5. *Curr Sex Health Rep*. 2014;6:259-264.

Reincke M, Sbiera S, Hayakawa A, Theodoropoulou M, Osswald A & Beuschlein F. Mutations in the deubiquitinase gene USP8 cause Cushing's disease. *Nat Genet.* 2015;47:31-38.

Reis FM, Vigano P, Arnaboldi E, Spritzer PM, Petraglia F & Di Blasio AM. Expression of prolactin-releasing peptide and its receptor in the human decidua. *Mol Hum Reprod.* 2002;8:356-362.

Remacha L, Pirman D, Mahoney CE, Coloma J, Calsina B, Curras-Freixes M, Leton R, Torres-Perez R, Richter S, Pita G, Herraes B, Cianchetta G, Honrado E, Maestre L, Urioste M, Aller J, Garcia-Uriarte O, Galvez MA, Luque RM, Lahera M, Moreno-Rengel C, Eisenhofer G, Montero-Conde C, Rodriguez-Antona C, Llorca O, Smolen GA, Robledo M & Cascon A. Recurrent Germline DLST Mutations in Individuals with Multiple Pheochromocytomas and Paragangliomas. *Am J Hum Genet.* 2019;104:651-664.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm H & ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015;17:405-424.

Richter S, Klink B, Nacke B, de Cubas AA, Mangelis A, Rapizzi E, Meinhardt M, Skondra C, Mannelli M, Robledo M, Menschikowski M & Eisenhofer G. Epigenetic Mutation of the Succinate Dehydrogenase C Promoter in a Patient With Two Paragangliomas. *J Clin Endocrinol Metab.* 2016;101:359-363.

Richter S, Peitzsch M, Rapizzi E, Lenders JW, Qin N, de Cubas AA, Schiavi F, Rao JU, Beuschlein F, Quinkler M, Timmers HJ, Opocher G, Mannelli M, Pacak K, Robledo M & Eisenhofer G. Krebs cycle metabolite profiling for identification and stratification of pheochromocytomas/paragangliomas due to succinate dehydrogenase deficiency. *J Clin Endocrinol Metab.* 2014;99:3903-3911.

Riddick DH, Luciano AA, Kusmik WF & Maslar IA. Evidence for a nonpituitary source of amniotic fluid prolactin. *Fertil Steril.* 1979;31:35-39.

Rocco A, Mori F, Baldelli R, Aversa A, Munizzi MR, Nardone MR, Fabbrini A & Falaschi P. Effect of chronic bromocriptine treatment on psychological profile of patients with PRL-secreting pituitary adenomas. *Psychoneuroendocrinology.* 1993;18:57-66.

Roland BL, Sutton SW, Wilson SJ, Luo L, Pyati J, Huvar R, Erlander MG & Lovenberg TW. Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. *Endocrinology.* 1999;140:5736-5745.

Rostomyan L, Daly AF, Petrossians P, Nachev E, Lila AR, Lecoq AL, Lecumberri B, Trivellin G, Salvatori R, Moraitis AG, Holdaway I, Kranenburg-van Klaveren DJ, Chiara Zatelli M, Palacios N, Nozieres C, Zacharin M, Ebeling T, Ojaniemi M, Rozhinskaya L, Verrua E, Jaffrain-Rea ML, Filippini S, Gusakova D, Pronin V, Bertherat J, Belaya Z, Ilovayskaya I, Sahnoun-Fathallah M, Sievers C, Stalla GK, Castermans E, Caberg JH, Sorkina E, Auriemma RS, Mittal S, Kareva M, Lysy PA, Emy P, De Menis E, Choong CS, Mantovani G, Bours V, De Herder W, Brue T, Barlier A, Neggers SJ, Zacharieva S, Chanson P, Shah NS, Stratakis CA, Naves LA & Beckers A. Clinical and genetic characterization of pituitary gigantism: an international collaborative study in 208 patients. *Endocr Relat Cancer.* 2015;22:745-757.

