Epigenetic Pathways in Human Disease: The Impact of DNA Methylation on Stress-Related Pathogenesis and Current Challenges in Biomarker Development

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1. Introduction

Genes implicated in glucocorticoid regulation play an important role in regulating the physiological impact of social and environmental stress, and have become a focal point for investigating the role of glucocorticoid regulation in the etiology of disease. We conducted a systematic review to critically assess the full range of clinical associations that have been reported in relation to DNA methylation of CRH, CRH-R1/2, CRH-BP, AVP, POMC, ACTH, ACTH-R, NR3C1, FKBP5, and HSD11β1/2 genes in adults. A total of 32 studies were identified. There is prospective evidence for an association between HSD11β2 methylation and hypertension, and functional evidence of an association between NR3C1 methylation and both small cell lung cancer (SCLC) and breast cancer. Strong associations have been reported between FKBP5 and NR3C1 methylation and PTSD, and biologically-plausible associations have been reported between FKBP5 methylation and Alzheimer’s Disease. Mixed associations between NR3C1 methylation and mental health outcomes have been reported according to different social and environmental exposures, and according to varying gene regions investigated. We conclude by highlighting key challenges and future research directions that will need to be addressed in order to develop both clinically meaningful prognostic biomarkers and an evidence base that can inform public policy practice.

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largely being regulated by CRH binding proteins (CRH-BP) (Westphal et al., 2005). CRH is transported via the hypophyseal portal system of blood vessels to the anterior pituitary gland, which causes the pituitary gland to secrete adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH then stimulates the production of glucocorticoids (e.g., cortisol) by the adrenal cortex. Glucocorticoids (GCs) are produced by the adrenal cortex and bind to glucocorticoid receptors in the anterior pituitary, hypothalamus, and the hippocampus to regulate production of CRH and ACTH, creating a negative feedback loop that stabilizes circulating levels of stress hormones within an appropriate physiological range. Adapted from: Schloesser et al. (2012).

Cortisol levels are further regulated by the HSD11β2 enzyme, which degrades cortisol to cortisone, whereas HSD11β1 catalyzes the conversion of the inactive cortisone to active cortisol (Tomlinson et al., 2004). The glucocorticoid receptor (GR) resides in the cell cytoplasm. When bound to glucocorticoids, the GR translocates to the nucleus in a complex of co-chaperones that regulates the synthesis of a number of immune, inflammatory, and metabolic proteins through two genomic mechanisms of action (Fig. 2). First, the GR complex may act as a transcription factor that activates the transcription of immune- and metabolic-related genes by binding directly to glucocorticoid response elements (GRs) in nuclear DNA through a process called transactivation. Second, the GR complex may also repress the transcription of genes that code for immunosuppressive and pro-inflammatory proteins such as cytokines and prostaglandins through a process called transrepression, wherein the GR complex interacts with other transcription factors such as AP-1 and NF-κB to reduce their transcriptional activity without contacting the DNA itself (Bateman et al., 2013; Schloesser and Burtger, 2013). Binding of the FKBP5 protein to this GR complex lowers the complex’s affinity for cortisol and makes nuclear translocation of the GR complex less efficient (Wochnik et al., 2005), while also promoting nuclear translocation of the non-active GR splice variant (isoform), and thus decreasing overall GR signaling (Zhang et al., 2008). In this way, overexpression of FKBP5 proteins can lead to glucocorticoid resistance and decreased glucocorticoid sensitivity (Binder, 2009). Expression of FKBP5 mRNA is induced by glucocorticoids, however, leading to an ultra-short negative feedback loop wherein greater circulating levels of glucocorticoids induce greater FKBP5 protein expression, thus regulating GR signaling within an appropriate range (Vermeer et al., 2003).

Glucocorticoids also exert non-genomic actions that can occur rapidly within a period of several minutes. This is thought to occur through the activation of signal transduction pathways, or through the interactions of glucocorticoids with cellular membranes (Strehl and Buttgereit, 2013; Kadmiel and Cidlowski, 2013). Through these genomic and non-genomic mechanisms of action, glucocorticoids exert a number of different effects in almost every tissue of the human body. Furthermore, through the above-mentioned interactions and pathways, the HPA axis and glucocorticoids interact with and regulate a number of fundamental physiological systems, including the nervous, cardiovascular, immune, musculoskeletal, visual, reproductive, and integumentary systems, and also play a role in regulating glucose and liver metabolism, mood and cognition, metabolic processes, and maintaining circadian rhythm (Smith and Vale, 2006; Kadmiel and Cidlowski, 2013; Kalsbeek et al., 2012). Not surprisingly, then, the HPA axis has received increasing attention over the past decade due to its critical role in regulating stress and its ability to influence a variety of health outcomes (Moisidias and Matthews, 2014; Kalsbeek et al., 2012; Turecki, 2014; Eades et al., 2014; Cameron, 2006; Conradt et al., 2013; Edelman et al., 2012; Lee et al., 2014; Wan et al., 2014).

Studies aimed at exploring epigenetic regulation of the genes that code for the hormones, proteins, and receptors within the HPA axis may further our understanding of the pathways through which glucocorticoid dysregulation might increase risk for a number of diseases. Although several studies and reviews have examined the impact of adverse childhood experiences, socioeconomic adversity, and other environmental stressors on epigenetic regulation (most commonly DNA methylation) of individual HPA axis genes, such as NR3C1, AVP, or FKBP5 (Palma-Gudiol et al., 2015; Daskalakis and Yehuda, 2014; Zannas et al., 2016; Needham et al., 2015), no comprehensive review exists that has examined the epigenetic regulation of all HPA axis genes within the entire glucocorticoid regulatory pathway. More importantly, no extant reviews systematically discuss the full range of clinical associations found in relation to epigenetic regulation of these genes. While one recent review has outlined the role of glucocorticoid sensitivity in various diseases (Quax et al., 2013), there also exists no comprehensive review that enumerates the full range of epigenetic pathways that might lead to glucocorticoid dysregulation, and ultimately, disease.
The purpose of this systematic review, therefore, is to critically examine the extant literature on DNA methylation of CRH, CRH-R1/2, CRH-BP, AVP, POMC, ACTH, ACTH-R, NR3C1, FKBP5, and HSD11B1/2 in relation to clinical outcomes in adults. In doing so, our aim is to also highlight current challenges in the field that will need to be addressed in order to develop clinically meaningful prognostic biomarkers, and to outline future research directions needed to create an evidence base that can inform public policy practice.

2. Methods

This systematic review was conducted according to Cochrane PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Liberati et al., 2009), and the software used to store information was RevMan 5.3.

2.1. Search Strategy and Selection Criteria

Three databases were used to retrieve studies for this review: PubMed, MEDLINE, and Google Scholar. Our searches were not limited by year of publication because the field of epigenetics is relatively new (the earliest study found was published in 2006).

We searched PubMed for “methylation”[MeSH Terms] OR “methylation”[All Fields] in conjunction with the following key search terms: “CRH” (26 results), “CRHR1” (11 results), “CRHR2” (3 results), (“ACTH” OR “adrenocorticotropic”) (68 results), (“ACTHR” OR “MC2”) (2 results), (“GR” OR “hGR” OR “GCR” OR “NR3C1”) (295 results), (“HSD11B1” OR “11β-hydroxysteroid dehydrogenase-1” OR “11-Beta-HSD1” OR “HSD11”) (5 results), (“HSD11B2” OR “11β-hydroxysteroid dehydrogenase-2” OR “11-Beta-HSD2” OR “HSD11”) (25 results), (“Pro-opiomelanocortin” or “POMC”) (100 results), (“tacrolimus binding protein 5”[Supplementary Concept] OR “FKBPS” OR “FK506 binding protein 5” OR “Tacrolimus Binding Proteins/genetics”[Mesh]) (50 results), (“CRHBP” OR “CRH-BP” OR “CRH binding protein”) (4 results), and (AVP OR Vasopressin) (59 results). In total, 648 articles were found on PubMed. No additional studies were found in our Google Scholar of MEDLINE searches. Bibliographic references of retrieved articles were scanned by the authors to identify any final articles to include, although no additional articles were found using this method.

The first and second authors screened the titles and the abstracts of all identified citations and selected potentially eligible studies. Full text articles were then included based on the following inclusion criteria: (1) evidence of quantitatively measured DNA methylation of any of the following: CRH, CRH-R1/2, CRH-BP, AVP, POMC, ACTH, ACTH-R, NR3C1, FKBP5, or HSD11B1/2; (2) recorded evidence of a quantifiable clinical association (e.g., diabetes, hypertension, depression) investigated in direct relation to DNA methylation levels in the above genes; (3) the study was reported in English; and (4) the study included adult human subjects (18 years or older) or used human-derived cell lines. A flow chart of this process is presented in Fig. 3.

We excluded studies reported in languages other than English, those using non-human subjects, qualitative studies, duplicates, editorials, case reports, and commentaries. Studies on the mineralocorticoid receptor gene (NR3C2) and both norepinephrine- and epinephrine-related genes were excluded because the focus of our review is on glucocorticoid regulation rather than mineralocorticoid regulation. We have also excluded studies on atrial natriuretic peptide (ANP) in this study, as although it is implicated in HPA activity, it is not directly involved in glucocorticoid regulation (Jessop, 1999). Finally, hormone secreting...
tumors and paraneoplastic syndromes that naturally dysregulate methylation status in a well-established manner (i.e., pituitary adenomas that secrete ACTH [Ceccato et al., 2015], thymic carcinoids [Mizoguchi et al., 2007], and Cushing syndrome [Newell-Price, 2003]) were excluded because of their ectopic tumor loci, which produce ACTH hormones outside of the HPA axis.

2.2. A Note on the Unique Structure of NR3C1

We did not seek in this review to give a detailed discussion of the methodological differences between studies, given that an insightful examination of this matter can be found in other reviews (Palma-Gudiel et al., 2015; Daskalakis and Yehuda, 2014). We also did not seek to give a detailed characterization of the structural classifications of all genes under study in this paper, since these have been dealt with in excellent detail elsewhere (Daskalakis and Yehuda, 2014; Zannas et al., 2015; Daskalakis and Yehuda, 2014). We also did not seek to give a detailed characterization of the structural classifications of all genes under study in this paper, since these have been dealt with in excellent detail elsewhere (Daskalakis and Yehuda, 2014; Zannas et al., 2015; Daskalakis and Yehuda, 2014). However, given that NR3C1 has a rather unique structure in relation to other HPA axis genes included in our review, it is worth providing more detailed information here in order to: (1) contextualize the unique gene region nomenclature used for NR3C1 throughout the research presented in this review; and (2) demonstrate how the unique structure of NR3C1 gives it a transcriptional complexity that may cause epigenetic regulation within different promoter regions to engender different responses to glucocorticoids in a tissue-specific manner.

NR3C1 contains 8 coding exons (exons 2–9) and a non-coding 5′ region consisting of 9 non-coding alternative first exons (1A–1I, excluding G) (Figs. 4–5). Each of these alternative first exons has its own promoter, which is immediately upstream from the exon itself, and the entire proximal promoter (highlighted in red in Figs. 4–5, encompassing exons 1D, 1J, 1E, 1B, 1F, 1C, and 1H) is located within a 3 kb CpG island. This non-coding 5′ region of NR3C1 is largely thought to be responsible for transcriptional regulation of GR protein levels (Turner et al., 2010).

Epigenetic research on NR3C1 to date has tended to focus exclusively on the CpG island within the proximal promoter region, with some notable exceptions (Shields et al., 2016). Although the GR protein is ubiquitously expressed throughout all tissues in humans, the different non-coding first exons within the 5′ region of NR3C1 are differentially expressed through alternative gene splicing within various tissues in the body (Fig. 6) (Turner and Muller, 2005; Presul et al., 2007; Cao-Lei et al., 2011). This suggests that these alternative first exons play a role in regulating tissue- and organ-specific responses to glucocorticoids, although the exact role of these first exons remains unknown (Turner et al., 2014).

