A candidate gene analysis of response to citalopram and escitalopram treatment in patients with Major Depressive Disorder and Generalized Anxiety Disorder

Laura Gedge

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Abstract

Objective: To determine whether genotype at the catechol-O-methyltransferase rs4680, dopamine D2 receptor rs1800497, serotonin receptor 1A rs6295 or serotonin transporter 5-HTTLPR single nucleotide polymorphisms is associated with response to citalopram and escitalopram treatment in patients with major depressive disorder and generalized anxiety disorder.

Methods: Twenty one patients with depression or anxiety who were treated with citalopram or escitalopram for greater than one year, and who stopped the medication for a period of time during which their symptoms returned, and upon re-commencing the medication their symptoms were again reduced, were classified as responders. Patients were assessed using the Sheehan Disability Scale and the Quick Inventory of Depressive Symptomology- self report. The control group consisted of 146 healthy participants. Genotype was determined at each of the candidate genes studied: catechol-O-methyltransferase, dopamine D2 receptor, serotonin receptor 1A and serotonin transporter. Chi squared tests were used to compare genotypic and allele frequencies between responders and controls.

Results: There was no significant difference in genotypic or allele frequencies between responders and controls at each of the genes analyzed.

Conclusions: This pilot study suggests that genotype at the catechol-O-methyltransferase, dopamine D2 receptor, serotonin receptor 1A and serotonin
transporter genes is not associated with response to citalopram and escitalopram treatment in patients with depression and anxiety. A larger sample size, along with a genome-wide scan are needed to identify genetic variants that predict medication response in future patients.
Co-Authorship

Dr. Roumen Milev and Dr. Ruzica Jokic of the Department of Psychiatry, Queen’s University contributed to this study. Dr. Milev and Dr. Jokic contributed to study design, and Dr. Milev also assisted with analysis and preparation of this document.
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Chapter 1: General Introduction

The initial course of antidepressant treatment is ineffective for over half of patients with depression and for 25% of patients with anxiety, resulting in a need to try other medications in an effort to find one that is effective (Thase et al., 2005). This extends suffering, continues functional disability, and increases both the risk of relapse and the risk that patients will abandon treatment. Research suggests that this considerable variation in individual treatment outcome might have a genetic basis.

Previous research has identified genes that affect mood, and as individual genomes differ, so do their depressions. Because the underlying causes of depression vary, the same drug that relieves symptoms for one person may have little or no effect on another. Candidate gene analysis studies have examined genes that may be involved in the pathophysiology of depression and in the mechanism of action of antidepressants, and genetic variations have been associated with treatment outcome.

It has been suggested that the catechol-O-methyltransferase (COMT) polymorphism - a substitution of methionine (met$^{158}$) for valine (val$^{158}$); the dopamine D2 receptor G to A substitution polymorphism; the serotonin 1A receptor G/C polymorphism and the serotonin transporter insertion/deletion polymorphism are associated with the development of depression and anxiety. Genotype at these polymorphisms has also been associated with antidepressant treatment outcome, including selective serotonin reuptake inhibitors, however results remain inconsistent and
contested. We believe that previous studies have examined a heterogeneous responder group that includes placebo responders, which may explain the lack of consistent significant findings. In this study, we use a strict criterion for classification of medication response in order to produce a more homogenous responder group, which may result in the finding of significant genetic similarities. Identifying a genotype that predicts treatment effectiveness would reduce the likelihood that patients with depression and anxiety will experience unsuccessful treatments in the future.
Chapter 2: Literature Review

2.1 Depression

2.1.1 Diagnosis

According to the diagnostic and statistical manual of mental disorders (DSM IV-TR), major depressive episodes consist of five or more of the following symptoms, occurring nearly every day for at least a two week period. At least one of the symptoms must be either (1) depressed mood or (2) loss of interest or pleasure, and these symptoms must cause clinically significant distress or functional impairment. The symptoms are (1) depressed mood most of the day, (2) markedly diminished interest or pleasure in all or almost all activity, (3) significant weight loss or decrease or increase in appetite, (4) insomnia or hypersomnia, (5) psychomotor agitation or retardation, (6) fatigue or loss of energy, (7) feelings of worthlessness, or excessive or inappropriate guilt, (8) diminished ability to think or concentrate or indecisiveness and (9) recurrent thoughts of death, suicidal ideation, or suicide attempt, or specific plan for committing suicide (American Psychiatric Association, 1994).

2.1.2 Epidemiology

According to a recent national survey conducted in the United States, the lifetime prevalence of MDD is 16.2% and the 12-month prevalence is 6.6% (Kessler et al., 2003; Kessler et al., 2005). The risk for onset of MDD begins to increase in the early teens and increases in an approximately linear fashion thereafter (Kessler et al., 2003). Women have a higher lifetime prevalence of MDD than men, as do respondents in the “other”
category of employment (including unemployed and disabled), those who were previouly married, and those living near poverty. Homemakers, those with an employment status of “other”, the never married, those with less than 12 months of education, and those living near poverty have a higher 12-month prevalence for MDD. The prevalence of lifetime MDD was lower among people who were retired and non-hispanic blacks (Kessler et al., 2003).

In 2004, unipolar depressive disorder was the third leading cause of disability worldwide, resulting in $65.5 \times 10^6$ disability-adjusted life years (DALYs) or 4.3% of total DALYs (burden of disease). In the Americas, unipolar depressive disorders are the first leading cause of disability, accounting for 7.5% of total DALYs (Ustun et al., 2004). The World Health Organization (WHO) predicts that by 2020, depression will become the second leading cause of DALYs lost worldwide, after ischemic heart disease.

### 2.2 Generalized anxiety disorder

#### 2.2.1 Diagnosis

The DSM IV-TR defines generalized anxiety disorder (GAD) as excessive anxiety about a number of events or activities, occurring more days than not, for at least 6 months. An individual with GAD finds it difficult to control the worry, and the anxiety and worry must be associated with at least 3 of the following symptoms: (1) restlessness or feeling keyed up or on edge, (2) being easily fatigued, (3) difficulty concentrating or mind going blank, (4) irritability, (5) muscle tension or (6) sleep disturbance. The anxiety,
worry, or physical symptoms must cause clinically significant distress or impairment in social or occupational functioning. To be diagnosed with GAD, the anxiety and worry must not be confined to features of an Axis I disorder, such as being embarrassed in public (as in social phobia), being contaminated (as in obsessive-compulsive disorder), being away from home or close relatives (as in separation anxiety disorder), gaining weight (as in anorexia nervosa), having multiple physical complaints (as in somatization disorder), or having a serious illness (as in hypochondriasis), and the anxiety and worry do not occur exclusively during post traumatic stress disorder (American Psychiatric Association, 1994).

2.2.2 Epidemiology

The world-wide one year and lifetime prevalence of any anxiety disorder is 10.6% and 16.6% respectively (Somers et al., 2006). Women have a higher prevalence of anxiety disorders than men. Generalized anxiety disorder is the most common anxiety disorder. The one year prevalence of GAD in Canada is 1.1% (Offord et al., 1996).

Patients with depression often present with co-morbid anxiety. A United States national survey found that 58% of patients with major depression also fulfilled the criteria for an anxiety disorder (Kessler et al., 1996). Furthermore, most patients with primary anxiety disorders also experience major depressive episodes. Sixty eight percent of individuals with co-morbid depression and anxiety were anxious for over 10 years before the eventual development of depression.
2.3 Pathophysiology of depression and anxiety

Patients with depression and anxiety share many overlapping symptoms including fatigue, impaired concentration, irritability, sleep disturbance, and somatization (Ninan et al., 1999). Depression and anxiety may also have a common pathophysiology (Weiss et al., 1994). Norepinephrine (NE) and serotonin (5-HT) dysregulation, abnormalities in corticotropin-releasing factor (CRF) stress responses, neurotrophic factors and cellular transduction factors may all be involved in the pathophysiology of depression and anxiety.