- Roszkó KL, Blouch E, Blake M, Powers JF, Tischler AS, Hodin R, Sadow P & Lawson EA. Case Report of a Prolactinoma in a Patient With a Novel MAX Mutation and Bilateral Pheochromocytomas. *J Endocr Soc.* 2017;1:1401-1407.
- Sandyk R, Bergsneider M & Iacono RP. Acute psychosis in a woman with a prolactinoma. *Int J Neurosci.* 1987;37:187-190.
- Sanger F & Thompson EOP. The amino-acid sequence in the glyceryl chain of insulin. 1. The identification of lower peptides from partial hydrolysates. *Biochem J.* 1953a;53:353-366.
- Sanger F & Thompson EOP. The amino-acid sequence in the glyceryl chain of insulin. 2. The investigation of peptides from enzymic hydrolysates. *Biochem J.* 1953b;53:366-374.
- Sanger F & Tuppy H. The amino-acid sequence in the phenylalanyl chain of insulin. 1. The identification of lower peptides from partial hydrolysates. *Biochem J.* 1951a;49:463-481.
- Sanger F & Tuppy H. The amino-acid sequence in the phenylalanyl chain of insulin. 2. The investigation of peptides from enzymic hydrolysates. *Biochem J.* 1951b;49:481-490.
- Sapkota S, Horiguchi K, Tosaka M, Yamada S & Yamada M. Whole-Exome Sequencing Study of Thyrotropin-Secreting Pituitary Adenomas. *J Clin Endocrinol Metab.* 2017;102:566-575.
- Sbardella E, Cranston T, Isidori AM, Shine B, Pal A, Jafar-Mohammadi B, Sadler G, Mihai R & Grossman AB. Routine genetic screening with a multi-gene panel in patients with pheochromocytomas. *Endocrine.* 2018;59:175-182.
- Scholl UI, Stolting G, Schewe J, Thiel A, Tan H, Nelson-Williams C, Vichot AA, Jin SC, Loring E, Untiet V, Yoo T, Choi J, Xu S, Wu A, Kirchner M, Mertins P, Rump LC, Onder AM, Gamble C, McKenney D, Lash RW, Jones DP, Chune G, Gagliardi P, Choi M, Gordon R, Stowasser M, Fahlke C & Lifton RP. CLCN2 chloride channel mutations in familial hyperaldosteronism type II. *Nat Genet.* 2018;50:349-354.
- Schulte HM, Allolio B, Gunther RW, Benker G, Winkelmann W, Ohnhaus EE & Reinwein D. Selective bilateral and simultaneous catheterization of the inferior petrosal sinus: CRF stimulates prolactin secretion from ACTH-producing microadenomas in Cushing's disease. *Clin Endocrinol (Oxf).* 1988;28:289-295.
- Sforza DM, Putman CM & Czeisler JR. Hemodynamics of Cerebral Aneurysms. *Annu Rev Fluid Mech.* 2009;41:91-107.
- Sharma ST & Nieman LK. Is prolactin measurement of value during inferior petrosal sinus sampling in patients with adrenocorticotropic hormone-dependent Cushing's Syndrome? *J Endocrinol Invest.* 2013;36:1112-1116.
- Sharma ST, Raff H & Nieman LK. Prolactin as a marker of successful catheterization during IPSS in patients with ACTH-dependent Cushing's syndrome. *J Clin Endocrinol Metab.* 2011;96:3687-3694.
- Sibal L, Ugwu P, Kendall-Taylor P, Ball SG, James RA, Pearce SH, Hall K & Quinton R. Medical therapy of macroprolactinomas in males: I. Prevalence of hypopituitarism at diagnosis. II. Proportion of cases exhibiting recovery of pituitary function. *Pituitary.* 2002;5:243-246.