3. Results

Thirty-two articles were identified as meeting our review inclusion and exclusion criteria. Full details of all studies retrieved in our review are given in Tables 1–5; however, we have limited our more detailed discussions in these sections to the high-quality and promising extant research that emerged from our review. Given the exceptional complexity of the HPA axis and its far-reaching interactions with a number of physiological systems, the plausible mechanisms and pathways through which epigenetic regulation of the HPA axis might contribute to disease are understandably varied. We will therefore also provide a critical discussion of the pathways implicated in the results reported across studies, where evidence exists, in order to contextualize the results reported for the many divergent conditions included in this review.

Potential associations between epigenetic regulation of HPA axis genes and clinical outcomes still remain relatively unmapped, as only DNA methylation of NR3C1, HSD11B2, and FKBP5 has been studied in direct relation to risk for any human disease, with the majority of research focusing on epigenetic regulation of NR3C1. Furthermore, the majority of studies vary in terms of gene region(s) or CpG loci selected for DNA methylation analysis (Table 1), although exon 1F or the 1F promoter

![Fig. 4. Mean and CpG site-specific methylation results reported for exon 1F and its promoter in NR3C1. First exon variants in red represent the proximal promoter region. ▼ or ▲ denote hypomethylation or hypermethylation, respectively. ▼ or ▲ arrows denote a correlation between observed methylation at a specific CpG site and decreased or increased NR3C1 expression levels, respectively. "Null" denotes no methylation found, or no difference found in methylation between healthy controls and those with the disease under investigation. Boxes around CpG site numbers represent NGFI-A transcription factor binding sites according to McGowan et al. (2009). CpG numbering taken from Palma-Gudiel et al. (2015). Figure is not to scale.](image-url)
have been investigated most frequently among NR3C1 methylation studies (17/24; Table 1, Figs. 4–5). No studies relevant to our inclusion criteria examined the relationship between DNA methylation of POMC, ACTH, ACTH-R, AVP, CRH, CRH-R1/2, or CRH-BP genes in relation to any clinical outcomes. Notably, three studies were retrieved on POMC methylation in relation to alcohol craving (Muschler et al., 2010), weight regain (Crujeiras et al., 2013), and malnutrition (Ehrlich et al., 2010), but these studies were excluded from the current review because they focused on intermediate outcomes instead of clinically-defined diseased states.

3.1. Cardiovascular Diseases

Three studies identified in our systematic review examined the associations between DNA methylation of either HSD11b2 or NR3C1 and cardiovascular diseases (Table 2).

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Fig. 5. Significant methylation results reported across the entire NR3C1 proximal promoter. Methylation results reported only for clinical outcomes that have been significantly associated with methylation status in at least one study. Diseases for which there have only been non-significant findings or for which the authors concluded that NR3C1 was not a likely biomarker are not included. First exon variants in red represent the proximal promoter region. ▼ or ▲ denote hypomethylation or hypermethylation, respectively, ↓ or ↑ arrows denote a correlation between observed methylation and decreased or increased NR3C1 expression levels, respectively. Methylation shown here represents either mean or CpG site-specific methylation, depending on the study method used, and arrows shown here are not meant to indicate methylation at specific CpG loci. Figure is not to scale.

*Mean methylation levels only reported across all 4 first exon promoters, and not within each promoter.

Fig. 6. Human tissue-specific gene expression of non-coding first exons within the NR3C1 proximal promoter, as reported by Turner and Muller (2005) and Presul et al. (2007). Dark blue to light blue gradients represent strong to weak expression, respectively. Note: exon 1J was discovered by Presul et al., and thus was not measured in Turner and Muller. Presul et al. did not measure exons 1E and 1H.
### Table 1
Genomic regions investigated in reviewed studies.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Gene</th>
<th>Genomic region tested</th>
<th>Nucleotide position</th>
<th>Exposure/Outcome</th>
</tr>
</thead>
</table>
| Friso et al. (2008)      | HSD11/2  | Promoter and exon 1 (2 CpG islands analyzed)                                          | Promoter: $-633$ to $-97^*$  
it Exon 1: $-77$ to $+460^*$                      | Glucocorticoid treatment/hypertension                   |
| Pizzolo et al. (2015)    | HSD11/2  | Promoter (2 regions analyzed)                                                          | $-692$ to $-595$, and $-419$ to $-177$                   | AME/hypertension                                       |
| Zhao et al. (2015)       | NR3C1    | Exon 1F promoter (22 CpG sites analyzed)                                              | $-3480$ to $-3352$                                        | Combat-related PTSD/subclinical atherosclerosis         |
| Nesson et al. (2014)     | NR3C1    | 6 regions in the proximal promoter:                                                   | $1J$: $-4287$ to $-4027$  
$1J$: $-4046$ to $-3764$  
$1B$: $-3549$ to $-3339$  
$1C$: $-2868$ to $-2665$  
$1C$: $-1997$ to $-1796$  
$1H$: $-2017$ to $-1796$ | None/breast cancer                      |
| Lien et al. (2006)       | NR3C1    | Exon 1C                                                                               | $-2677$ to $-2371$                                         | None/breast cancer                                      |
| Kay et al. (2011)        | NR3C1    | 1D promoter (26 CpG sites analyzed)                                                    | (not reported)                                            | None/small cell lung cancer                            |
| Abhquis et al. (2008)    | NR3C1    | Promoter                                                                              | (not reported)                                            | None/colorectal cancer                                  |
| Lind et al. (2006)       | NR3C1    | Proximal promoter                                                                      | $-477$ to $-25$                                           | None/colon cancer                                       |
| Wu et al. (2007)         | NR3C1    | Exon 2                                                                                | $+168$ to $+242$                                           | None/ovarian cancer                                     |
| Kang et al. (2008)       | NR3C1    | Promoter                                                                              | (not reported)                                            | None/gastric cancer                                      |
| Hiraki et al. (2011)     | NR3C1    | 1B and 1C promoters                                                                   | (not reported)                                            | None/gastric cancer                                     |
| Sanchez-Vega and Gandhi (2009) | NR3C1 | Promoter                                                                              | (not reported)                                            | None/multiple myeloma                                   |
| Ronco et al. (2010)      | HSD11/2  | Promoter (2 CpG islands analyzed)                                                     | $\text{(not reported)}^{1,3}$  
Epigenome-wide (485,577 unique sites) | Cadmium exposure/choriocarcinoma                        |
| Smyth et al. (2014)      | FKBP5    |                                                                                      | $\text{(not reported)}^{1,3}$  
\text{Promoter: chr6:35,654,363}  
\text{Enhancer: chr6:35,570,224}  
\text{CpG island: chr6:35,655,607--35,656,856)} | None/chronic kidney disease                             |
| Vangeel et al. (2015)    | NR3C1    | Exon 1F and 1F promoter (47 CpG sites analyzed)                                        | $-3471$ to $-2101$                                        | Childhood trauma/chronic fatigue syndrome               |
| Chen et al. (2015)       | NR3C1    | Exon 1D, 1E, 1F, and 1H promoters                                                     | (not reported)                                            | SLE/Childhood physical and sexual abuse/PTSD           |
| Klengel et al. (2013)    | FKBP5    | $\text{CpG island near the TSS (190 CpG sites analyzed)}  
\text{Promoter: chr6:35,798,260--35,798,490}  
\text{Intron 2: chr6:35,715,732--35,716,027}  
\text{Intron 5: chr6:35,686,677--35,687,097}  
\text{Intron 7: chr6:35,677,658--35,677,924}  
\text{18: 479 bp}  
\text{1C: 364 bp}  
\text{(exact positions not reported)} | Childhood trauma/chronic fatigue syndrome               |
| Labonte et al. (2014)    | NR3C1    | 1B promoter, including exons 1 J and 1E (29 CpG sites analyzed)                        | $-3521$ to $-3156$                                        | PE/PTSD                                               |
| Yehuda et al. (2013)     | NR3C1    | 1F promoter and exon 1F (39 CpG sites analyzed)                                        | $-3236$ to $-3030$                                        | Rwandan genocide/PTSD                                   |
| Yehuda et al. (2015a)    | NR3C1    | 1F promoter and exon 1F (39 CpG sites analyzed)                                        | $-3521$ to $-3156$                                        | DEX treatment/PTSD                                      |
| Vukovicic et al. (2014)  | NR3C1    | 1F promoter and exon 1F (8 CpG sites analyzed)                                         | $-3266$ to $-3030$                                        | None/Alzheimer’s disease                                |
| Blair et al. (2013)      | FKBP5    | $\text{Promoter: chr6:35,798,260--35,798,490}  
\text{Intron 2: chr6:35,715,732--35,716,027}  
\text{Intron 7: chr6:35,666,288--35,666,763}  
\text{18: 479 bp}  
\text{1C: 364 bp}  
\text{(exact positions not reported)} | None/Alzheimer’s disease                                      |
Table 1 (continued)

<table>
<thead>
<tr>
<th>Authors</th>
<th>Gene</th>
<th>Genomic region tested</th>
<th>Nucleotide position</th>
<th>Exposure/Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moser et al. (2007)</td>
<td>NR3C1</td>
<td>Small piece of 1F promoter and exon 1F (74 bp around the NGFI-A binding site (9 CpG sites analyzed))</td>
<td>−3281 to −3177</td>
<td>None/Parkinson’s disease, presenile and senile dementia-Alzheimer’s type, dementia</td>
</tr>
<tr>
<td>Na et al. (2014)</td>
<td>NR3C1</td>
<td>1F promoter and exon 1F (5 CpG sites analyzed)</td>
<td>−3279 to −3039</td>
<td>Early parental death/depression</td>
</tr>
<tr>
<td>Melas et al. (2013)</td>
<td>NR3C1</td>
<td>1F promoter and exon 1F (47 CpG sites analyzed)</td>
<td>−3480 to −3126</td>
<td>Prenatal exposure to famine/depression, diabetes, cardiovascular disease</td>
</tr>
<tr>
<td>Veenendaal et al. (2012)</td>
<td>NR3C1</td>
<td>1C promoter and partial exon 1C</td>
<td>−3035 to −2704</td>
<td>None/MDD</td>
</tr>
<tr>
<td>Alt et al. (2010)</td>
<td>NR3C1</td>
<td>3 regions in the proximal promoter:</td>
<td>294 bp (exact position not reported)</td>
<td>Childhood averse events, lifetime history of depression/MD</td>
</tr>
<tr>
<td>Höhne et al. (2015)</td>
<td>FKBP5</td>
<td>Intron 7 (7 CpG sites analyzed)</td>
<td>chr6:35,666,288–35,666,763</td>
<td>Childhood maltreatment; BPD</td>
</tr>
<tr>
<td>Fries et al. (2015)</td>
<td>FKBP5</td>
<td>Area surrounding glucocorticoid response elements found in 3 regions:</td>
<td>chr6:35,666,288–35,666,763</td>
<td>None/BN, BPD, Suicidality</td>
</tr>
<tr>
<td>Dammann et al. (2011)</td>
<td>NR3C1</td>
<td>Exon 1B</td>
<td>chr6:35,666,288–35,666,763</td>
<td>None/borderline personality disorder (BPD)</td>
</tr>
<tr>
<td>Perroud et al. (2011a)</td>
<td>NR3C1</td>
<td>1F promoter and exon 1F (first 8 CpG sites of exon 1F analyzed)</td>
<td>chr6:35,666,288–35,666,763</td>
<td>Childhood maltreatment; BPD, MDD</td>
</tr>
<tr>
<td>Steiger et al. (2013)</td>
<td>NR3C1</td>
<td>1B promoter (includes exons 1J and 1E)</td>
<td>chr6:35,666,288–35,666,763</td>
<td>None/BN, BPD, Suicidality</td>
</tr>
</tbody>
</table>

Note: NR3C1 base pair numbering reflects position relative to ATG start codon in exon 2. HSD11β2 and FKBP5 base pair numbering is relative to the transcription start site in exon 1.