The monoamine hypothesis of depression proposes that depression results from a functional deficiency of the brain monoaminergic transmitters norepinephrine (NE), serotonin (5-HT), and/or dopamine (DA). Evidence for this hypothesis comes from the observation that the administration of the antihypertensive drug reserpine, which causes a depletion of pre-synaptic stores of NE, 5-HT, and DA, induces a syndrome resembling depression (Schildkraut, 1965; Matussek, 1972; Coppen, 1967). Monoaminergic systems are responsible for many behavioural symptoms, such as mood, vigilance, motivation, fatigue, and psychomotor agitation or retardation. Therefore, the symptoms of depression may arise from altered synthesis, storage, or release of the neurotransmitters, as well as from disturbed sensitivity of their receptors or subcellular messenger functions (Stahl, 1998). NE and 5-HT systems are dysregulated with depression and anxiety disorders, with more data suggesting increased NE transmission or receptor supersensitivity and
decreased 5-HT transmission in these systems.

Researchers have attempted to demonstrate reduced monoamine availability in patients with depression by measuring neurotransmitters and/or their metabolites in post-mortem brain tissues and body fluids, such as cerebrospinal fluid (CSF), blood, and urine (Potter et al., 1985). Decreased levels of the NE metabolite α-methoxy-4-hydroxyphenylglycol (MHPG) have been found, indicating NE turnover in brain. This supports the hypothesis of a deficient noradrenergic system, however results are inconsistent and have not been found for all depressed patients (Potter et al., 1985; Schatzberg et al., 1995). In contrast, increases in MHPG have been correlated with anxiety (Sevy et al., 1989) and hypersecretion of NE in plasma and CSF has been reported in depression and anxiety (Roy et al., 1988; Sevy et al., 1989; Wyatt et al., 1971). In addition, studies have found increases in α2 receptors (Meana et al., 1992) and β-adrenergic receptors (Mann et al., 1996; Mann, 1999) in depressed patients as well in post-mortem tissue, suggesting a dysregulation and possible supersensitivity of NE transmission in depression.

Moreover, research suggests that a deficiency of brain serotonergic activity increases vulnerability to depression and possibly anxiety (Maes and Meltzer, 1995; Mann, 1999). Decreased 5-HT levels have been found in the CSF of patients with anxiety (Brewerton et al., 1995). However, decreased levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) have not been found in all depressed patients, indicating that reduced serotonergic transmission is not exclusively associated with
depression (Maes et al., 1994; Cheetham et al., 1991; Leonard et al., 2000).

Alterations in 5-HT receptor populations have been associated with depression and anxiety. Increased 5-HT$_2$ binding (Mann et al., 1986; Arango et al., 1992) and increased 5-HT$_{1A}$ binding (Matsubara et al., 1991) have been found in the cortex of post-mortem tissue, possibly indicating the up-regulation of receptors in the presence of decreased pre-synaptic transmission. Reduced serotonin uptake and reduced serotonin transporter binding has been found in platelets (Kaplan and Mann, 1982; Nemeroff, 1988; Owens and Nemeroff, 1994) and in the brain of depressed individuals (Malison et al., 1998). Reduced serotonin transporter binding has also been observed in generalized anxiety disorder (Ity et al., 1994).

It is also possible that impaired activity of the enzymes responsible for the synthesis and degradation of monoamines leads to the development of depression, however results are inconsistent. Tyrosine hydroxylase and tryptophan hydroxylase are essential for NE and 5-HT synthesis, respectively, and have been found to be either up- or down-regulated in post-mortem brain samples of depressed patients, suggesting that transmitter synthesis alone is not associated with depression (Leonard et al., 2000).

Addition of $\alpha$-methylparatyrosine, which inhibits the NE-synthesizing enzyme tyrosine hydroxylase, leads to a depletion of NE in the synapse (Miller et al., 1996). The application of a tryptophan-free amino acid mixture, which induces a rapid cerebral depletion of tryptophan and decreases 5-HT concentrations, can be applied to investigate
the impact of monoamine concentration on depression (Neumiester et al., 2000). The depletion of monoamines did not induce or worsen the symptoms of depression in healthy controls or unmedicated patients, indicating that monoamine deficiency alone does not lead to the development of depression.

In summary, alterations in the NE and 5-HT systems likely lead to the symptoms of depression and anxiety. Patients vary in their noradrenergic abnormalities, however it is plausible that varied mechanisms of dysregulation, for example, the combination of increased or decreased release with increased or decreased receptor sensitivity, may lead to the same overall functional changes. The majority of the literature supports the hypothesis that overall increased NE activity and decreased 5-HT activity leads to depression and anxiety.

In addition, a down-regulation of the gamma-aminobutyric acid (GABA) system may be involved in the pathophysiology of anxiety disorders. GABA is the primary inhibitory neurotransmitter in the CNS and counterbalances the actions of the excitatory neurotransmitter glutamate. Studies measuring GABA concentrations in plasma or CSF have found lower levels of GABA in patients with anxiety, however other studies have found no significant difference in GABA concentrations between patients with GAD and healthy controls (Goddard et al., 2001). Patients with GAD show reduced benzodiazepine binding to the GABA<sub>A</sub> receptor in various brain regions compared to controls, suggesting that anxiety is associated with benzodiazepine receptor abnormalities (Tiihonen et al., 1997; Malizia et al., 1998; Bremner et al., 2000). Individuals with anxiety may under-
produce an endogenous benzodiazepine agonist or overproduce an endogenous benzodiazepine inverse agonist. The administration of a benzodiazepine antagonist to patients with anxiety induces a panic attack compared to healthy control subjects who do not experience any anxious symptoms (Nutt et al., 1990). Administering an inverse benzodiazepine agonist also induces a severe panic attack in patients with anxiety (Dorow et al., 1983).

The limbic system, which mediates stress, fear, anger and other emotions, is important in depression and anxiety. The amygdala functions to mediate sensory thalamic and cortical stimuli in relation to previously learned aversive stimuli such as fear, stress and pain, or appetitive stimuli such as approach, motivational and hedonic (Lang et al., 1998). Upon recognition of previous associations, the appropriate conditioned response pathway is activated, such as the central nucleus of amygdala in stress/fear responses or the bed nucleus of the stria (BNST) in fear/anxiety responses (LeDoux, 1996). Activation of the central nucleus of the amygdala initiates neural and endocrine stress responses including corticotropin-releasing factor (CRF) and cortisol release (Davis, 1998a). The hippocampus is involved in memory functioning. During low stress, the hippocampus attenuates the amygdala response and potentiates extinction of aversive memories, whereas during periods of higher stress or alertness, the hippocampus increases long-term potentiation (LTP) related to aversive contexts and potentiates amygdala responsiveness (Gray, 1987; Mongeau et al., 1997). The prefrontal cortex is involved in regulating affect, providing cognitive control over stress and fear responsiveness along with anger, anxiety, and frustration tolerance (Lang et al., 1998;
LeDoux, 1996). The limbic cortex (LC) is activated by novel, aversive, or stressful stimuli (Robbins and Everitt, 1995). NE from the LC stimulates potentially stressful memories and fear/stress responses in the amygdala (Cahill and McGaugh, 1998). NE also directly activates CRF activity in the amygdala (Feldman and Weidenfeld, 1998; Van Bockstaele et al., 1999). High NE activity can lead to an over-activation of the limbic stress/fear pathways over cortical pathways to promote survival if rapid fight or flight response is needed (Valentino et al., 1993). In contrast, 5-HT from the raphe nuclei mediates tolerance to aversive experience in the amygdala, decreasing the likelihood of a fight or flight response (Stutzmann et al., 1998). Serotonin may decrease context conditioning to aversive stimuli in the hippocampus, and allow an individual to tolerate aversive situations (Mongeau et al., 1997).