- Sinha YN. Structural variants of prolactin: occurrence and physiological significance. *Endocr Rev.* 1995;16:354-369.
- Smith CR & Norman MR. Prolactin and growth hormone: molecular heterogeneity and measurement in serum. *Ann Clin Biochem.* 1990;27:542-550.
- Snellen M, Power J, Blankley G & Galbally M. Pharmacological lactation suppression with D2 receptor agonists and risk of postpartum psychosis: A systematic review. *Aust N Z J Obstet Gynaecol.* 2016;56:336-340.
- Song ZJ, Reitman ZJ, Ma ZY, Chen JH, Zhang QL, Shou XF, Huang CX, Wang YF, Li SQ, Mao Y, Zhou LF, Lian BF, Yan H, Shi YY & Zhao Y. The genome-wide mutational landscape of pituitary adenomas. *Cell Res.* 2016;26:1255-1259.
- Spratt DI, O'Dea LS, Schoenfeld D, Butler J, Rao PN & Crowley WF Jr. Neuroendocrine-gonadal axis in men: frequent sampling of LH, FSH, and testosterone. *Am J Physiol.* 1988;254:E658-666.
- Steiner AL, Goodman AD & Powers SR. Study of a kindred with pheochromocytoma, medullary thyroid carcinoma, hyperparathyroidism and Cushing's disease: multiple endocrine neoplasia, type 2. *Medicine (Baltimore).* 1968;47:371-409.
- Suh HK & Frantz AG. Size heterogeneity of human prolactin in plasma and pituitary extracts. *J Clin Endocrinol Metab.* 1974;39:928-935.
- Sulkowski PL, Sundaram RK, Oeck S, Corso CD, Liu Y, Noorbakhsh S, Niger M, Boeke M, Ueno D, Kalathil AN, Bao X, Li J, Shuch B, Bindra RS & Glazer PM. Krebs-cycle-deficient hereditary cancer syndromes are defined by defects in homologous-recombination DNA repair. *Nat Genet.* 2018;50:1086-1092.
- Tabarin A, Corcuff JB, Rashedi M, Angibeau R, Caille JM, Ducassou D, Dufy B & Roger P. Multihormonal response to corticotropin-releasing hormone in inferior petrosal sinus blood of one patient with Cushing's disease: comparison with in vitro secretion of the tumoral corticotropes. *Acta Endocrinol (Copenh).* 1992;127:284-288.
- Talwalker PK, Ratner A & Meites J. In vitro inhibition of pituitary prolactin synthesis and release by hypothalamic extract. *Am J Physiol.* 1963;205:213-218.
- Tanizaki Y, Jin L, Scheithauer BW, Kovacs K, Roncaroli F & Lloyd RV. P53 gene mutations in pituitary carcinomas. *Endocr Pathol.* 2007;18:217-222.
- Thondam SK, Alusi S, O'Driscoll K, Gilkes CE, Cuthbertson DJ & Daousi C. Impulse control disorder in a patient on long-term treatment with bromocriptine for a macroprolactinoma. *Clin Neuropharmacol.* 2013;36:170-172.
- Thrane K, Eriksson H, Maaskola J, Hansson J & Lundeberg J. Spatially Resolved Transcriptomics Enables Dissection of Genetic Heterogeneity in Stage III Cutaneous Malignant Melanoma. *Cancer Res.* 2018;78:5970-5979.
- Toledano Y, Lubetsky A & Shimon I. Acquired prolactin deficiency in patients with disorders of the hypothalamic-pituitary axis. *J Endocrinol Invest.* 2007;30:268-273.

Toledo RA, Burnichon N, Cascon A, Benn DE, Bayley JP, Welander J, Tops CM, Firth H, Dwight T, Ercolino T, Mannelli M, Opocher G, Clifton-Bligh R, Gimm O, Maher ER, Robledo M, Gimenez-Roqueplo AP & Dahia PL. Consensus Statement on next-generation-sequencing-based diagnostic testing of hereditary pheochromocytomas and paragangliomas. *Nat Rev Endocrinol*. 2017;13:233-247.

Tordjman K, Stern N, Ouaknine G, Yossiphov Y, Razon N, Nordenskjold M & Friedman E. Activating mutations of the Gs alpha-gene in nonfunctioning pituitary tumors. *J Clin Endocrinol Metab*. 1993;77:765-769.