3.1.1. Hypertension

In a sample of 32 Italian patients who were treated with glucocorticoids, those who developed clinically-diagnosed hypertension had greater HSD11β2 promoter methylation, and a higher urinary tetrahydrocortisol/tetrahydrocortisone (THF/THE) ratio, both of which indicate lower HSD11β1 protein activity (Friso et al., 2008). This prospective study may highlight a potential new mechanism in the pathogenesis of glucocorticoid-induced hypertension, and may furthermore indicate HSD11β2 promoter methylation as a potentially useful biomarker to characterize hypertensive patients. Excess levels of cortisol can effect regulation of sodium absorption in the kidneys, alongside aldosterone, and thus can have a direct impact on salt-induced hypertension (Hunter et al., 2014). Hypermethylation of the HSD11β2 promoter, however, disrupts its ability to carry out cortisol to cortisone conversion through decreasing HSD11β2 protein expression, which leads to greater levels of cortisol relative to cortisone (i.e., a higher tetrahydrocortisol [THF] to tetrahydrocortisone [THE] ratio) and promotes hypertension (Ferrari et al., 2001; Udali et al., 2013).

Indeed, related research has shown that changes in HSD11β2 protein activity significantly affects blood pressure levels in healthy adults (Ferrari et al., 2001), and that HSD11β2 protein activity is decreased in the presence of greater HSD11β2 promoter methylation in human cells (Alikhani-Koopaei et al., 2004). Future prospective research in healthy adults is needed, however, to more precisely characterize the significance of this relationship in the development of hypertension. Furthermore, it will be important for future research to investigate whether early life or current stress play a role in the epigenetic regulation of HSD11β2 in relation to hypertension.

3.1.2. Subclinical Atherosclerosis

Among 84 American veteran monzygotic twin pairs (168 participants in total), intra-pair increases in flow-mediated dilation (FMD; determined using bi-mode ultrasound), a marker of subclinical atherosclerosis, was associated with both mean NR3C1 1F promoter hypermethylation and site-specific methylation at 12 out of 22 CpG sites investigated (Zhao et al., 2015). Even after adjustment for post-traumatic stress disorder (PTSD) symptoms, a 1% increase in the intra-pair difference in mean DNA methylation was associated on average with a 2.83% increase in the intra-pair difference in FMD. Another study included in our review, however, found no association between NR3C1 1C promoter methylation and a range of other factors associated with coronary heart disease, including blood pressure, as well as glucose and insulin levels (Veenendaal et al., 2012). Future research on subclinical atherosclerosis and other risk factors for cardiovascular disease may do well to investigate the 1F promoter, specifically, in NR3C1.

3.2. Cancer

Identifying epigenetic modifications associated with cancer is a rapidly expanding field of study. Our review shows that DNA methylation of NR3C1 has been associated with a variety of cancer outcomes (Table 3), although there is a lack of in vivo studies that could confirm the preliminary conclusions reported by studies using in vitro cell lines. Furthermore, corroboration between observed tumor tissue methylation and methylation levels in peripheral blood, which would be needed to implement non-invasive testing, has not yet been established. These results could then be more appropriately used towards the identification of biomarkers and the development of interventions. Among the studies reviewed, there is currently only strong evidence that NR3C1 methylation might be implicated in breast cancer (Nesset et al., 2014) and small cell lung cancer (SCLC) (Kay et al., 2011). Evidence from these studies indicates that promoter methylation of NR3C1 may contribute to tumorigenesis through decreasing NR3C1 expression, leading to downstream inhibition of the tumor suppressing and anti-proliferative capabilities of the GR for these cancers.

3.2.1. Breast Cancer

One study using matched normal and tumor breast tissue from 59 Canadian breast cancer patients examined methylation in a genomic region
spanning almost the entire proximal promoter of \textit{NR3C1} (Nesset \textit{et al.}, 2014). The authors found that while non-tumor tissues were uniformly unmethylated, 15% (8/59) of breast cancer tumor tissues were methylated. Specifically, one region containing exons 1J–E and their promoters, as well as a second region containing exon 1B and the 1F promoter, both showed methylation in the highest number of tumors (7/59 and 5/59, respectively). There was also a statistically significant 4.6-fold decrease in \textit{NR3C1} expression in tumors methylated at exon 1B and the 1F promoter, compared with tumors unmethylated at this region. A previous study, however, found \textit{NR3C1} exon 1C to be uniformly unmethylated in breast tissue samples taken from 118 GR-immunonegative breast cancer carcinoma subjects from Taiwan (Lien \textit{et al.}, 2006), although this study only investigated a small gene region (exon 1C), which is not as heavily expressed in breast tissue, and did not study any of the regions shown to be methylated in the more recent paper.

DNA methylation of exon 1B within the proximal promoter of \textit{NR3C1} therefore seems to be a promising early candidate for a possible breast cancer biomarker. This is all the more biologically plausible because Nesset \textit{et al.} (2014) experimentally determined that exon 1B is the predominant \textit{NR3C1} first exon variant expressed in human breast tissue. Importantly, high levels of \textit{NR3C1} protein (GR) expression and high concentrations of cortisol in breast tissue have been shown to have an anti-proliferative effect in cancerous breast tissue (Vilasco \textit{et al.}, 2011). Accordingly, Nesset \textit{et al.} demonstrated that in all patients except one (58/59) from their study, all breast cancer tumors showed lower expression compared with normal tissue, with an average decrease in expression of 13.81-fold. This decrease in \textit{NR3C1} expression was also observed in the study by Lien \textit{et al.} (2006) included in this review. Combined, these findings suggest that decreased \textit{NR3C1} expression appears to be a common and important factor in breast cancer tumor development. Furthermore, the results reported by Nesset \textit{et al.} indicate that promoter methylation of \textit{NR3C1} might be one significant mechanism that leads to decreases in gene and GR protein expression.

### Table 2

<table>
<thead>
<tr>
<th>Authors</th>
<th>Study design</th>
<th>Gene</th>
<th>Method</th>
<th>Tissue type</th>
<th>Exposure/Outcome</th>
<th>Sample</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Friso \textit{et al.} (2008)</td>
<td>Prospective</td>
<td>HSD11/J2</td>
<td>Methylation-specific PCR</td>
<td>Peripheral blood (PBMC)</td>
<td>Glucocorticoid treatment/hypertension</td>
<td>N = 57 Italian subjects. 32 glucocorticoid-treated patients normotensive at baseline (mean age: 54.56 years; 4 men); 25 essential hypertensive patients (EH) (mean age: 51.92 years; 14 men)</td>
<td>Glucocorticoid-treated patients who developed hypertension had:</td>
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<tr>
<td>Pizzolo \textit{et al.} (2015)</td>
<td>Cross-sectional</td>
<td>HSD11/J2</td>
<td>Pyrosequencing</td>
<td>Peripheral blood (PBMC)</td>
<td>AME/hypertension</td>
<td>N = 12 members of the same Italian family, 2 proband brothers (7 and 13 years old) with apparent mineralocorticoid excess (AME: A221G missense mutation) and 10 relatives (age range: 46–71 years), 6 of whom had the A221G mutation</td>
<td>Among the 6 family members heterozygous for 221AG:</td>
</tr>
<tr>
<td>Zhao \textit{et al.} (2015)</td>
<td>Cross-sectional</td>
<td>NR3C1</td>
<td>Pyrosequencing</td>
<td>Peripheral blood (leukocytes)</td>
<td>Combat-related PTSD/Subclinical atherosclerosis</td>
<td>N = 168 US veterans (84 monozygotic [MZ] twin pairs) (mean age: 55.1 years). The majority of twins were Caucasian (94%) and free of history of cardiovascular disease at enrollment</td>
<td>Except for LD1, there was no significant association between \textit{NR3C1} 1F promoter methylation and standard coronary risk factors (e.g., triglycerides, blood pressure)</td>
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<td>• Intra-pair difference in methylation was significantly and positively associated with intra-pair difference in flow-mediated dilation (FMD; determined using bi-mode ultrasound) at 50% (12/22) of studied CpG sites, and with mean DNA methylation across all studied CpG sites in the 1F promoter</td>
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<td>• On average, 1% increase in the intra-pair difference in mean DNA methylation was associated with 2.83% increase in the intra-pair difference in FMD</td>
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<td>• Methylation variation at each individual CpG site only conferred a small risk to disease, but the authors identified three methylation blocks (−3365 to −3371, −3418 to −3445, and −3456 to −3470 bp from ATG) that were significantly associated with FMD in their sample</td>
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<td>• FMD-related methylation changes may therefore be systematically organized, rather than randomly distributed</td>
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<tr>
<td>Authors</td>
<td>Study design</td>
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<tr>
<td>Nesset et al.</td>
<td>Case-control; in vitro cell lines</td>
<td>NR3C1</td>
<td>Methylation-specific PCR</td>
<td>Breast tissue</td>
<td>None/breast cancer</td>
<td>N = 59 breast cancer patients from Ontario, Canada</td>
<td>• Exon 1B is the predominant NR3C1 first exon expressed in normal and cancerous breast tissue&lt;br&gt;• 8 (15%) breast cancer tumors showed elevated methylation. The 1B promoter was methylated in 7/8 of these. &lt;br&gt;• No methylation was observed in normal breast tissue &lt;br&gt;• No difference in NR3C1 expression between tumors methylated and unmethylated at the NR3C1 proximal promoter as an aggregate &lt;br&gt;• However, tumors methylated at the 1B promoter showed a 4.6-fold decrease in NR3C1 expression compared with tumors unmethylated at this region</td>
</tr>
<tr>
<td>Lien et al.</td>
<td>Cross-sectional</td>
<td>NR3C1</td>
<td>Methylation-specific PCR</td>
<td>Breast tissue</td>
<td>None/breast cancer</td>
<td>N = 118 GR-immunonegative carcinoma specimens (106 IDCs, 8 ILCs, 1 mucinous carcinoma, 3 DCIS) taken from human breast samples in Taipei City, Taiwan</td>
<td>• NR3C1 was strongly expressed in metaplastic carcinomas (94.4%), but was not expressed in the majority of non-metaplastic carcinomas (58.2%)&lt;br&gt;• NR3C1 exon 1C was uniformly unmethylated in non-metaplastic carcinomas</td>
</tr>
<tr>
<td>Kay et al.</td>
<td>Case-control (cell line and tissue); prospective</td>
<td>NR3C1</td>
<td>Bisulfate sequencing</td>
<td>SCLC cell lines</td>
<td>Small cell lung cancer (SCLC)</td>
<td>Cases: 14 Small Cell Lung Cancer (SCLC) cell lines: Cor L24, Cor L27, Cor L31, Cor L32, Cor L42, Cor L47, Cor L51, Cor L88, Cor L99, Cor L103, DMS 79, DMS 153, H12, and H 148&lt;br&gt;Cell line controls: human lung epithelial carcinoma cells A549, human embryonic kidney cells HEK-293, human cervical carcinoma cells HeLa, and human osteosarcoma cells U2OS (European Collection of Cell Cultures, Wiltshire, UK). Non--small cell lung cancer (NSCLC) lines NCI-H1558 and -H727 (European Collection of Cell Cultures, Wiltshire, UK) and NC-2-H23, -H441, -H1299 (American Type Culture Collection, USA)&lt;br&gt;Peripheral blood controls: human peripheral blood mononuclear cells (PBMC) from health donors</td>
<td>• There was a significant difference in methylation levels between SCLC cases and controls at several CpG sites in the 1C promoter (CpG 69 individually, CpG 68 and 69 in combination) as well as a significant difference in methylation levels across the whole 1C promoter region (CpGs1-69) and for all 1C CpGs excluding 68 and 69 &lt;br&gt;• There was a significant association between number of methylated CpGs and NR3C1 protein expression within the panel of 14 SCLC cell lines &lt;br&gt;• After 72 h of treatment with 5′Azadeoxycytidine, NR3C1 mRNA levels increased dramatically in SCLC cells while there was no change in expression in HEK and A549 control cells, and a decrease in NR3C1 in U2OS control cells &lt;br&gt;While 3/4 SCLC cell lines showed augmented NR3C1 expression after 5′Azadeoxycytidine treatment, only 1/4 NSCLC cell lines showed increased NR3C1 expression</td>
</tr>
<tr>
<td>Ahlquist et al.</td>
<td>Case-control</td>
<td>11 genes, including NR3C1</td>
<td>Quantitative methylation-specific PCR</td>
<td>Colorectal cancer (CRC) and normal colon mucosa tissue</td>
<td>None/colorectal cancer</td>
<td>Cases: 63 adenomas from 52 Norwegian individuals (median age: 67), and 52 carcinomas from 51 patients in 7 hospitals in Oslo, Norway (median age: 70 years) &lt;br&gt;Controls: 21 normal colon mucosa samples (N1) from 20</td>
<td>• NR3C1 showed an increasing methylation frequency from adenomas to carcinomas&lt;br&gt;• NR3C1 methylation was significantly higher among microsatellite instable (MSI) than among microsatellite stable (MSS) carcinomas (P = 0.001)</td>
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Table 3 (continued)