In depression and anxiety, the stress response by NE and 5-HT is dysregulated. Norepinephrine transmission is overactive and 5-HT transmission is underactive. These neurotransmitter alterations contribute to the over-activation of the amygdala, hippocampal, and cortical pathways, activating the stress/fear response and under-activating higher cortical areas involved in inhibiting these pathways. Therefore, the same intensity of a stressful stimulus that leads to minimally increased arousal in a normal system, leads to significant arousal, vigilance and activation of the fight or flight response pathways in patients with depression and anxiety.

In addition to monoamine abnormalities, hormonal abnormalities such as altered levels of cortisol, growth hormone (GH), or thyroid hormones as well as dysfunctions in
the hypothalamic-pituitary-adrenal (HPA) axis and/or the regulation of thyroid function may be related to depression and anxiety. The fear-stress response is activated by the central nucleus of the amygdala (Davis, 1998a; LeDoux, 1998) or the bed nucleus of the stria terminalis (BNST), a similar region of the 'extended amygdala' in the case of anxiety (Davis, 1998b). Activation of the fear-stress response leads to the release of corticotropin releasing factor (CRF) in numerous brain areas and mobilizes the CNS response to stress (Nemeroff, 1996). In addition, direct activation of the paraventricular nucleus (PVN) of the hypothalamus leads to CRF release that stimulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. Seventy-five percent of patients with MDD have an overactive HPA, characterized by hypercortisolemia (Laird and Benefield, 1995; Nemeroff, 1998; Ninan, 1999). HPA axis dysfunction is also involved in some cases of GAD (Avery et al., 1985; Tiller et al., 1988). A significant subpopulation of depressed patients hypersecrete cortisol when they are in a depressed state but not after they have recovered from depression (Rubin et al., 1989). Neuroimaging studies have found a correlation between increased amygdala activation and plasma cortisol levels in depression (Abercrombie et al., 1996; Drevets, 1997). Some depressed patients hypersecrete the hypothalamic corticotropin-releasing hormone (CRH), display inadequate glucocorticoid feedback, increased cortisol levels, and impaired suppression of the HPA axis in response to exogenous glucocorticoid administration (Holsboer 1996, 1986, 1982, 2000). Patients with depression have increased CRF concentrations in their lumbar cerebrospinal fluid compared to healthy subjects and to patients with other psychiatric disorders (Banki 1987, 1992a; Nemeroff 1984; Wilderlov 1988). In post-mortem studies, increased CRF concentrations and
increased CRF mRNA expression have been observed in the hypothalamic tissue of depressed patients compared to healthy controls, suggesting that depression involves an alteration of neural circuitry mediating the stress response pathways (Raadsheer, 1994, 1995). Decreased CRF-receptor binding in the frontal cortex of suicide victims is likely due to chronic CRF hypersecretion (Nemeroff, 1988). Therefore, impaired corticosteroid receptor signalling may be involved in the pathogenesis of depression (Holsboer, 2000). Antidepressant treatment has been shown to reduce and normalize central CRF activity. Treatment with fluoxetine results in a decrease in cerebrospinal fluid CRF concentrations (DeBellis, 1993) and patients who remain recovered during a 6 month antidepressant treatment have reduced cerebrospinal fluid CRF concentrations compared to patients who were not in recovery who still displayed elevated levels of CRF (Banki, 1992b).

Alterations in thyroid function have been associated with MDD and the administration of triiodothyronine (T3) is an effective adjunctive treatment for many patients (Duval et al., 1999; Altshuler et al., 2001). The administration of thyroid hormone increases cortical serotonin release (Gur et al., 1999) and may act as a co-transmitter to NE in the adrenergic nervous system (Gordon et al., 1999). However, the exact mechanism of this interaction is not clear. Serotonin function was reduced in patients without hypothalamus-pituitary-thyroid axis abnormalities, suggesting that non-serotonergic mechanisms might be involved in the reduced secretion of thyroid-stimulating hormone (TSH; Duval et al., 1999).

Immunosuppression or immune activation may play a role in the development of
depression (Licino and Maes, 1995). Depressed patients display increased plasma cytokine and acute phase protein concentrations in their blood (Maes, 1997). Furthermore, exposure to stressful life events leads to impairment in cellular immune function, such as lymphocyte and killer T cell activity (Connor, 1998). Cytokines influence various CNS functions that are dysregulated in MDD, such as sleep, food intake, cognition, temperature and neuroendocrine regulation (Sternberg, 1997; Rothwell, 1995). The administration of the cytokine interleukin-1 (IL-1) into the CNS produces stress-like effects on behaviour, monoamine transmitters, HPA axis activity, and immune function. IL-1 also regulates the 5-HT transporter gene (Connor and Leonard, 1998). Moreover, healthy individuals treated with exogenous cytokines such as interleukin-2 (IL-2) and interferon-α (IFN-α) develop depression-like symptoms, such as depressed mood, increased somatic problems, and low motivation (Connor and Leonard, 1998). These effects then disappear when cytokine therapy is terminated, suggesting that cytokines may be involved in producing these symptoms.

It is hypothesized that the plasticity of neurons is involved in the pathophysiology and treatment of MDD. MDD may develop from the inability of neurons to adapt to aversive stimuli, and antidepressants may act by correcting this dysfunction (Duman et al., 1999). Neurotrophic factors are endogenous growth factors that play an important role in the adult nervous system. Brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) have been shown to promote the function and growth of 5-HT–containing neurons in the brain, and low levels of these neurotrophins may be associated with depression (Altar, 1999). Animal studies have shown that antidepressant treatment,
including specific inhibitors of 5-HT or NE uptake as well as monoamine oxidase inhibitors (MAOIs), elevates BDNF mRNA levels in the rat hippocampus (Duman, 1997). Post-mortem studies show that patients treated with antidepressants display increased BDNF expression (Chen et al., 2001).

Moreover, neuroimaging studies using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have contributed knowledge regarding the pathophysiology of depression and anxiety. Researchers suggest that hippocampal atrophy is associated with MDD and GAD. Magnetic resonance imaging studies have shown that smaller hippocampal volumes are found in patients treated for major depression than in non-depressed comparison subjects (Bremner, 2000). As well, women with a history of recurrent depression have significantly reduced hippocampal volumes and a correlation was found between the total duration of depressive episodes and the extent of atrophy (Sheline et al., 1996). Hippocampal atrophy has also been found in patients with anxiety. Decreased neuronal density and atrophy in the hippocampus leads to decreased hippocampal activity, decreasing its ability to inhibit amygdala stress/fear responsiveness. Functional imaging studies also show reduced blood flow in the frontal lobe and basal ganglia of patients with MDD (Bench et al., 1992). Changes in frontal cortical activity have been found in depressed states (Drevets, 1998; Mayberg et al., 1999). Dorsal prefrontal cortical (PFC) activity is suppressed (Dolan et al., 1993, 1994; Biver et al., 1994; Drevets, 1998), and ventral PFC and orbital cortex are activated in depressed patients compared to controls (Baxter et al., 1987; Drevets et al., 1992; Price et al., 1996). Increased activation of ventral and orbital areas is also found in patients with
anxiety disorders (Baxter et al., 1987; Rauch et al., 1994). Antidepressant treatment normalizes these abnormalities, increasing dorsal PFC activity, and decreasing ventral PFC and orbital activity. The amygdala is also abnormally activated in patients with depression (Drevets, 1998) and its activity has been correlated with the severity of depression (Drevets et al., 1992, 1995; Abercrombie et al., 1996). Amygdala hypertrophy has been found to be associated with different depressive syndromes (Bremner et al., 2000; Altshuler et al., 1998). Patients who have been treated for major depression display amygdala volumes that are 25% larger (23% on the left and 27% on the right) than those of healthy comparison subjects (Bremner et al., 2000). Amygdala activity decreases towards normal levels following antidepressant treatment (Drevets, 1998).