Toussaint L, Indelicato DJ, Muren LP, Li Z, Lassen-Ramshad Y, Kirby K, Pedro C, Mikkelsen R, Di Pinto M, Hoyer M & Stokkevag CH. Temporal lobe sparing radiotherapy with photons or protons for cognitive function preservation in paediatric craniopharyngioma. *Radiother Oncol*. 2020;142:140-146.

Trivellin G, Daly AF, Faucz FR, Yuan B, Rostomyan L, Larco DO, Schernthaner-Reiter MH, Szarek E, Leal LF, Caberg J-H, Castermans E, Villa C, Dimopoulos A, Chittiboina P, Xekouki P, Shah N, Metzger D, Lysy P, Ferrante E, Strebkova N, Mazerkina N, Zatelli M, Lodish M, Horvath A, de Alexandre R, Manning A, Levy I, Keil M, Sierra Mde L, Palmeira L, Coppieters W, Georges M, Naves L, Jamar M, Bours V, Wu T, Choong C, Bertherat J, Chanson P, Kamenický P, Farrell W, Barlier A, Quezado M, Bjelobaba I, Stojilkovic S, Wess J, Costanzi S, Liu P, Lupski J, Beckers A & Stratakis C. Gigantism and acromegaly due to Xq26 microduplications and GPR101 mutation. *N Engl J Med*. 2014;371:2363-2374.

Truong AT, Duez C, Belayew A, Renard A, Pictet R, Bell GI & Martial JA. Isolation and characterization of the human prolactin gene. *EMBO J*. 1984;3:429-437.

Tseng PL, Wu WH, Hu TH, Chen CW, Cheng HC, Li CF, Tsai WH, Tsai HJ, Hsieh MC, Chuang JH & Chang WT. Decreased succinate dehydrogenase B in human hepatocellular carcinoma accelerates tumor malignancy by inducing the Warburg effect. *Sci Rep*. 2018;8:3081.

Tsunoda A, Okuda O & Sato K. MR height of the pituitary gland as a function of age and sex: especially physiological hypertrophy in adolescence and in climacterium. *AJNR Am J Neuroradiol*. 1997;18:551-554.

Turner TH, Cookson JC, Wass JA, Drury PL, Price PA & Besser GM. Psychotic reactions during treatment of pituitary tumours with dopamine agonists. *Br Med J (Clin Res Ed)*. 1984;289:1101-1103.

Valenca GT, Glass PG, Negreiros NN, Duarte MB, Ventura LM, Mueller M & Oliveira-Filho J. Past smoking and current dopamine agonist use show an independent and dose-dependent association with impulse control disorders in Parkinson's disease. *Parkinsonism Relat Disord*. 2013;19:698-700.

Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, Banks E, Garimella KV, Altshuler D, Gabriel S & DePristo MA. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinformatics*. 2013;43:11.10.1-11.10.33.

Vasquez A, Atallah-Yunes N, Smith FC, You X, Chase SE, Silverstone AE & Vikstrom KL. A role for the aryl hydrocarbon receptor in cardiac physiology and function as demonstrated by AhR knockout mice. *Cardiovasc Toxicol*. 2003;3:153-163.

Vaz-Drago R, Custodio N & Carmo-Fonseca M. Deep intronic mutations and human disease. *Hum Genet*. 2017;136:1093-1111.

Venselaar H, Te Beek TA, Kuipers RK, Hekkelman ML & Vriend G. Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. *BMC Bioinformatics*. 2010;11:548.

Verbalis JG, Nelson PB & Robinson AG. Reversible panhypopituitarism caused by a suprasellar aneurysm: the contribution of mass effect to pituitary dysfunction. *Neurosurgery*. 1982;10:604-611.

Verhelst J, Abs R, Maiter D, van den Bruel A, Vandeweghe M, Velkeniers B, Mockel J, Lamberigts G, Petrossians P, Coremans P, Mahler C, Stevenaert A, Verlooy J, Raftopoulos C & Beckers A. Cabergoline in the treatment of hyperprolactinemia: a study in 455 patients. *J Clin Endocrinol Metab*. 1999;84:2518-2522.