<table>
<thead>
<tr>
<th>Authors</th>
<th>Study design</th>
<th>Gene</th>
<th>Method</th>
<th>Tissue type</th>
<th>Exposure/Outcome</th>
<th>Sample</th>
<th>Results</th>
</tr>
</thead>
</table>
| Kang et al.      | Case-control     | 17 genes, including NR3C1 | Quantitative methylation-specific PCR (MethyLight) | Gastric cancer cell lines and normal gastric mucosa tissue | Gastric cancer | Cell line cases: 8 gastric cancer (GC) cell lines treated with 5-aza-deoxycytidine and or Trichostatin A | • NR3C1 was methylated in GC at a level three times higher than GCN, although this difference was not significant  
  • No significant difference in methylation level in NR3C1 |
| Wu et al. (2007) | Cross-sectional  | 13 genes, including NR3C1 | Methylation-specific PCR, bisul fate sequencing | Ovarian cancer cell lines and ovarian tumor tissue | Ovarian cancer | Cell lines: 4 ovarian carcinoma cell lines, ES-2, OV-90, OVCAR-3, and SKOV-3 (American Type Culture Collection, Manassas, USA) | NR3C1 promoter was unmethylated in ovarian carcinoma tissues and in all cell lines |
| Lind et al.      | Cross-sectional  | Multiple genes, including NR3C1 | Methylation-specific PCR, bisul fate sequencing | Cell lines, CRC tissue, and normal colon mucosa tissue | Colon cancer | Cell lines: 20 colon cancer cell lines were included: 9 from microsatellite instable (MSI) tumors (Co115, HCT15, HCT116, LoVo, LS174T, RKO, SW48, TC7, and TC71); 11 from microsatellite stable (MSS) tumors (ALA, Colo320, EB, FRL, HT29, IS1, IS2, IS3, LS1034, SW480, and V9P). 10 cell lines from prostate tissue (N = 3), testicular germ cell tumors (N = 3), and ovary tissues (n = 4) were included, as well as a clinical series of prostate (N = 20) and kidney (N = 20) carcinomas from the University Hospital of Porto, and a series of testicular germ cell tumors (N = 42) from patients admitted to the Norwegian Radium Hospital, Oslo  
  Tissue sample cases: DNA from patients in Norwegian hospitals: 53 colorectal carcinomas (25 MSS and 28 MSI) from 52 patients (mean age 68 years), 63 adenomas (61 MSS and 2 MSI) from 52 patients (mean age 67 years)  
  Tissue sample controls: Normal mucosa samples from 22 colorectal cancer patients (mean age 64 years) taken from distant sites from the primary carcinoma, and 22 normal colorectal mucosa samples from cancer-free individuals (mean age 54 years, including 8 individuals ≥ 60 years) | Cell line results:  
  • The promoters of ADAMTS1, CRABP1, and NR3C1 were hypermethylated in 17/20 (85%), 18/20 (90%), and 7/20 (35%) colon cancer cell lines, respectively.  
  • Promoter hypermethylation of NR3C1 was also significantly associated with reduced gene expression in cancer cell lines.  
  • NR3C1 was unmethylated in subsets of cancer cell lines from prostate, testis, and kidney tissues (N = 82).  
  Tissue results:  
  • The primary carcinomas represented in the nine pairs of matched normal and cancer tissue were methylated in 4/7 (57%) for NR3C1, whereas the corresponding normal mucosa samples were unmethylated  
  • The methylation frequency of NR3C1 was also significantly higher in MSI carcinomas (12/28; 43%) than in MSS carcinomas (1/23; 4%)  
  • NR3C1 methylation was higher among tumors from females (11/27; 41%) than in those from males (2/24; 8%), and 77% (10/13) of NR3C1 methylated tumors were located in the right side of the colon |

NR3C1 was also significantly associated with reduced gene expression in cancer cell lines.
thus disrupting the anti-proliferative capabilities of the GR and contributing to breast cancer tumorigenesis.

Nesset et al. also note that the levels of methylation that they found in NR3C1 (15%) were comparable to promoter methylation levels reported for another known breast cancer tumor suppressor gene, BRCA1 (9–41%) (Birgisdottir et al., 2006; Parrella et al., 2004; Li et al., 2006; Baylin et al., 2001), indicating that future investigation might be warranted into whether NR3C1 itself may act as a tumor suppressor gene for breast cancer. The majority of methylated breast cancer tumors in the sample from Nesset et al. were also ER+/PR+/Her2-, and thus future research will be needed to investigate the role that these subtypes might play in the interaction between breast cancer tumorigenesis and NR3C1 methylation. As a final note, since only 15% of breast cancer tumors showed hypermethylation of NR3C1, while decreased NR3C1 expression was observed in almost all breast cancer tumors, future research should also aim to further identify other mechanisms through which NR3C1 and GR expression are downregulated in breast cancer tumors.

### 3.2.2. Small Cell Lung Cancer

Kay et al. (2011) conducted an analysis of 14 SCLC cell lines in comparison with a variety of other cancer control cell lines, including non-

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<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Hiraki et al.</td>
<td>Case-control</td>
<td>NR3C1</td>
<td>Quantitative methylation-specific PCR (MethyLight)</td>
<td>Gastric cancer cell lines and normal gastric mucosa tissue</td>
<td>Gastric cancer</td>
<td>Cell lines: 8 gastric cancer cell lines, HSC45, HSC57, KATO III, MKN1, MKN7, MKN28, MKN45, and MKN74</td>
<td>Cell lines: 8 gastric cancer cell lines, HSC45, HSC57, KATO III, MKN1, MKN7, MKN28, MKN45, and MKN74 showed no significant difference in methylation levels between the two</td>
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<tr>
<td>Sanchez-Vega and Gandhi (2009)</td>
<td>Cross-sectional</td>
<td>NR3C1</td>
<td>5-aza-deoxycytidine (decitabine) treatment</td>
<td>Myeloma cell lines</td>
<td>Multiple Myeloma</td>
<td>Myeloma cell line (MM.1) taken from peripheral blood cells of a US patient in the leukemia phase of multiple myeloma (MM) being treated with glucocorticoids (GCs), 1 GC-sensitive cell line, MM.15, and two GC-resistant cell lines, MM.1RE (early phenotype) and MM.1RL (late phenotype)</td>
<td>Six genes (BNIP3, CHFR, CYP1B1, MINT25, RASSF2, and SFRP2) showed significantly higher methylation in primary cancer tissues than the adjacent normal gastric mucosa, but methylation of NR3C1 showed no significant difference in methylation levels between the two</td>
</tr>
<tr>
<td>Ronco at. (2010)</td>
<td>Case-control</td>
<td>HSD11β2</td>
<td>Bisulfate sequencing</td>
<td>Choriocarcinoma cell lines</td>
<td>Choriocarcinoma</td>
<td>Cases: human choriocarcinoma cells, JEG-3 (HTB-36), from The American Type Culture Collection (ATCC, Manassas, VA) exposed to a low dose of cadmium (Cd2+)</td>
<td>Treating MM.1 cell lines with the demethylating agent 5-aza-deoxycytidine (decitabine) did not change the levels of expression of full length NR3C1 mRNA, thus ruling out the possibility of promoter methylation as a mechanism for decline in NR3C1 mRNA expression in MM.1RL</td>
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small cell lung cancer (NSCLC) cells and peripheral mononuclear blood cells taken from healthy donors. They found that there was a significant difference in methylation levels between SCLC cases and controls at several CpG sites in the 1C promoter, as well as a significant difference in methylation levels across the whole 1C promoter region. They also observed a significant association between the number of methylated CpGs and GR expression within the panel of 14 SCLC cell lines. As the only cell line study in our review to employ prospective methods, Kay et al. were able to provide causal support of this association by demonstrating that after treatment with a demethylating agent (decitabine), NR3C1 mRNA and GR protein levels increased dramatically in SCLC DMS79 cells, but not in controls or NSCLC cells. The authors therefore suggest that DNA methylation plays a role in regulating NR3C1 expression in human SCLC cells, but not NSCLC cells, and predict that this increase in expression would restore glucocorticoid sensitivity to these cells. Decitabine has been actively employed, along with its sister drug azacitidine, in the treatment of other cancers that rapidly divide such as acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) (Tseng et al., 2015; Falini et al., 2015). As such, further studies are needed to confirm the benefits of decitabine to regulate NR3C1 expression in SCLC cancers in vivo.

Importantly, Kay et al. also showed that this increase in NR3C1 and GR expression after decitabine treatment led to several further changes in the SCLC cell lines, including increased cortisol sensitivity, and increases in cleaved caspase-3 activity and decreases in BCL-2 gene expression, both of which indicate cell apoptosis. Indeed, after 5 days of decitabine treatment, the SCLC cell lines underwent cell death. Combined, these data suggest that demethylation of NR3C1 leads to increased gene and protein expression, which in turn promote SCLC cell death in a similar manner to that for conventional SCLC tumor suppressor genes. Although demethylating agents are currently an underutilized spectrum of drugs, methylation of those genes more promising biomarker candidates for early, non-invasive detection of colorectal cancer tumors. In another study included in our review, NR3C1 promoter methylation was similarly observed in colon cancer cell lines and colon cancer tissues (while also shown to be unmethylated in non-cancerous tissue from the same individuals), and this methylation was significantly associated with reduced gene expression (Lind et al., 2006). However, the authors were unable to determine if the observed methylation was specific to colon cancer, and two other genes, ADAMTS1 and CRABP, showed much higher methylation frequencies. Therefore, future research is needed to confirm the extent to which NR3C1 methylation is involved in colon cancer tumorigenesis.