2.4 Pharmacological treatments for depression and depression with co-morbid anxiety

There are many different pharmacological treatments available for the treatment of MDD. Antidepressants are the major class of medications used in the treatment of a major depressive episode. The Canadian Network for Mood and Anxiety Treatment has published guidelines for the treatment of MDD. The guidelines indicate that the first-line treatment options for MDD include Selective Serotonin Reuptake Inhibitors (SSRIs) and venlafaxine, the second line options are amitriptyline and clomipramine and third line options include Tricyclic antidepressants (TCAs) and Monoamine Oxidase Inhibitors (MAOIs) (Kennedy et al., 2001). Venlafaxine, citalopram and escitalopram can be used to treat both MDD and GAD and are recommended for patients with co-morbid depression and anxiety.
2.4.1 Antidepressants

There are different classes of antidepressants, specifically, Selective Serotonin Reuptake Inhibitors (SSRIs), Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs), Norepinephrine Dopamine Reuptake Inhibitors (NDRIs), Tricyclic antidepressants (TCAs) and Monoamine Oxidase Inhibitors (MAOIs). SSRIs include fluoxetine, paroxetine, sertraline, fluvoxamine, citalopram and escitalopram. Fluoxetine also has a low affinity as a norepinephrine reuptake inhibitor (Owens et al., 1997; Eli Lilly, 1998). Both paroxetine and sertraline also have a moderate affinity as a norepinephrine reuptake inhibitor and a slight affinity as a dopamine reuptake inhibitor (Owens et al., 1997; Pfizer, 2004). Two common SNRIs are venlafaxine and duloxetine, and bupropion is one of a few NDRIs. Venlafaxine is a potent serotonin reuptake inhibitor, and at higher doses, is a potent norepinephrine reuptake inhibitor (Owens et al., 1997; Roseboom and Kalin, 2000). Venlafaxine is an efficacious treatment for depression as well as for depression with co-morbid anxiety (Khan et al., 1998). Bupropion is a dual reuptake inhibitor of both norepinephrine and dopamine and does not have any serotonergic effects (Stahl et al., 2004). The TCAs include clomipramine, imipramine, desipramine, and amitriptyline, and the most common MAOIs are phenelzine and moclobemide (Stahl, 1998). Amitriptyline is a norepinephrine and serotonin reuptake inhibitor and also has affinity for muscarinic, histaminergic (H1) and adrenergic (a1) receptors (Owens et al., 1997; Guaiana et al., 2007). Two commonly used antidepressants that do not fit into any of the above categories are mirtazapine and trazodone. Trazodone is a heterocyclic antidepressant. It is a relatively weak serotonin reuptake inhibitor, compared to SSRIs. It has affinity for histamine H1, 5-HT1A and 5-HT2 receptors (Owens et al., 1997; Maes et al., 1997).
2.4.2 Selective serotonin reuptake inhibitors (SSRIs)

The serotonin transporter 5-HTT is localized at pre-synaptic terminals of serotonergic neurons and terminates the neurotransmitter’s effect by binding and removing 5-HT from the synapse. Selective serotonin reuptake inhibitors (SSRI) are a class of antidepressants that act by inhibiting the action of 5-HTT, extending the duration of 5-HT in the synapse and reducing 5-HT release in the long-term. Drugs belonging to this class suppress the activity of serotonin reuptake transporters (SERTs), which regulate extracellular serotonin (5-HT) concentrations in the brain (Bel and Artigas, 1992, 1993, 1995; Dreshfield et al., 1996; Moret and Briley, 1996). Through sustained inhibition, SSRIs desensitize the pre-synaptic 5-HT autoreceptors found at serotonergic nerve terminals, enhancing the synaptic efficacy of the serotonergic system (Haddjeri et al., 1998; Pejchal et al., 2002).

2.4.3 Citalopram and escitalopram

Citalopram and escitalopram are two related SSRIs, with little affinity for other receptors including norepinephrine (Owens, 1997, Sanchez, 2004). Citalopram is a 1:1 racemic mixture of S(+)-citalopram and R(-)-citalopram, however only the S-enantiomer is responsible for the pharmacological effects of the drug (Hyttel et al., 1992; Sanchez et al., 2003a, 2003b; Sanchez and Kreilgaard, 2004; Sanchez et al., 2004). Escitalopram is the S-enantiomer of citalopram only.

Escitalopram is clinically more effective than citalopram and all other SSRIs (Murdoch, 2005; Montgomery et al., 2007). The R-enantiomer partially counteracts the
actions of the S-enantiomer (Sanchez et al., 2004). The S-enantiomer alone binds to the high affinity, primary serotonin-binding site, however both enantiomers bind to a lower affinity allosteric site and affect the binding of escitalopram to the primary site (Mansari et al., 2007). The therapeutically inactive R-enantiomer competes with the serotonin-enhancing S-enantiomer at a low-affinity allosteric site on serotonin reuptake transporters (SERTs), and reduces the effectiveness of the S-enantiomer at the primary, high affinity serotonin-binding site. The R-citalopram inhibits the S-enantiomer occupancy of SERT to a greater extent with multiple dosing because the metabolism and elimination of R-citalopram is slower, thereby reducing the effectiveness of escitalopram at the primary SERT-binding site (Klein et al., 2007; Meyer et al., 2004, Sanchez and Kreilgaard, 2004; Sanchez et al., 2004).

### 2.5 Personalized medicine

The initial course of antidepressant treatment is ineffective for over half of patients with clinical depression and for 25% of patients with anxiety, resulting in a need to try other medications in an effort to find one that is effective (Thase et al., 2005). This extends suffering, continues functional disability, and increases both the risk of relapse and the risk that patients will abandon treatment. Research suggests that this considerable variation in individual treatment outcome might have a genetic basis.

Previous research has identified genes that affect mood, and as individual genomes differ, so do their depressions. Because the underlying causes of depression
vary, the same drug that relieves symptoms for one person may have little effect on another. Candidate genes of pharmacokinetic and pharmacodynamic pathways of antidepressants have been investigated, and associations between several candidate genes and response to antidepressants have been found. Researchers have looked for polymorphisms in genes coding for either pharmacokinetic pathways—processes influencing the delivery of a drug to the target such as absorption, distribution, metabolism and elimination, or pharmacodynamic pathways—processes influencing the relationship between drug concentration and the resulting effect.

Personalized medicine or individualized treatment based on genetic polymorphism information can design the appropriate treatment for a patient according to his genotype, matching the right drug to the right patient. A biological marker of likely treatment effectiveness would reduce the likelihood that patients with depression will experience unsuccessful treatments.