Vilar L, Fleseriu M & Bronstein MD. Challenges and pitfalls in the diagnosis of hyperprolactinemia. *Arq Bras Endocrinol Metabol*. 2014;58:9-22.

Vilar L, Freitas MC, Naves LA, Casulari LA, Azevedo M, Montenegro R Jr., Barros AI, Faria M, Nascimento GC, Lima JG, Nobrega LH, Cruz TP, Mota A, Ramos A, Violante A, Lamounier Filho A, Gadelha MR, Czepielewski MA, Glezer A & Bronstein MD. Diagnosis and management of hyperprolactinemia: results of a Brazilian multicenter study with 1234 patients. *J Endocrinol Invest*. 2008;31:436-444.

Villa C, Lagonigro MS, Magri F, Koziak M, Jaffrain-Rea ML, Brauner R, Bouligand J, Junier MP, Di Rocco F, Sainte-Rose C, Beckers A, Roux FX, Daly AF & Chiovato L. Hyperplasia-adenoma sequence in pituitary tumorigenesis related to aryl hydrocarbon receptor interacting protein gene mutation. *Endocr Relat Cancer*. 2011;18:347-356.

Vinkers DJ & van der Wee NJ. A case of mania after long-term use of quinagolide. *Gen Hosp Psychiatry*. 2007;29:464.

Wang F, Gao H, Li C, Bai J, Lu R, Cao L, Wu Y, Hong L, Wu Y, Lan X & Zhang Y. Low levels of PRB3 mRNA are associated with dopamine-agonist resistance and tumor recurrence in prolactinomas. *J Neurooncol*. 2014;116:83-88.

Webster J, Piscitelli G, Polli A, Ferrari CI, Ismail I & Scanlon MF. A comparison of cabergoline and bromocriptine in the treatment of hyperprolactinemic amenorrhea. *N Engl J Med*. 1994;331:904-909.

Weintraub D, Hoops S, Shea JA, Lyons KE, Pahwa R, Driver-Dunckley ED, Adler CH, Potenza MN, Miyasaki J, Siderowf AD, Duda JE, Hurtig HI, Colcher A, Horn SS, Stern MB & Voon V. Validation of the questionnaire for impulsive-compulsive disorders in Parkinson's disease. *Mov Disord*. 2009;24:1461-1467.