NR3C1 methylation does not seem likely to serve as an early detection biomarker for ovarian (Wu et al., 2007) or gastric cancers (Kang et al., 2008; Hiraki et al., 2011) because of non-significant methylation patterns found, or because other genes have been identified that show much more significant patterns of DNA methylation. Interestingly, however, Sanchez-Vega and Gandhi (2009) showed that histone H3 was trimethylated on Lys4 (3 mKd) in exons 1C, 2, and 3, and throughout intron B of NR3C1 RNA in glucocorticoid-sensitive myeloma cells, but not glucocorticoid resistant cells. Thus, the authors concluded that while NR3C1 promoter methylation is likely not implicated in the development of glucocorticoid resistance in myeloma cells, histone modification—another epigenetic mechanism—likely plays a role, and may influence glucocorticoid resistance.

3.3. Renal, Metabolic, and Inflammatory Diseases

Table 4 summarizes the 3 studies included in our review that investigated FKBP5 and NR3C1 methylation in relation to renal, metabolic, and inflammatory diseases.

3.3.1. Chronic Kidney Disease

In an epigenome-wide methylation analysis of 407 white participants from the UK (Smyth et al., 2014), FKBP5 emerged as a strong biomarker candidate for chronic kidney disease (CKD), with significantly lower methylation observed in CKD cases versus healthy controls at three CpG sites (one in an enhancer in intron 4, and two in the 5′UTR region in the north and south shores of a CpG island). Hypomethylation of FKBP5 was not associated with a change in gene expression, although this study had an extremely small subsample for expression analyses (2 CKD cases versus 2 healthy controls). The Klengel et al. (2013) PTSD study described later in this review, however, has documented an association between decreased FKBP5 methylation and increased stress-dependent gene transcription of FKBP5.

3.3.2. Other Inflammatory Diseases

NR3C1 methylation has also been investigated in relation to several inflammatory diseases. In a study of 95 women from Belgium (Vangeel et al., 2015), mean and CpG site-specific NR3C1 1F promoter hypomethylation was seen in those with chronic fatigue syndrome (CFS; clinically diagnosed according to U.S. CDC criteria [Fukuda et al., 1994]) compared with healthy controls. In a study of 30 Chinese patients (Chen et al., 2015), there were significantly higher mean methylation levels across four NR3C1 first exon promoters (1D, 1E, 1F, and 1H) between systemic lupus erythematosus (SLE; clinically diagnosed according to American College of Rheumatology revised criteria [Hochberg, 1997]) and healthy control patients, but no results were reported for each of the individual promoters. Site-specific CpG methylation varied greatly between SLE individuals in this region, with no patterns of common CpG site methylation emerging among the entire SLE group. There was also an inverse association in the SLE group between the overall mean methylation status of the 4 promoters and GR mRNA expression, which is the active 3′ GR isoform. Caution should be taken in interpreting these associations, however, as a recent epigenome-wide methylation analysis of Japanese monzygotic twins discordant for SLE identified differential methylation and expression of 5 genes that may be important in the pathogenesis of SLE, but NR3C1 was not among these (Furukawa et al., 2013).

3.4. Mental Health Outcomes

Table 5 summarizes the 16 studies that have investigated NR3C1 and FKBP5 methylation status in relation to mental health outcomes. To date, DNA methylation of other genes along the HPA axis has not been investigated in relation to any mental health outcomes.

3.4.1. Post-traumatic Stress Disorder

One study showed a strong association between FKBP5 methylation and PTSD (Klengel et al., 2013). Specifically, those from the U.S. Grady Trauma Project Cohort (N = 1963) who experienced child abuse (measured via self-report by the CTQ [Fink et al., 1995]) with a known risk allele (rs1360780) for PTSD, but not those with the protective allele, showed greater adult PTSD symptoms (measured via self-report by the mPSS scale [Falsetti et al., 1993; Foa and Tolin, 2000]), and also greater
lifetime PTSD (clinically-diagnosed via the CAPS scale [Weathers et al., 2001]; N = 519). In subsequent analyses among a mostly African American subsample of 76 adults from the Grady Cohort, child abuse-exposed risk allele carriers showed an average decrease of 12.3% in DNA methylation in a bin of 3 CpGs arranged around glucocorticoid response elements in intron 7 (bin 2) compared to those abused without the risk allele or those not abused with or without the risk allele. This same site showed no association with adult trauma (measured by the TEI [Binder et al., 2008]), pointing to a sensitive window in childhood for such epigenetic programming. These results were further replicated in a smaller cohort of African American women (N = 57).

The authors of this study also determined that childhood abuse was associated with increased expression of 76 GR-responsive immune system genes, and that FKBP5 demethylation at intron 7 bin 2 was associated with altered GR sensitivity in peripheral blood. This latter result suggested an enhancement of the ultra-short feedback loop between the GR and FKBP5, which leads to increased GR resistance. Lastly, demethylation of FKBP5 intron 7 in a subset of the replication cohort was associated with altered GR sensitivity in peripheral blood. This result was further replicated in a smaller cohort of African American women (N = 57).

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Table 4

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<tr>
<th>Authors</th>
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<tr>
<td>Smyth et al. (2014)</td>
<td>Case-control</td>
<td>FKBP5</td>
<td>Illumina methylation assay (Infinium 450 K BeadChip)</td>
<td>Peripheral blood (leukocytes)</td>
<td>None/chronic kidney disease</td>
<td>N = 407 individuals of white ancestry from the United Kingdom</td>
<td>• Based on epigenome-wide methylation analyses, FKBP5 and 5 other genes (CUX1, ELOD1, INHBA-AS1, PTPRN2, and PRKAC2) were strong candidates for kidney disease biomarkers</td>
</tr>
<tr>
<td>Vangeel et al. (2015)</td>
<td>Case-control</td>
<td>NR3C1</td>
<td>Base-specific cleavage/MALDI-TOF mass spectrometry (EpiTYPER)</td>
<td>Peripheral blood (whole blood)</td>
<td>Childhood trauma/chronic fatigue syndrome</td>
<td>N = 95 women from Antwerp, Belgium</td>
<td>• Found a significantly lower mean methylation level in exon 1F and the 1F promoter in those with CFS versus controls</td>
</tr>
<tr>
<td>Chen et al. (2015)</td>
<td>Case-control</td>
<td>NR3C1</td>
<td>Methylation-specific PCR</td>
<td>Peripheral blood (PBMC)</td>
<td>None/SLE</td>
<td>N = 30 Chinese patients</td>
<td>• CGG site methylation differed between SLE cases and controls for each of the 4 first exon promoters studied, however site-specific results are only reported for the 1D promoter</td>
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• Controls: 19 healthy controls (mean age: 43.2 years)

• Controls: 152 individuals with no evidence of renal disease (median age: 36 years; 76 male)

• Three CpG sites in FKBP5 (one in an enhancer region of intron 4, and two in the 5’UTR region) were identified that showed significantly decreased methylation in cases compared to controls

• None of the six genes identified demonstrated significant changes in gene expression

• Immune diseases.

• Fatigue syndrome trauma/chronic kidney disease (CKD) cases: 255 individuals with chronic kidney disease (CKD) (median age: 36.2 years; 132 male)

• Controls: 15 newly emerging systemic lupus erythematosus (SLE) patients (mean age: 27.9 years; 13 female) recruited from a Chinese hospital. All SLE patients were steroid-naive

• Controls: 15 healthy volunteers (mean age: 27.9 years)

• Controls: 15 healthy volunteers (mean age: 43.2 years)

• Controls: 15 healthy volunteers (mean age: 44.2 years).

• Controls: 15 healthy volunteers (mean age: 36 years; 76 male)

• Controls: 15 healthy volunteers (mean age: 36 years; 76 male)

• Controls: 15 healthy volunteers (mean age: 36 years; 76 male)

• Controls: 15 healthy volunteers (mean age: 36 years; 76 male)
also associated greater volume of the right hippocampal head—a change that indicates greater stress hormone system reactivity—thus showing a connection between abuse-related methylation changes in peripheral blood and effects in the central nervous system (greater detail given in Table 5).

This paper represents a serious advancement in understanding the functional epigenetic pathways contributing to PTSD. Based on these results, the authors have put forward a model of PTSD pathogenesis in which FKBP5 intron 7 demethylation—induced by long-lasting cortisol exposure (Yang et al., 2012) as a result of childhood abuse and risk allele status—leads to a tightening of the ultra-short feedback loop between the GR and FKBP5 by local activation of the GR complex in peripheral tissue (which prolongs the stress cascade). On a systems level, these changes lead to GR resistance, alterations to stress hormone, neuronal, and other GR-responsive systems, and ultimately, higher risk for PTSD (see Fig. 7).

Although not as rich in functional analyses, the results reported on NR3C1 methylation levels and PTSD show the most uniform trend for NR3C1 methylation in relation to any mental health outcome in the literature—all studies show lower methylation despite examining different NR3C1 promoter regions (see Figs. 4–5).

One study of 46 individuals from Canada showed that those with lifetime PTSD (assessed via the CAPS scale) showed lower overall NR3C1 methylation at the 1B and 1C promoters compared with controls, with site-specific hypomethylation found in the 1B and 1C promoters (Labonte et al., 2014). One CpG site in the 1C promoter region, however, was significantly hypermethylated. Furthermore, an association was found between overall and site-specific hypomethylation in the NR3C1 1B promoter and both higher total mRNA expression levels for NR3C1 and higher relative mRNA expression levels for the 1B promoter. Cortisol levels were positively associated with methylation levels at CpG sites 11 and 13 in exon 1B. No association was found, however, between total methylation in the 1C promoter and relative NR3C1 1C mRNA expression or cortisol levels.

Another study of 16 racially diverse combat veterans being treated for PTSD with prolonged exposure (PE) therapy in New York found that methylation levels could predict treatment response (Yehuda et al., 2013). Specifically, those with higher methylation of the 1F promoter had significantly lower post-treatment PTSD symptom severity (clinician-assessed via CAPS) and a significantly greater reduction in PTSD symptom severity from pre- to post-treatment, suggesting that higher NR3C1 methylation may be a useful marker for better PTSD prognosis and may even mediate disease progression. The authors also investigated FKBP5 methylation, although unlike NR3C1, FKBP5 promoter methylation did not predict treatment outcome or symptom severity. However, higher FKBP5 promoter methylation associated cross-sectionally with measures of cortisol at pre-treatment and ACTH at post-treatment. This led the authors to conclude that FKBP5 promoter methylation may be associated with changes in HPA axis activity connected to changes in PTSD symptom expression, rather than upstream regulation of cortisol.

Furthermore, among a racially diverse sample of 122 combat veterans recruited from two hospitals in New York, those with PTSD (clinician-assessed via CAPS) showed significantly lower overall methylation of the NR3C1 1F promoter, compared with those without PTSD, with site-specific significant differences at CpGs 23 and 39 (Yehuda et al., 2015a). 1F promoter methylation was not associated with NR3C1 1F expression, except at CpG site 23, however lower 1F promoter methylation levels were associated with greater cortisol decline in response to dexamethasone treatment, and associated with poorer sleep quality, higher peritraumatic dissociation, and higher psychiatric distress. Early life trauma exposure (measured by the ETI [Bremner et al., 2000]) was not associated with 1F promoter methylation.

Lastly, in a study of 224 survivors of the Rwandan genocide (Vukojevic et al., 2014), lower methylation levels at a CpG site embedded in a transcription factor (NGFI-A) binding site of the NR3C1 1F promoter were significantly associated with greater PTSD symptom severity (assessed via the PDS [Foà et al., 1997]) related to re-experiencing traumatic events (intrusive memories) in men but not in women. This methylation was not associated with the severity of PTSD symptoms related to avoidance and hyperarousal, indicating that the methylation changes at this site were likely related to the memory aspects of PTSD. Higher methylation levels at this site were also significantly associated with a lower lifetime PTSD risk in men but not in women. Importantly, however, the authors did not find a significant difference in methylation at this CpG site between the Rwandan genocide and healthy Swiss samples, meaning that methylation at this site may be a more stable trait that preexisted genocide trauma, or reflects a trauma or stress earlier in life.