2.6 Candidate Genes in depression

Pharmacogenetic studies in depression have focused on candidate genes that may be involved in the pathophysiology of depression and in the mechanism of action of antidepressants. The Sequenced Treatment Alternatives to Relieve Depression study (STAR*D) is a multi-site, prospective, randomized, clinical trial of outpatients with MDD who were treated with citalopram. This study provides DNA from a clinically representative cohort of approximately 2000 adults with MDD, which other researchers
can use in genetic studies. A large multi-centre integrated project called the Genome-based Therapeutic Drugs for Depression (GENDEP) investigated 117 SNPs in 10 candidate genes involved in the molecular mechanisms of antidepressant action (Uher et al., 2009).

The study of candidate genes implicated in the mechanisms of action or pharmacokinetics of antidepressants has identified an association between genetic variation and outcome of antidepressant treatment. Variations in the serotonin transporter gene (5-HTT) have been associated with antidepressant response in patients with MDD. The serotonin transporter gene is located at 17q11.1-17q12 (Lesch et al., 1993). Two polymorphisms in this gene are possible: one in the promoter region (5-HTTLPR) and one in intron 2 (STin2) which is related to transcriptional regulation (MacKenzie and Quinn, 1999). An insertion or deletion can occur in 5-HTTLPR. A deletion produces a short (S) allele that is 44 bp shorter than the long (L) allele. The S variant is associated with reduced transcriptional activity of the 5-HTT gene promoter, leading to lower expression of 5-HTT sites and reduced serotonin uptake (Heils et al., 1996). The L allele is associated with a better response to fluvoxamine (Smeraldi et al., 1998) and the S allele is associated with poor response to antidepressant treatment with SSRIs (Pollock et al., 2000; Zanardi et al., 2000; Arias et al., 2003). However these finding are true for Caucasians and are not applicable to Asians. Asians with the S/S genotype respond better to antidepressants (Kim et al., 2000, 2006a, b; Yoshida et al., 2002). A GENDEP study found that individuals with MDD who were carriers of the L allele in the 5-HTTLPR showed greater improvement after escitalopram treatment than individuals who were
homozygous for the S allele (Huezo-Diaz et al., 2009).

Furthermore, individuals can have a variable number tandem repeat (VNTR) in STin2, having either 9, 10 or 12 copies of a 16-17 base pair repeat element. The 12-repeat variant increases transcription compared to the 10-repeat variant (Fiskerstrand et al., 1999). In Caucasians in the GENDEP study, patients with MDD with the STin2 10/12 genotype showed a less favourable response to SSRI treatment than individuals with other STin2 genotypes. The STAR*D study found that the STin2 12/12 genotype is associated with remission after citalopram treatment (Mrazek et al., 2009).

Polymorphisms in the serotonin transporter gene are also associated with anxiety. A polymorphism in the serotonin transporter gene 5-HTTLPR has been associated with anxiety as well as with response to SSRIs in patients with generalized social anxiety disorder (Lesch et al., 1996; Mazzanti et al., 1998; Murakami et al., 1999; Greenberg et al., 2000; Stein et al., 2006). Carriers of the S allele show poorer response to SSRIs (Stein, 2006).

Variations in the serotonin receptor genes have also been associated with antidepressant response in patients with MDD. Polymorphisms occur in the 5-HT1A, 5-HT2A and 5-HT6 receptors. HTR2A encodes the serotonin 2A receptor, which is down-regulated by citalopram. The GG genotype of the –1438A/G polymorphism in HTR2A is associated with better treatment response to citalopram (Choi et al., 2005). Within the STAR*D sample of patients, individuals homozygous for the A allele at one SNP in
HTR2A had an 18% reduction in absolute risk of having no response to citalopram treatment, compared with those homozygous for the G allele (McMahon et al., 2006).

Guanine nucleotide binding proteins (G-proteins) are important in signal transduction pathways due to their role in regulating transmembrane coupling of many receptors with internal second messenger systems. They transmit signals from receptors to effector proteins and are thereby involved in regulating cellular responses to almost all neurotransmitters, including serotonin and noradrenaline. G-proteins are composed of three subunits. A polymorphism in exon 10 of the G-protein β3 subunit (C825T) is associated with depression as well as response to antidepressant treatment (Zill et al., 2000).

Tryptophan hydroxylase (TPH) is involved in the synthesis of serotonin from tryptophan (Walther and Bader, 2003). There is an A218C polymorphism in the tryptophan hydroxylase 1 (TPH1) gene located in a potential GATA transcription factor-binding site, potentially influencing TPH gene expression. The A218C polymorphism is associated with response to the SSRIs fluvoxamine and paroxetine in both Korean and Caucasian patients with MDD, but not Japanese patients (Serretti et al. 2001a, b; Peters et al., 2004, Yoshida et al., 2002). The SNP G1463 in the tryptophan hydroxylase 2 (TPH2) gene, involved in the synthesis of serotonin, has been linked to depression. Individuals with the mutant allele (A) of the TPH2 polymorphism G1463A produce very low levels of serotonin and are relatively resistant to the SSRIs Prozac and Zoloft, however this variant is very rare in the population (Zhang et al., 2005). The SNP rs10879346 in the
TPH2 gene is associated with short-term response to antidepressant treatment in patients with MDD (Tzvetkov et al., 2008). The G1287A polymorphism is present in the noradrenaline transporter gene. This polymorphism is associated with response to norepinephrine reuptake inhibitors, but not with SSRI response. Individuals with MDD with the GG genotype show greater nortriptyline treatment response rates (Kim et al., 2006a, b).

Genes involved in the HPA axis are associated with medication response. Variations in the promoter region (SNP rs4713916), intron 2 (SNP rs1360780) and 3’ untranslated region (SNP rs3800373) of the FK506 binding protein 5 gene (FKBP5) are associated with antidepressant response (Binder et al., 2004). Individuals with the TT genotype at rs1360780 had also experienced more previous depressive episodes. Carriers of the T allele of rs1360780 or the C allele of rs3800373 have a better chance of responding to antidepressants than non-carriers (Kirchheiner et al., 2008). A polymorphism in the glucocorticoid receptor gene (ER22/23EK) has been associated with susceptibility to developing MDD and with a faster response to antidepressant treatment (Van Rossum et al., 2006). A corticotropin-releasing hormone receptor 1 haplotype is associated with response to antidepressant treatment in patients with co-morbid depression and anxiety (Licino et al., 2004).

Polymorphisms in candidate genes have been associated with citalopram response. The serotonin transporter promoter polymorphism 5-HTTLPR is associated with adverse effects of citalopram (Hu et al., 2007), markers in the serotonin 2A receptor
HTR2A are associated with treatment outcome of citalopram (McMahon et al., 2006), markers within kainate-sensitive ionotropic glutamate receptor GluR6 (GRIK2) and ionotropic glutamate receptor AMPA 3 (GRIA3) are associated with citalopram treatment emergent suicidal ideation (Laje et al., 2007), polymorphisms in the CREB1 (cyclic adenosine monophosphate response element binding protein 1) are associated with treatment-emergent suicidality among men with depression (Perlis et al., 2007) and variation in a kainic acid-type glutamate receptor (GRIK4) is associated with citalopram response (Paddock et al., 2007).

Genetic variation in the HTR2A gene is associated with the outcome of citalopram treatment in patients with MDD. HTR2A encodes the serotonin 2A receptor, which is down-regulated by citalopram. Individuals homozygous for the A allele had an 18% reduction in absolute risk of having no response to treatment, compared with those homozygous for the other allele (McMahon et al., 2006). The STin2 12/12 genotype of 5-HTT is associated with remission after citalopram treatment (Mrazek et al., 2009).

Furthermore, an association between the tryptophan hydroxylase-1 gene A218C polymorphism and citalopram response was found in a Korean population (Ham et al., 2007).