- Weintraub D, Koester J, Potenza MN, Siderowf AD, Stacy M, Voon V, Whetteckey J, Wunderlich GR & Lang AE. Impulse control disorders in Parkinson disease: a cross-sectional study of 3090 patients. *Arch Neurol*. 2010;67:589-595.
- Wells SA, Asa SL, Dralle H, Elisei R, Evans DB, Gagel RF, Lee N, Machens A, Moley JF, Pacini F, Raue F, Frank-Raue K, Robinson B, Rosenthal MS, Santoro M, Schlumberger M, Shah M & Waguespack SG & American Thyroid Association Guidelines Task Force on Medullary Thyroid Carcinoma. Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma. *Thyroid*. 2015;25:567-610.
- Wermer P. Genetic aspects of adenomatosis of endocrine glands. *Am J Med*. 1954;16:363-371.
- Whitworth J, Skytte AB, Sunde L, Lim DH, Arends MJ, Happerfield L, Frayling IM, van Minkelen R, Woodward ER, Tischkowitz MD & Maher ER. Multilocus Inherited Neoplasia Alleles Syndrome: A Case Series and Review. *JAMA Oncol*. 2016;2:373-379.
- Wierinckx A, Delgrange E, Bertolino P, François P, Chanson P, Jouanneau E, Lachuer J, Trouillas J & Raverot G. Sex-Related Differences in Lactotroph Tumor Aggressiveness Are Associated With a Specific Gene-Expression Signature and Genome Instability. *Front Endocrinol (Lausanne)*. 2018;9:706.
- Wittert GA, Crock PA, Donald RA, Gilford EJ, Boolell M, Alford FP & Espiner EA. Arginine vasopressin in Cushing's disease. *Lancet*. 1990;335:991-994.
- Wu WX, Brooks J, Glasier AF & McNeilly AS. The relationship between decidualization and prolactin mRNA and production at different stages of human pregnancy. *J Mol Endocrinol*. 1995;14:255-261.
- Wu Y, Berends MJ, Sijmons RH, Mensink RG, Verlind E, Kooi KA, van der Sluis T, Kempinga C, van dDer Zee AG, Hollema H, Buys CH, Kleibeuker JH & Hofstra RM. A role for MLH3 in hereditary nonpolyposis colorectal cancer. *Nat Genet*. 2001;29:137-138.
- Wu ZB, Zheng WM, Su ZP, Chen Y, Wu JS, Wang CD, Lin C, Zeng YJ & Zhuge QC. Expression of D2RmRNA isoforms and ERmRNA isoforms in prolactinomas: correlation with the response to bromocriptine and with tumor biological behavior. *J Neurooncol*. 2010;99:25-32.
- Xekouki P, Pacak K, Almeida M, Wassif CA, Rustin P, Nesterova M, de la Luz Sierra M, Matro J, Ball E, Azevedo M, Horvath A, Lyssikatos C, Quezado M, Patronas N, Ferrando B, Pasini B, Lytras A, Tolis G & Stratakis C. Succinate dehydrogenase (SDH) D subunit (SDHD) inactivation in a growth-hormone-producing pituitary tumor: a new association for SDH? *J Clin Endocrinol Metab*. 2012;97:E357-366.
- Xekouki P, Szarek E, Bullova P, Giubellino A, Quezado M, Mastroyannis SA, Mastorakos P, Wassif CA, Raygada M, Rentia N, Dye L, Cougnoux A, Koziol D, Sierra Mde L, Lyssikatos C, Belyavskaya E, Malchoff C, Moline J, Eng C, Maher Lr, Pacak K, Lodish M & Stratakis C. Pituitary adenoma with paraganglioma/pheochromocytoma (3PAs) and succinate dehydrogenase defects in humans and mice. *J Clin Endocrinol Metab*. 2015;100:E710-719.
- Yagnik G, Jahangiri A, Chen R, Wagner JR & Aghi MK. Role of a p53 polymorphism in the development of nonfunctional pituitary adenomas. *Mol Cell Endocrinol*. 2017;446:81-90.

Yamaji T, Ishibashi M, Teramoto A & Fukushima T. Hyperprolactinemia in Cushing's disease and Nelson's syndrome. *J Clin Endocrinol Metab.* 1984;58:790-795.

Yanovski JA, Cutler GB Jr., Doppman JL, Miller DL, Chrousos GP, Oldfield EH & Nieman LK. The limited ability of inferior petrosal sinus sampling with corticotropin-releasing hormone to distinguish Cushing's disease from pseudo-Cushing states or normal physiology. *J Clin Endocrinol Metab.* 1993;77:503-509.

Yoo F, Kuan EC, Heaney AP, Bergsneider M & Wang MB. Corticotrophic pituitary carcinoma with cervical metastases: case series and literature review. *Pituitary.* 2018;21:290-301.

Zhang Q, Peng C, Song J, Zhang Y, Chen J, Song Z, Shou X, Ma Z, Peng H, Jian X, He W, Ye Z, Li Z, Wang Y, Ye H, Zhang Z, Shen M, Tang F, Chen H, Shi Z, Chen C, Chen Z, Shen Y, Wang Y, Lu S, Zhang J, Li Y, Li S, Mao Y, Zhou L, Yan H, Shi Y, Huang C & Zhao Y. Germline Mutations in CDH23, Encoding Cadherin-Related 23, Are Associated with Both Familial and Sporadic Pituitary Adenomas. *Am J Hum Genet.* 2017;100:817-823.

Zovickian J, Oldfield EH, Doppman JL, Cutler GB Jr. & Loriaux DL. Usefulness of inferior petrosal sinus venous endocrine markers in Cushing's disease. *J Neurosurg.* 1988;68:205-210.