Although the literature on PTSD from this review lends the strongest support to a connection between NR3C1 methylation and mental health, the results reported are mixed on the connection between this methylation and adverse childhood experiences. For instance, Yehuda et al. (2015a) reported no association between NR3C1 1F promoter methylation and early life trauma exposure (measured by the ETI) in combat veterans with PTSD, and Vukojevic et al. (2014) found no difference in NR3C1 1F promoter methylation between those who had survived the Rwandan genocide and Swiss controls. These results indicate that great care needs to be taken in future research to identify if trauma at different points in life or slightly different forms of trauma have differential effects on NR3C1 methylation and development of PTSD.

3.4.2. Alzheimer’s Disease

One study examining medial temporal gyrus brain tissue from 15 elderly U.S. adults found that one CpG site in intron 7 and two CpGs in the promoter of FKBP5 showed significant hypomethylation in Alzheimer’s disease (AD) cases versus healthy controls (Blair et al., 2013). One CpG in intron 2, however, showed significant hypermethylation, mRNA expression analyses in a separate and larger sample (N = 59) showed that FKBP5 expression was significantly higher in AD cases versus healthy controls, and that increased FKBP5 expression was significantly associated with Braak staging—a clinical tool used to assess the degree of pathology in AD (Braak and Braak, 1991)—even when evaluating the oldest samples alone. These results show a promising initial association between altered FKBP5 methylation levels and AD. Further research in larger, more adequately powered samples is needed, however, to directly identify the genomic region of FKBP5 wherein methylation is functionally associated with the differing FKBP5 expression levels observed between AD cases and healthy controls. This same study also experimentally determined in mouse models that overexpression of FKBP5 preserves the soluble species of tau proteins that have been linked to AD (Lasagna-Reeves et al., 2012; de Calignon et al., 2010; Santarcuz et al., 2005; de Calignon et al., 2012), and so if FKBP5 methylation levels at certain sites can be linked with FKBP5 expression, then these results would point towards a biologically plausible role of FKBP5 methylation in leading to AD and expediting pathogenesis through encouraging the production and accumulation of toxic tau proteins.

Research to date on NR3C1 and AD has showed no differences in methylation between healthy controls and those with AD, Parkinson’s disease, or dementia (Moser et al., 2007). This study, however, suffered from a small sample size (5 controls vs. 27 cases).

3.4.3. Depression

Results reported on depression are mixed (see Figs. 4–5), although several different types of tissue were analyzed between studies. Among 117 participants from Korea (Na et al., 2014), those with major depressive disorder (MDD; clinically–diagnosed using the Korean version of the SCID-IV [Hahn et al., 2000]) had significantly lower blood NR3C1 methylation at CpGs 3–4 of the 1F promoter compared with healthy controls, and among those with MDD, those with higher perceived stress showed even lower methylation at CpG3 of the 1F promoter.
Conversely, in an analysis of 1668 Swedish women with a low expression variant of MAOA (MAOA-L) (Melas et al., 2013), parental loss in early in life was associated with increased saliva DNA methylation levels at CpG site 35 of the 1F promoter, and increased risk of developing depression (self-reported using the MDI [Bech et al., 2001]) compared to those with the MAOA-H genotype. A study of 759 participants from the Dutch famine birth cohort (Veenendaal et al., 2012) also found that increased blood NR3C1 methylation was associated with higher levels of anxiety and depression (self-reported using the anxiety and depression subscales from a Dutch translation of the HADS [Zigmond and Snaith, 1983]), but at the 1C promoter.

In contrast to the above studies, another study using hippocampal tissue from 12 brain tissue donors in the Netherlands found no difference in tissue methylation levels between MDD donors (confirmed post-mortem by a psychiatrist examining medical records according to DSM-IV criteria) and healthy controls across a number of regions in NR3C1 (exon 1J and 1B, and promoters 1J, 1E, 1B, and 1F) (Alt et al., 2010).

To summarize, then, depression has been associated with: (a) lower blood methylation of the exon 1F promoter (Na et al., 2014); (b) higher saliva methylation of the exon 1F promoter (Melas et al., 2013) and higher blood methylation of the 1C promoter (Veenendaal et al., 2012); and (c) no change in hippocampal methylation at exon 1J, and the 1B and 1F promoters (Alt et al., 2010). The discrepancies in findings between studies, however, may be largely driven by the different forms of stress or adversity that were analyzed, the different promoter regions analyzed, or perhaps even the different tissue types analyzed. While Na et al. (2014) demonstrated that current perceived stress (measured via the PSS [Cohen et al., 1983]) in depressive patients was associated with lower NR3C1 1F promoter methylation, Melas et al. (2013) found that early childhood trauma, measured as early parental death, parental divorce, or early childhood financial constraints (assessed via original measures in the PART questionnaire), was associated with increased methylation of the 1F promoter and depression. Veenendaal et al. (2012), on the other hand, found an association between increased NR3C1 1C promoter methylation and depression, but no association between NR3C1 1C promoter methylation and early childhood adversity when looking at gestational exposure to famine as a measure of early childhood adversity.

It is important to point out here that none of the above studies on depression measured NR3C1 expression or cortisol levels. Although Na et al. (2014) suggest that NR3C1 methylation may occur in a compensatory pattern opposite of HPA activity, the authors could not determine whether the HPA axis was hypoactive in their MDD sample. Therefore, the functional and biological mechanisms connecting HPA axis gene methylation levels and depression remain largely unknown. This may also account for the discrepancy in the results reported, as methylation of specific CpG sites in NR3C1 may simply prove to have no functional contribution towards depression. Future research on depression should aim to elucidate which regions of NR3C1 may simply prove to have no functional role in the relationship between early childhood adversity, NR3C1 methylation, and depression through at least measuring cortisol and NR3C1 expression levels.

One study examined FKBP5 methylation in relation to depression (Höhne et al., 2015). Among 96 German participants from the Early Developmental Stages of Psychopathology (EDSP) study, subjects with the FKBP5 rs1360780 T risk allele genotype and a lifetime history of major depression (MD; self-reported via the M-CIDI [Wittchen et al., 1998]) had a 10% higher DNA methylation rate in intron 7 of FKBP5 than healthy controls with the same genotype, although posthoc comparisons did not reach significance and only showed a non-significant trend. The authors note that this was likely due to a small, inadequately powered sample. The same study found altered cortisol and expression levels after a stress test in healthy adults but not in depressed cases, which suggests that methylation of another region of FKBP5 may be more functionally important in depression. Future research should investigate early life stress (and, if necessary, also measure and compare levels of current stress), use greater sample sizes, and explore different regions of FKBP5 known to be sensitive to psychosocial or early life stress.

3.4.4. Bipolar Disorder

One study examined the role of FKBP5 methylation in bipolar disorder (BD) (Fries et al., 2015). Among 68 Brazilian adults, those with BD (clinically diagnosed according to DSM-IV Axis I criteria) showed significantly higher methylation of one CpG in intron 7 of FKBP5 compared with their siblings, and significantly higher methylation of one CpG in intron 2 compared with non-related healthy controls. No association was found, however, between DNA methylation and basal FKBP5 mRNA and protein levels.

3.4.5. Borderline Personality Disorder and Eating Disorders

The results concerning NR3C1 methylation and borderline personality disorder (BPD) are also mixed. In a sample of 36 patients from Switzerland, significantly increased methylation of NR3C1 exon 1B was found at CpG1 and CpG5 in BPD patients (clinically diagnosed according to DSM IV criteria), but the low level of methylation changes observed relative to other candidate genes led the authors to conclude that NR3C1 methylation is likely not a suitable biomarker for BPD (Dammann et al., 2011). A further study of 101 Swiss subjects with BPD (clinically assessed via the SCID-II [First et al., 1994]) and 99 MDD controls (clinically assessed via the French version of the DICS [Preisig et al., 1999]) showed that those with BPD and no sexual abuse had significantly greater NR3C1 exon 1F methylation compared with those with MDD and no sexual abuse (Perroud et al., 2011a).

In another sample of 96 U.S. women between the ages of 17 and 48, women with bulimia nervosa (BN; self-reported according to DSM-IV criteria) with comorbid BPD (self-reported via the SCID-II) showed elevated, but non-significant methylation at CpG sites 10 and 21 in the promoter of NR3C1 exon 1C, compared with non-eating disordered controls (Steiger et al., 2013). BN women with comorbid suicidality, on the other hand, showed significantly elevated methylation in CpG sites 10, 22, and 29 in the 1C promoter, compared to non-eating disordered controls. BN women with comorbid BPD also displayed significantly lower methylation at the 1H promoter when compared to BN women with no BPD or non-eating disordered individuals. These results suggest that NR3C1 methylation may act as an epigenetic process regulating comorbid BPD and suicidality expression in BN, rather than being directly implicated in the pathogenesis of BPD, although they do provide indication that 1H hypomethylation, specifically, may be associated with BPD.