Genetic variations have recently been associated with escitalopram treatment response. The GENDEP study found that HTR2A as well as variations in the glucocorticoid receptor gene are associated with response to escitalopram treatment.
(Uher et al., 2009). Individuals with MDD who are carriers of the L allele in the 5-HTTLPR show greater improvement after escitalopram treatment than individuals who are homozygous for the S allele (Huezo-Diaz et al., 2009).

2.7 Pharmacogenetics

Pharmacogenetics aims to identify variations in genetic factors, specifically in single nucleotide polymorphisms (SNPs) and copy number variations (CNVs), that contribute to variations in disease susceptibility, drug metabolism, and drug response. A single nucleotide polymorphism (SNP) is a small variation in the DNA sequence.

Most of the study of pharmacogenetics to date has focused on genetic polymorphisms in drug metabolizing enzymes. The isoforms CYP3A, CYP2D6, CYP2C19, and CYP2C9 of cytochrome P-450 isoenzymes have been found to be important in the metabolism of antidepressants (Wieczorek, 2001; Steimer et al., 2001; Staddon et al., 2002). Different drugs are metabolized by different enzymes, and variations in these genes can lead to three possible phenotypes: poor metabolizers (PM), normal metabolizers (NM), and extensive metabolizers (EM) (Nebert and Dieter, 2000). Individuals who are PM have an increased risk of a toxic reaction, whereas drugs may not reach therapeutic plasma concentrations in individuals who are EM. Therefore, individuals with different metabolic phenotypes require different doses of medication to reach a therapeutic, but not toxic effect.
2.9 Research rationale and hypothesis

The large individual variation in antidepressant treatment outcome remains poorly understood. Medication response may have a genetic basis and several polymorphisms in candidate genes have been associated with antidepressant response, however few results have been replicated.

Most studies have assessed response to medication after 6 weeks of treatment, with 13 weeks as the longest time period after which response was assessed. It is possible that assessing medication response after 6 or 13 weeks may include placebo responders in the medication response group, thereby creating a heterogeneous response group as opposed to a homogenous group of true responders. Therefore, it may be too early to properly assess medication response after 13 weeks, and different results may be generated if treatment response is determined after one or more years of medication treatment. Furthermore, a decrease in clinical scale score after commencing a medication may not be the most effective way to determine whether or not an individual is a true responder to the medication. Medication response may be better indicated by the return of symptoms after stopping the medication and a corresponding decrease of symptoms after re-commencing the medication. Creating strict criteria for classification of medication response may result in a more homogenous responder group, which may result in the finding of significant genetic similarities.

The objective of this study is to identify genetic variants underlying the considerable individual differences in response to antidepressant treatment. A candidate
gene analysis of response to citalopram and escitalopram treatment will be performed. Genotype at the catechol-O-methyltransferase rs4680, dopamine D2 receptor rs1800497, serotonin receptor 1A rs6295 and serotonin transporter promoter polymorphisms will be compared between patients with MDD and/or GAD who respond to citalopram or escitalopram treatment and healthy controls.

Catechol-O-methyltransferase (COMT) has been associated with schizophrenia, anxiety and response to SSRIs in patients with MDD. Catechol-O-methyltransferase is an enzyme involved in the catabolism of dopamine, adrenalin, and noradrenalin. COMT is expressed in brain tissue and plays a major role in dopamine breakdown in frontal cortex (Hong et al., 1998; Lloyd et al., 1975). A functional polymorphism in the COMT gene accounts for a variation in enzymatic activity. The COMT gene (located in 22q11.1–q11.2) contains a G to A missense mutation, resulting in a substitution of methionine (met) for valine (val) at codon 158 of the membrane-bound isoform of the protein (SNP rs4680). The \text{val}^{158} \text{met} (G/A) functional polymorphism results in less efficient catecholamine catabolism compared to individuals who are homozygous for the \text{val}^{158} allele (G/G), as the \text{met}^{158} allele has about one-third to one-fourth of the activity of the \text{val}^{158} allele (Lachman et al., 1996; Lotta et al., 1995; Weinshilboum et al., 1999). Individuals with the \text{val} allele (G) have increased COMT activity and lower pre-frontal extracellular dopamine compared with those with the \text{met} (A) substitution.

It is possible that the COMT polymorphism is associated with depression and anxiety. The worrier/warrior hypothesis proposes that \text{val}^{158} alleles may be associated
with advantages in processing aversive stimuli (warrior strategy), while met^{158} alleles may be associated with advantages in memory and attention tasks (worrier strategy; Heinz, 2006). It is possible that individuals with val^{158} alleles may have improved dopaminergic transmission and better performance under conditions of stress when dopamine release is increased, while individuals with met^{158} alleles may have less efficient neurotransmission and worse performance. Individuals with greater numbers of met^{158} alleles show increased limbic and pre-frontal activation to unpleasant stimuli, potentially contributing to lower emotional resilience against negative mood states and resulting in the development of depression or anxiety (Heinz and Smolka, 2006; Smolka et al., 2005).

The dopamine D2 receptor gene (DRD2) encodes a G protein-coupled receptor located on post-synaptic dopaminergic neurons, which plays a central role in reward-mediating mesocorticolimbic pathways (Hauge et al., 1991). Variation occurs at the SNP rs 1800497, located over 10,000 base pairs downstream from the termination codon in the 3' flanking region of DRD2, and in the recently discovered ankyrin repeat and kinase domain containing 1 (ANKK1) gene which is closely linked to DRD2 (Grandy et al., 1989; Neville et al., 2004). The DRD2 gene is located on chromosome 11 at q22-q23. The Taq1A polymorphism is a G to A substitution. The A1 (A) allele is associated with low D2 receptor availability and reduced expression of dopamine D2 receptors in the striatum (Pohjalainen et al., 1998). The A1 (A) allele has also been associated with low dopamine density and lower mean relative glucose metabolic rate in dopaminergic regions in the brain (Noble et al., 2007). Individuals with the A1/A2 genotype (A/G)
display significant reduction in D2 receptor availability reflecting an alteration in receptor density compared to the A2/A2 genotype (G/G) (Pohjalainen et al., 1998).

DRD2 may have a role in the development of depression and anxiety. The DRD2 polymorphism has been found to moderate the effect of stressful life events on depressive symptoms and individuals with the A2/A2 genotypes may be more vulnerable to depression than others (Elovainio et al., 2007). In individuals with the A2/A2 genotype, the occurrence of stressful life events is associated with the development of depressive symptoms, but not in individuals with the A1/A1 or A1/A2 genotype (Elovainio et al., 2007).

5-hydroxytryptamine receptor 1A (5-HTR1A; serotonin receptor 1A) may influence the development of MDD and antidepressant treatment outcome. Activation of the 5-HT1A receptors leads to the hyperpolarization of 5-HT neurons, decreasing their firing rate and the release of serotonin. Alterations of 5-HT1A levels are observed in patients with MDD, anxiety and bipolar disorder. The SNP rs6295, also called C(-1019)G, located in the regulatory region of the HTR1A promoter impacts transcriptional regulation of the gene through altered binding of the transcription factors human nuclear deformed epidermal autoregulatory factor-1 (DEAF-1)-related (NUDR)/Deaf-1 and Hairy/Enhancer-of-split-5 (Hes5). The G allele is associated with increased 5-HTR1A protein and binding (Lemonde et al., 2003). Positron emission tomography (PET) shows that both healthy and depressed patients with the G allele have increased 5-HT1A autoreceptor density, which leads to decreased amygdala reactivity (Parsey et al, 2006;
The serotonin transporter gene (SLC6A4), located on chromosome 17q11.1-17q12, plays a key role in serotonergic neurotransmission and its protein is the target of many antidepressants. An insertion/deletion polymorphism occurs in the promoter region of SLC6A4, referred to as 5-HTTLPR. A deletion (S allele) reduces the efficiency of transcription, resulting in decreased expression of the serotonin transporter. 5-HTTLPR modulates the amygdala's reactivity to fear-provoking stimuli, which may relate the S/S genotype to susceptibility of depression (Hariri et al., 2005; Pezawas et al., 2005). The role of the 5-HTTLPR genotype in the development of depression remains unclear. Some studies have found that the S/S genotype is associated with depression (Kunugi et al., 1997; Bellivier et al., 1998; Gutierrez et al., 1998; Ramasubbu et al., 2006), however others have not reached this conclusion (Collier et al., 1996; Stober et al., 1996; Furlong et al., 1998). A recent meta-analysis found that 5-HTTLPR genotype was not associated with depression and no interaction effect between genotype and stressful life events on depression was observed (Risch et al., 2006). The 5-HTTLPR polymorphism has been associated with anxiety traits and GAD (Ohara et al., 1998; Lesch, 1999; You et al., 2005). A large Spanish study found that the S/S genotype is associated with depression, independent of age, gender or GAD co-morbidity (Cervilla et al., 2006).
It is hypothesized that a particular genotype is associated with response to citalopram and escitalopram treatment in patients with MDD and/or GAD. We expect that variation in the COMT, DRD2, 5-HTR1A and SLC6A genes is predictive of treatment outcome.
Chapter 3: Methods

This study was approved by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board.

3.1 Participants

Twenty-one participants signed written informed consent prior to participating and were included in the analysis. Participants were recruited from general practitioners offices and from advertisements in the community of Kingston, Ontario. Participants were enrolled between August 2009 and April 2010, and were 18 years of age or older. Participants were currently taking citalopram or escitalopram for the treatment of depression and/or anxiety and have been taking the medication for a period of at least one year. Subjects must have experienced improvement in their symptoms to be eligible for the study. Furthermore, after experiencing improvement in their symptoms, they must have stopped the medication for a period of time during which their depressive or anxious symptoms returned. Participants must have then re-commenced the medication, after which time their symptoms improved once again. Participants were only taking citalopram or escitalopram, and were not taking any other antidepressants, antipsychotics, anxiolytics or medications for the treatment of mood disorders. One hundred and forty six participants were included as healthy control subjects who do not have depression or anxiety. Control subjects were recruited from Kingston, Ontario and were mainly Caucasian. Many control subjects were recruited from a control population of an autism pharacogenetics study, who have no history of autism or psychiatric illness.
3.2 Clinical measures

Participants were assessed using the Sheehan Disability Scale and the Quick Inventory of Depressive Symptomology- Self Report (QIDS-SR).

3.3 Sample collection

A venous blood sample was drawn from each participant.

3.4 DNA extraction

Genomic DNA was isolated and purified using the Qiagen Flexigene extraction kit for whole blood. Briefly, lysis buffer is added to the sample and cell nuclei and mitochondria are pelleted by centrifugation. The pellet is re-suspended and incubated in denaturation buffer containing a chaotropic salt and QIAGEN Protease to remove contaminants. Isopropanol is added to precipitate DNA. DNA is recovered by centrifugation, washed in 70% ethanol, dried, and re-suspended in hydration buffer (10 mM Tris.Cl, pH 8.5).

3 ml of whole blood is added to 7.5 ml of FG1 buffer (lysis buffer) and mixed by inverting the tube five times. The samples are centrifuged for 5 min at 2000 x g in a swing-out rotor. The supernatant is discarded and the tube is inverted on a clean sheet of absorbent paper for 2 minutes while ensuring that the pellet remains in the tube. 1.5ml of FG2 buffer (denaturation buffer)/QIAGEN Protease is added and the sample is vortexed until the pellet is completely homogenized. Next, the tube is inverted 3 times, placed in a water bath and incubated at 65°C for 10 minutes. 1.5 ml of 100% isopropanol is the added and the tube is mixed by inversion until the DNA precipitate becomes visible as
threads or as a clump. The tube is then centrifuged for 3 minutes at $2000 \times g$ and the supernatant is discarded. The tube is briefly inverted onto a clean piece of absorbent paper. 1.5 ml of 70% ethanol is then added and the sample is vortexed for 5 seconds followed by centrifugation for 3 minutes at $2000 \times g$. The supernatant is discarded and the tube is inverted on a clean piece of absorbent paper for 5 minutes. The DNA pellet is air-dried until all the liquid has evaporated (at least 5 minutes). Next, 300 μl of FG3 buffer (hydration buffer) is added and the sample is vortexed for 5 seconds at low speed. The DNA is then dissolved by incubating for 1 hour at 65°C in a water bath.

### 3.5 Genotyping

COMT, DRD2 and HTR1A SNPs were genotyped using TaqMan assays according to the manufacturer's protocol (Applied Biosystems, California). 0.75μl of TaqMan Universal PCR Master Mix was added to 0.0375μl of 40x SNP genotyping assay (containing 2 primers for amplification of the polymorphic sequence and 2 probes for distinguishing between the 2 alleles) and 0.7125μl of distilled water, for a total volume of 1.5 μl per well. The mixture was centrifuged and added to the DNA reaction plate. DNA was amplified using PCR and the fluorescence signal generated by PCR indicates which alleles are present in the sample. After PCR amplification, allele discrimination plate read was performed using the Applied Biosystems Real-Time PCR System. The Sequence Detection System (SDS) Software uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample. Each probe contains a reporter dye at the 5' end of the probe. VIC dye is linked to the 5' end of the allele 1 probe and FAM dye is linked to the 5' end of the allele 2 probe. An increase in
VIC dye fluorescence indicates homozygosity for allele 1, whereas an increase in FAM dye fluorescence indicates homozygosity for allele 2 and an increase in both VIC and FAM dye fluorescence indicates allele 1 and 2 heterozygosity.

The 5-HTTLPR polymorphism was genotyped using PCR amplification followed by gel electrophoresis. PCR used 0.15 μl of distilled water, 0.5 μl of 10x PCR buffer, 0.5 μl of 10x dNTPs, 0.5 μl of HTTLPR forward primer, 0.5 μl of HTTLPR reverse primer, 0.15 μl of 50mM MgCl$_2$, 0.15 μl of dGTP, 0.5 μl of BSA, 0.05 μl Taq Polymerase, 3 μl of reaction cocktail and 2 μl of DNA template per reaction (Invitrogen). Cycling parameters were 94°C for 4 minutes, 94°C for 45 seconds, 62°C for 45 seconds, 72°C for 1 minute and 72°C for 6 minutes, for 38 cycles. The gel (Invitrogen) was run for approximately 1.5 hours at 200 volts.

3.6 Statistical analysis

Statistical analysis of data was done using S.P.S.S. 17.0.1 for PC (SPSS Inc, Chicago, IL). Chi-squared tests were employed to compare genotypic and allele frequencies between the responder group and control group. A one-way ANOVA with a significance of 0.05 was used to compare the age, gender, ethnicity, QIDS scale score and diagnosis with genotype in the responder group. A multiple logistic regression was used to determine whether or not genotype is associated with medication response to predict the probability that an individual with a particular genotype will respond to the medication. Logistic regression was also used examine gene-gene interactions on medication response. Due to small sample size, regression results are not valid and are
therefore not reported here.
Chapter 4: Results

4.1. Participants

One hundred and forty six healthy participants comprised the control group. Twenty one participants were included in the responder group of the study. Their mean (± SD) age was 32 ± 14 years ranging from 18 to 60 years of age. There were 13 female Caucasians, 4 male Caucasians, 1 female Asian, 1 female Hispanic, 1 male Hispanic and 1 female Egyptian. Seven patients were taking citalopram and 14 were taking escitalopram. The mean (± SD) length of time participants had been taking the medication was 2.17 ± 1.34 years. Five participants were taking the medication for the treatment of MDD alone, 6 for GAD alone and 10 for MDD with co-morbid GAD (Table 1).