A recent epigenome-wide methylation study of 96 Swiss subjects with BPD did not find high DNA methylation levels of NR3C1 in relation to methylation levels of 14 other candidate genes identified (Prados et al., 2015), however future research should try to determine if NR3C1 methylation plays a more prominent role in development of BPD among those who have experienced early childhood adversity. One study not included in this review (Martin-Blanco et al., 2014) found that among 281 Spanish subjects with BPD (clinically assessed via a Spanish version of the SCID-II [Gómez-Beneyto et al., 1994]), there was a positive association between mean DNA methylation of NR3C1 exon 1F and childhood physical abuse (measured via the CTQ). Site-specific methylation at three CpGs also showed a significant association with physical abuse and emotional neglect in childhood (measured via the CTQ), and with unemployment. Greater BPD severity scores were also associated with exon 1F methylation at 4 different CpG sites. The study by Perroud et al. (2011a), included in our review, also showed that among those with BPD, increased severity of childhood sexual abuse and childhood physical neglect (measured by the CTQ) were significantly associated with increased NR3C1 1F promoter methylation, and that those with one reported experience of abuse had less methylation than those reporting two or more experiences of...
### Table 5: Associations between FKBP5/NR3C1 methylation and mental health outcomes.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Study design</th>
<th>Gene</th>
<th>Method</th>
<th>Tissue type</th>
<th>Exposure/Outcome</th>
<th>Sample</th>
<th>Results</th>
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</table>
| Klengel et al. (2013) | Case-control; in vitro cell lines | FKBP5  | Pyrosequencing                            | Peripheral blood (whole blood) | Childhood abuse/PTSD          | N = 76 mostly African American U.S. adults from the Grady trauma project | Cases: 30 individuals that experienced both physical and sexual child abuse (mean age: 41.46 years; 28 females; 27 African American, 2 white, 1 mixed ethnicity)  
Controls: 46 individuals without any childhood trauma (mean age: 40.97 years; 36 females; 45 African American, 1 other ethnicity)  
• The authors confirmed that a known a risk allele (rs1360780) for PTSD after childhood trauma affects chromatin conformation and transcription  
• Exposure to childhood trauma significantly increased the risk of lifetime PTSD in risk allele carriers, but not in carriers of the protective genotype  
• Child abuse-exposed risk allele carriers showed an average decrease of 12.3% in DNA methylation in intron 7 (bin 2) of FKBP5 compared to those abused without the risk allele or those not abused with or without the risk allele  
• In hippocampal progenitor cell lines, intron 7 bin 2 also showed significant demethylation after treatment with dexamethasone. This demethylation remained unchanged after 20 days in a culture steroid-devoid medium, which indicates that there is a stable epigenetic memory of GC-induced demethylation  
• Demethylation of intron 7 bin 2 was shown to associate with higher DEX IC₅₀ in peripheral blood from a replication cohort (N = 34), suggesting an enhancement of the ultrashort feedback loop between FKBP5 and the glucocorticoid receptor (GR) that leads to increased GR resistance  
• FKBP5 hypomethylation was significantly associated with greater volume of the right hippocampal head |
| Labonte et al. (2014)   | Case-control | NR3C1  | Base-specific cleavage/MALDI-TOF mass spectrometry (EpiTYPER) | Peripheral blood (T-lymphocytes) | None/PTSD                   | N = 46 individuals in Montréal, Canada                                 | Cases: 30 individuals meeting DSM-IV criteria for lifetime PTSD (average age: 43.4 years; 15 female)  
Controls: 16 individuals with a negative trauma history (average age: 36.9 years; 8 female), but no lifetime trauma, and no current psychiatric diagnosis  
• Individuals with lifetime PTSD showed lower morning cortisol release, and higher mRNA expression of total NR3C1 and the 1B and 1C promoters  
• Lower overall methylation levels in PTSD individuals were found in the 1B and 1C promoter regions  
• Site-specific hypomethylation was found at 1B CpG sites 2–4, 11, 13, and 14, and 1C CpG sites 40–41. CpG 51 in the 1C promoter was significantly hypermethylated in the PTSD group  
• There was a negative association between overall methylation levels in the 1B promoter and NR3C1 total and 1B relative mRNA expression levels, with site-specific associations at 1B CpG sites 2–4, 11, and 13  
• Methylation levels at 1B CpG sites 11 and 13 were positively associated with cortisol levels. There was no association between total methylation in the 1C promoter and relative NR3C1 1C mRNA expression or cortisol levels |
| Yehuda et al. (2013)    | Pro-spective | NR3C1, FKBP5 | Bisulfate sequencing (nested PCR) | Peripheral blood (PBMC) | PE/PTSD                  | N = 16 combat veterans (14 men, 2 women) being treated for PTSD in the Bronx, NY who completed prolonged exposure (PE) psychotherapy. Participants had no significant illness that would interfere with interpretation of biological data, no regular use of benzodiazepines or oral steroids, a BMI <30, smoked less than two packs per day, no substance abuse or dependence within the last 6 months, and no lifetime history of schizophrenia, schizoaffective disorder, bipolar disorder, obsessive compulsive disorder | NR3C1:  
• Responders to PE at pre-treatment had greater average number of methylated CpG sites in the 1F promoter than non-responders  
• Higher pre-treatment levels of methylation were significantly associated with both lower post-treatment PTSD symptom severity and a greater reduction in symptom severity from pre- to post-treatment  
• Higher post-treatment methylation also predicted lower self-reported, but not clinician-rated, PTSD symptoms later at follow-up.  
• Pre-treatment methylation was positively associated with post-treatment 24 h–urinary cortisol levels |
• FKBP5 methylation showed a decrease in PE treatment responders at follow-up, but an increase in non-responders
• Higher FKBP5 methylation at pre-treatment was associated with lower cortisol levels at pre-treatment
• Post-treatment FKBP5 methylation was also associated with lower ACTH levels
• Lower FKBP5 methylation at follow-up was associated with greater clinical-rated PTSD symptom severity at post-treatment, and both higher urinary and plasma cortisol levels at post-treatment

Yehuda et al. (2015a)

- Case-control
- NR3C1
- Bisulfate sequencing (nested PCR)
- Peripheral blood (PBMC)
- DEX treatment/PTSD
- N = 122 male combat veterans recruited from two hospitals in New York City
  - Cases: 61 veterans with PTSD (mean age: 34.2 years)
  - Controls: 61 veterans without PTSD (mean age: 33 years)

- Vukojevic et al. (2014)

- Case-control
- NR3C1
- Pyrosequencing
- Saliva DNA
- Rwandan genocide/PTSD
- N = 224 adults from Rwanda and Switzerland
  - Cases: 152 survivors from the 1994 Rwandan genocide (69 females, 83 males; median age: 35 years) who lived as refugees in Uganda (93 with lifetime PTSD, and 59 individuals without PTSD)
  - Controls: 72 healthy young Swiss subjects (47 females, 25 males; median age: 23 years)

- Blair et al. (2013)

- Case-control
- FKBP5
- Pyrosequencing
- Brain tissue (medial temporal gyrus)
- Alzheimer’s disease
  - Cases: 10 elderly adults with Alzheimer’s disease (AD; mean age: 85.6 years; 5 males)
  - Controls: 5 elderly adults without AD (mean age: 92.2 years; 4 males)

- FKBP5:
  • Significantly lower methylation rates in the 1F promoter were observed across the 39 CpG sites in the PTSD individuals compared with controls, even after controlling for co-variates
  • Site-specific hypomethylation was also observed at CpG sites 23 and 39 for PTSD individuals
  • There was no overall significant association between 1F promoter methylation and expression, however methylation at CpG site 23 was negatively associated with NR3C1 1F expression
  • Lower 1F promoter methylation levels were associated with greater cortisol decline in response to Dexamethasone (DEX) treatment, and associated with poorer sleep quality, higher peritraumatic dissociation, and higher psychiatric distress
  • Early life trauma exposure was not associated with 1F promoter methylation

- Methylation levels at the CpG 3 site (embedded in an NGFI-A binding site) in the 1F promoter were negatively associated with the severity of PTSD symptoms related to re-experiencing traumatic events in men but not in women
- Higher methylation levels at CpG 3 were also significantly associated with a lower lifetime PTSD risk in men but not in women
- No significant difference in CpG 3 methylation between the Rwandan and Swiss samples
- In a subset of 24 healthy (12 females and 12 males) subjects, methylation at the CpG 3 site associated significantly with both exon 1F and total NR3C1 expression

- AD cases showed significant hypomethylation at 3 CpG sites (one in intron 7 and two in the promoter) and hyper-methylation at 1 CpG site (in intron 2).
- Among another set of brain samples diagnosed with AD (N = 26; mean age: 85.7 years) and age-matched control samples (N = 33; mean age: 84.2 years), FKBP5 expression was significantly higher in AD samples compared with controls.
- Expression was much higher in adults with AD aged 70–80 years compared to adults > 80 years.
- Expression for older (> 80 years) AD and non-demented adults were the same.
- Increased FKBP5 expression levels were significantly associated with increased Braak staging.

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<tr>
<th>Authors</th>
<th>Study design</th>
<th>Gene</th>
<th>Method</th>
<th>Tissue type</th>
<th>Exposure/Outcome</th>
<th>Sample</th>
<th>Results</th>
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<tbody>
<tr>
<td>Moser et al. (2007)</td>
<td>Case-control</td>
<td>NRC3I</td>
<td>Methylation-specific PCR</td>
<td>Post-mortem hippocampal tissue</td>
<td>None/Parkinson’s disease, presenile and senile dementia-Alzheimer’s type, dementia</td>
<td>( N = 32 ) subjects from Germany</td>
<td>- CpG sites 2–8 (including 2 in an NGFI-A binding site) were unmethylated in humans</td>
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<td>Cases: 27 subjects (12 males/20 females, mean age 80-1 years/82-6 years) with various diagnoses: Parkinson’s disease (nine), presenile dementia-Alzheimer’s type (two), senile dementia-Alzheimer’s type (SDAT; eight), dementia (eight) Controls: 5 subjects without any diagnoses</td>
<td>Two CpG sites were methylated: CpG1 was unmethylated</td>
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<tr>
<td>Na et al. (2014)</td>
<td>Case-control</td>
<td>NR3C1</td>
<td>Pyrosequencing</td>
<td>Peripheral blood (doesn’t specify)</td>
<td>MDD, structural brain alterations</td>
<td>( N = 117 ) participants from Soeul, Korea with no history of comorbid axis I or II disorders, no psychiatric features, no history of taking antidepressants or history of primary neurologic diseases, and no organic brain lesions in patients with MDD compared to healthy controls</td>
<td>- Patients with MDD had significantly lower methylation at CpGs 3–4 in the 1F promoter compared to controls</td>
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<td>Melas et al. (2013)</td>
<td>Nested case-control</td>
<td>NR3C1</td>
<td>Base-specific cleavage/MALDI-TOF mass spectrometry (EpiTYPER)</td>
<td>Saliva DNA</td>
<td>Early parental death/depression</td>
<td>( N = 1668 ) participants from the PART study in Stockholm County, Sweden (male median age: 56; female median age: 58)</td>
<td>- MAOA-L females with childhood adversities had a higher risk of developing depression compared to those with the MAOA-H genotype</td>
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<td>Cases: 392 individuals diagnosed with major depression, mixed anxiety depression, or dysthymia (285 females and 107 males) Controls: 1276 individuals with no symptom of depression, anxiety (708 females and 568 males)</td>
<td>Controls: 72 healthy individuals (51 male)</td>
<td>- Females who lost a parent early in life had increased DNA methylation levels at CGC site 35 of the NR3C1 1F promoter</td>
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<td>Controls: 440 participants who hadn’t been exposed to famine</td>
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<td>- MAOA polymorphisms mediated NR3C1 methylation: early parental death was associated with increased NR3C1 methylation levels only in MAOA-L individuals</td>
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<td>Veenendaal et al. (2012)</td>
<td>Case-control</td>
<td>Four genes, including NR3C1</td>
<td>Methylation-specific PCR (leukocytes)</td>
<td>Peripheral blood</td>
<td>Prenatal exposure to famine/depression, diabetes, cardiovascular disease</td>
<td>( N = 759 ) participants from the Dutch famine birth cohort (mean age: 58 years; 349 [46%] men)</td>
<td>- Exposure to famine during gestation was not associated with NR3C1 1C promoter methylation</td>
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<td>Cases: 319 participants who had been exposed to famine in utero</td>
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<td>- Increased 1C promoter methylation status was associated with higher levels of anxiety and depression, lower self-perceived health, lower body mass index (BMI), higher levels of physical activity, and non-smoking</td>
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<td>Controls: 440 participants who hadn’t been exposed to famine</td>
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<td>- 1C promoter methylation was not associated with glucose or insulin levels, nor with a range of factors associated with coronary heart disease: HDL, LDL, cholesterol, triglycerides, blood pressure, and intima-media thickness</td>
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<td>- Low methylation levels in 3 promoter regions were found in both MDD and the control group, with the exception of promoter 18</td>
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<td>- No significant difference in methylation patterns was found between MDD and control groups</td>
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<td>Alt et al. (2010)</td>
<td>Case-control</td>
<td>NR3C1</td>
<td>Pyrosequencing</td>
<td>Hippocampus</td>
<td>None/MDD</td>
<td>( N = 12 ) brain tissue donors from Amsterdam, Netherlands</td>
<td>- Subjects with the FKBP5 rs1360780 T risk allele genotype and a lifetime history of MD had a 10% higher DNA methylation rate in intron 7 than healthy controls with the same FKBP5 genotype, although posthoc comparisons did not reach significance and only showed a non-significant trend</td>
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<td>Controls: 6 brain samples from donors without diagnosed central nervous system (CNS) disease or long-term psychotropic medication (median age: 72.5 years; 3 females)</td>
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</table>
Power analyses revealed that the sample was not adequately powered for the methylation analyses.

After an experimental psychosocial stress test, there were no genotype differences in cortisol response or mRNA expression in the remitted depression cases.

Compared with siblings, BD patients showed significantly increased methylation of one CpG in intron 7 of FKBP5, and increased FKBP5 protein levels.

Compared to healthy controls, BD patients showed significantly increased methylation of one CpG in intron 2 compared, with a trend towards significance of increased FKBP5 protein levels.

A significant association was also found between FKBP5 protein and post-dexamethasone cortisol levels, and patients with BD showed higher post-dexamethasone cortisol levels.

No association was found between DNA methylation and basal FKBP5 mRNA and protein levels.

Increased methylation of NR3C1 was found at 8 CpG sites (CPGs 1–8). Significantly increased methylation of NR3C1 was found at CpG1 and CpG5 in BPD patients.

Childhood physical neglect and increased severity of childhood sexual abuse were significantly associated with increased exon 1F methylation in BPD cases.

There was a significant and positive dose-response association between the number of types of childhood abuse and neglect and methylation status.

In the whole sample (BPD and MDD groups), sexually abused, emotionally abused, emotionally neglected, physically abused, and physically neglected subjects had higher methylation status.

MDD subjects without sexual abuse showed significantly lower methylation than BPD subjects.

With the exception of CpG 1, all CpGs showed a association with the number of types of abuse and neglect at a level of significance of \( P < 10^{-6} \).

BN women with comorbid PTSD showed elevated (non-significant, \( p = 0.07 \)) methylation in CpG positions 10 and 21 in the 1C promoter compared with NED controls, and significantly lower methylation in the 1H promoter when compared to NED or BN without BPD groups.

BN women with comorbid suicidality showed significantly elevated methylation in CpG positions 10, 22, and 29 in the 1C promoter, compared to those obtained in NED controls.
4. Discussion

This is the first systematic review to examine DNA methylation status for multiple genes along the HPA axis that are involved in glucocorticoid regulation. While other reviews have examined the association between early life stressors and HPA axis gene methylation, this review critically maps the terrain of clinical relevance for emerging epigenetics research concerning HPA axis genes. In this critical review, we synthesize current, clinically important epigenetics research in order to highlight the potential utility of using DNA methylation of HPA axis genes as a potential biomarker useful to advance early detection methods for psychiatric illness. In the remainder of this review, we highlight key challenges and future research directions that will need to be addressed in order to develop both clinically meaningful prognostic biomarkers and an evidence base that can inform public policy practice.

4.1. Poor Socio-demographic Characterization of Study Populations

One of our deepest concerns about current epigenetics study design, at least within the studies included in this review, is a general lack of detailed reporting regarding the socio-demographic characteristics of study populations. This is an important point, since epigenetic modifications of HPA axis genes are highly dependent on specific socioeconomic, social, and psychosocial factors (Needham et al., 2015; Yehuda et al., 2015b). Of the 30 articles reviewed here that used blood or tissue samples for their methylation analyses (excluding 2 in vitro only studies), 23 did not report any detailed information about the race/ethnicity of their sample populations. Furthermore, only half of the studies conducted within the U.S. reported information on race/ethnicity. Given the findings reported in this review on hypertension and cancer, however, such information is clinically relevant. African Americans bear a disproportionate burden of hypertension (Yoon et al., 2015), and African American women have a prevalence of hypertension 2–3 times greater than white women (Gillum, 1996; Nwankwo et al., 2013). There is also a significant difference in cancer mortality between African American and white populations in the U.S. (Siegel et al., 2015; NCI, 2008). Future researchers should therefore endeavor to design studies with samples from minority and disadvantaged populations in order to better understand whether the more severe clinical disease risk that these populations experience is a result of variable epigenetic regulation in the face of unique environmental and psychosocial stressors. Similar efforts should be undertaken to better characterize the socioeconomic characteristics of study samples.

4.2. Lack of Consistency in Gene Regions Studied and CpG Nomenclature

To date, the full potential of existing epigenetics research on HPA axis genes has been limited by several factors that need to be addressed in order to realize the full clinical and public health benefit of this work. These include: (1) investigating different and non-overlapping gene regions between studies on the same disease, without also replicating results of prior studies on previously measured CpG sites, creating a
patchwork of somewhat unrelated results; (2) inconsistent CpG numbering and nomenclature between studies, making it difficult to easily compare data across studies; and (3) failure to select gene regions to study based on theoretical grounds pertinent to the disease phenotype studied (as opposed to merely studying the most cited, but perhaps less relevant region).

Regarding our first concern, the exact gene regions examined for DNA methylation analyses varied significantly between the studies on NR3C1 reviewed here. Some looked at the entire promoter of a gene, while others focused on only 5 CpG sites with a single promoter region, making comparison between findings difficult. Furthermore, some studies examining the same outcome or disease didn’t look at overlapping promoter regions within the same genes at all (Figs. 4–5), making comparisons and identifying trends a tenuous exercise, at best. There is, however, consistency within studies investigating methylation of FKBP5, as nearly all studies included in our review investigated the same regions outlined in Klengel et al. (2013) (Table 1).

Second, the inconsistency between studies on NR3C1 methylation regarding how CpG sites are identified and labeled makes understanding overlap in CpG sites investigated and synthesizing findings between studies difficult. Future studies would therefore benefit from explicit selection of gene promoter regions or CpG sites that have been studied previously, and from a discussion of how these regions overlap with previous research in order to more easily amass a comprehensive evidence base. If researchers wish to also investigate novel sites, then simultaneously investigating regions that overlap with previous research would give much more contextual significance to any findings observed in these new sites. We believe the field as a whole could benefit from adopting the CpG numbering for NR3C1 found in Palma-Gudiel et al. (2015), as these numberings reflect position within the entire NR3C1 proximal promoter, and have been assigned according to their review of existing research on epigenetic modifications of NR3C1.

Our third concern stems from the tissue-specificity observed between expression of the various NR3C1 first exons (Fig. 6). Future studies looking at NR3C1 methylation should be sure to investigate the most pertinent region of the NR3C1 promoter for the disease in question, instead of merely looking at mean methylation across the entire promoter or simply looking at exon 1F or its promoter because it is the most widely cited in the literature.

4.3. Measuring the Functional Effects of DNA Methylation

It is crucial that all future studies measure NR3C1 expression and glucocorticoid hormone levels, at least, in order to determine which CpG sites are functionally sensitive in relation to a given pathology. Further functional studies of more pathology-specific markers similar to those done in the studies included in this review on hypertension (Friso et al., 2008), small cell lung cancer (Kay et al., 2011), Alzheimer’s (Blair et al., 2013), and PTSD (Klengel et al., 2013) will be especially useful. This will also be important for understanding the contradictory results found in studies on depression and other mental health outcomes. Furthermore, given that one of the prominent mechanisms through which DNA methylation silences gene expression is inhibition of transcription factor binding, future research on any of the genes reviewed here should also be certain to include specific CpG loci known to be located in transcription factor binding sites throughout the NR3C1 proximal promoter (Turner et al., 2010), the 4 CpG islands found in HSD11β2 (Aliakhani-Koopeai et al., 2004), or in distal intronic glucocorticoid response elements (GREs) in FKBP5 through which the GR complex regulates FKBP5 expression (Paakinaho et al., 2010).

4.4. Accounting for Genotype-specific Associations and Cell Type Heterogeneity in Blood Samples

Several studies included in our review showed that adverse childhood experiences only associated with depression (Melas et al., 2013; Höhne et al., 2015) or PTSD (Klengel et al., 2013) among those with a known risk genotype. Furthermore, DNA methylation of FKBP5 and NR3C1 in these studies was only associated with these exposures and outcomes among those with the risk genotype. It will be crucial for future studies to interpret the methylation results of any studies that have not investigated genotype-specific associations for exposures or outcomes where there is a known risk genotype. In particular, the rs1360780 risk genotype in FKBP5 plays a clear role in modulating stress reactivity (Zannas et al., 2016; Klengel et al., 2013), and further research should endeavor to more precisely elucidate the complex interplay between environmental stress, genotype, and epigenetic regulation of FKBP5.

Cell type heterogeneity may also be an important source of confounding in DNA methylation studies using blood samples (Bock, 2012; Adalsteinsson et al., 2012; Lam et al., 2012), and statistical adjustment for cell composition in blood samples is increasingly becoming a standard methodological characteristic for high-quality studies on DNA methylation of NR3C1, in particular, in relation to environmental exposures (Madrigano et al., 2012; Burris et al., 2013; Kim et al., 2016). Only 3 studies included in our review, however, made statistical adjustments to account for cell composition in their analyses (Smyth et al., 2014; Yehuda et al., 2015a; Höhne et al., 2015). This will be an increasingly crucial methodology to include in future research, although it will require consideration early in the process of planning candidate gene research, since complete blood counts (CBCs) can only be done when blood samples are first drawn. For epigenome-wide and high-throughput studies, however, statistical methods have been developed to determine cell composition in blood samples without the need for a CBC or a reference dataset (Houseman et al., 2014; Houseman et al., 2016; Shannon et al., 2017).

4.5. More Comprehensively Understanding the Epigenetic Plasticity of HPA Axis Genes

Although recent research included in our review has shown that early childhood is a sensitive period in life during which stable epigenetic regulation of FKBP5 can occur and persist into adulthood independent of adulthood stressors (Klengel et al., 2013), the epigenetic plasticity exhibited by NR3C1 needs to be further studied to better understand the contribution of various temporal stressors and influences to the etiology of disease across the life course. Attenuating influences in later life may reverse prior NR3C1 methylation, which in part may explain the divergences in findings for NR3C1 1F promoter methylation and depression among those experiencing current stress (Na et al., 2014) versus early life stress (Melas et al., 2013). This very plasticity, which on the one hand creates methodological challenges, is in fact what makes epigenetics such a promising tool to inform developments in clinical and public health practice.

4.6. Building Consensus Regarding Optimal Social/psychosocial Measures to be Used in Studies

The link between adverse childhood experiences (ACE) and NR3C1 or FKBP5 methylation has been well established in numerous systematic reviews and recent studies (Palma-Gudiel et al., 2015; Daskalakis and Yehuda, 2014; Needham et al., 2015; Yehuda et al., 2015b; Turecki and Meaney, 2014; Tyrka et al., 2015). Indeed, our review demonstrates that there now exists evidence for an association between such NR3C1 or FKBP5 methylation resulting from early childhood stress and the development of mental health disorders later in life, although the temporality of stressors, as well as the number and type of adversities experienced, seem to have a strong influence on the functional role that NR3C1 or FKBP5 methylation play in the emergence of specific mental health disorders later in life.

Insights gained from epigenetics research on HPA axis genes involved in glucocorticoid regulation could be significantly advanced.
through building a consensus in the scientific community on which measures, instruments, or scales of early childhood trauma or adversity, adult psychosocial stress, and environmental and social stressors optimally capture critical exposures likely to affect human health across the lifespan. Our review revealed that NR3C1 methylation can significantly vary by type and number of abuses experienced in childhood, for example, but extant studies used varying measures of childhood trauma or adversity, making comparisons of results across studies difficult. Future studies are needed that might replicate the results reported here using the same combination of outcomes, NR3C1 promoter region(s), and exact measures of childhood trauma or adversity in new populations to test the strength of established associations.

5. Conclusion

We are hopeful that the findings reported in this review will reveal new insights useful to molecular biologists, clinicians, and researchers alike who might consider DNA methylation status as a potential disease- or gene-specific biomarker to advance our understanding of disease etiology and perhaps even treatment. Strong functional relationships, in particular, need to be examined using more robust study designs in order to prevent reporting associations where none actually exist. In order for this to be accomplished, the results reported in this review will require further prospective exploration across diverse populations to form a conclusive evidence base regarding the directionality and magnitude of clinical associations reported to date. Despite these challenges, the results of this review indicate that investigating DNA methylation of HPA axis genes involved in glucocorticoid regulation is an important and growing field for better understanding the etiology—and ultimately, how to detect and treat—of a number of key diseases affecting population health, including hypertension, breast cancer, small cell lung cancer, chronic kidney disease, Alzheimer’s, PTSD, depression, and borderline personality disorder.

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Conflicts of Interest

The authors report no conflicts of interest.

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