4.2 Clinical Measures

The mean (± SD) work/school life, social life and family/home life section scores of the SDS were 3.5 ± 2.7 ranging from 0 to 10; 3.6 ± 2.8 ranging from 0 to 10; and 3.1 ± 2.7 ranging from 0 to 7, respectively. The mean (± SD) number of days lost due to symptoms was 0.5 ± 1.2 ranging from 0 to 4, and the mean (± SD) number of days unproductive due to symptoms was 1.5 ± 2.3 ranging from 0 to 7. The mean (± SD) QIDS-SR score was 11.95 ± 7.0 ranging from 0 to 26.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Responder Group (N=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
<td></td>
</tr>
<tr>
<td>MDD</td>
<td>5</td>
</tr>
<tr>
<td>GAD</td>
<td>6</td>
</tr>
<tr>
<td>MDD and GAD</td>
<td>10</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
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</tr>
<tr>
<td>Asian</td>
<td>1</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2</td>
</tr>
<tr>
<td>Middle Eastern</td>
<td>1</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
</tr>
<tr>
<td>Citalopram</td>
<td>7</td>
</tr>
<tr>
<td>Escitalopram</td>
<td>14</td>
</tr>
</tbody>
</table>
4.3 Catechol-\textit{O}-methyltransferase gene polymorphism rs4680

The allele frequencies in the COMT polymorphism were: (A) 62% in responders and 59% in controls; (G) 38% in responders and 41% in controls. The genotypic frequencies were: (G/A) 48% in both responders and controls; (G/G) 38% in responders and 34% in controls; (A/A) 14% in responders and 18% in controls. There was no significant difference in genotypic frequency between responders and controls ($\chi^2 = 0.23$, df = 2, p = 0.89). The frequency of the G allele ($\chi^2 = 0.06$, df = 1, p = 0.82) or the A allele ($\chi^2 = 0.05$, df = 1, p = 0.83) did not significantly differ between responders and controls (Table 2). There was no significant difference between genotype and ethnicity (F= 1.46, p= 0.26), age (F= 1.5, p= 0.25), gender (F= 1.47, p= 0.26), diagnosis (F= 0.64, p= 0.54) or QIDS score (F= 0.86, p= 0.44) in the responder group.

4.4 Dopamine D2 receptor gene polymorphism  rs1800497

The allele frequencies in the DRD2 SNP were: (G) 85% in responders and 69% in controls; (A) 15% in responders and 31% in controls. The genotypic frequencies were: (G/A) 30% in responders and 41% in controls; (G/G) 70% in responders and 46% in controls; (A/A) 0% in responders and 4% in controls. There was no significant difference in genotypic frequencies between responders and controls ($\chi^2 = 5.33$, df = 2, p = 0.07). The frequency of the G allele ($\chi^2 = 0.82$, df = 1, p = 0.37) or the A allele ($\chi^2 = 2.18$, df = 1, p = 0.14) did not significantly differ between responders and controls (Table 3). There was no significant difference between genotype and ethnicity (F= 2.86, p= 0.11), gender (F= 0.29, p= 0.60), age (F= 0.63, p= 0.44), diagnosis (F= 0.01, p= 0.91) or QIDS score (F= 1.46, p= 0.24) in the responder group.
Table 2. Allele and genotype (frequency) distributions of catechol-\textit{O}-methyltransferase gene polymorphism rs4680 in responders to citalopram and escitalopram treatment compared with controls.

<table>
<thead>
<tr>
<th></th>
<th>Allele</th>
<th>Genotype</th>
<th></th>
<th></th>
<th></th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>A</td>
<td>Total alleles</td>
<td>G/A</td>
<td>G/G</td>
<td>A/A</td>
</tr>
<tr>
<td>Responders</td>
<td>26 (62%)</td>
<td>16 (38%)</td>
<td>42</td>
<td>10 (48%)</td>
<td>8 (38%)</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>Control</td>
<td>164 (59%)</td>
<td>116 (41%)</td>
<td>280</td>
<td>68 (48%)</td>
<td>49 (34%)</td>
<td>26 (18%)</td>
</tr>
</tbody>
</table>
Table 3. Allele and genotype (frequency) distributions of dopamine D2 receptor gene polymorphism rs1800497 in responders to citalopram and escitalopram treatment compared with controls.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Genotype</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>A</td>
<td>Total alleles</td>
<td>G/A</td>
<td>G/G</td>
</tr>
<tr>
<td>Responders</td>
<td>34 (85%)</td>
<td>6 (15%)</td>
<td>40</td>
<td>6 (30%)</td>
<td>14 (70%)</td>
</tr>
<tr>
<td>Control</td>
<td>188 (69%)</td>
<td>84 (31%)</td>
<td>272</td>
<td>56 (41%)</td>
<td>62 (46%)</td>
</tr>
</tbody>
</table>
4.5 Serotonin receptor 1A gene polymorphism rs6295

The allele frequencies in the HTR1A polymorphism were: (G) 55% in responders and 52% in controls; (C) 45% in responders and 48% in controls. The genotypic frequencies were: (G/C) 70% in responders and 55% in controls; (G/G) 20% in responders and 24% in controls; (C/C) 10% in responders and 21% in controls. There was no significant difference in genotypic frequency between responders and controls ($\chi^2 = 1.89$, df = 2, $p = 0.39$). The frequency of the G allele ($\chi^2 = 0.52$, df = 1, $p = 0.47$) or the C allele ($\chi^2 = 1.66$, df = 1, $p = 0.20$) did not significantly differ between responders and controls (Table 4). There was no significant difference between genotype and ethnicity ($F = 0.36$, $p = 0.70$), gender ($F = 0.34$, $p = 0.72$), age ($F = 0.08$, $p = 0.92$), diagnosis ($F = 2.4$, $p = 0.12$) or QIDS score ($F = 3.45$, $p = 0.06$) in the responder group.

4.6 Serotonin transporter gene polymorphism 5-HTTLPR

The 5-HTTLPR allele frequencies were: (S) 50% in responders and 48% in controls; (L) 50% in responders and 52% in controls. The genotypic frequencies were: (S/L) 50% in responders and 37% in controls; (S/S) 25% in responders and 28% in controls; (L/L) 25% in responders and 35% in controls. There was no significant difference in genotypic frequency between responders and controls ($\chi^2 = 1.21$, df = 2, $p = 0.55$). The frequency of the S allele ($\chi^2 = 0.59$, df = 1, $p = 0.44$) or the L allele ($\chi^2 = 0.90$, df = 1, $p = 0.34$) did not significantly differ between responders and controls (Table 5). There was no significant difference between genotype and ethnicity ($F = 2.3$, $p = 0.13$), gender ($F = 0.0$, $p = 1.0$), age ($F = 1.42$, $p = 0.27$), diagnosis ($F = 1.17$, $p = 0.33$) or QIDS score ($F = 0.30$, $p = 0.74$) in the responder group.
Table 4. Allele and genotype (frequency) distributions of serotonin receptor 1A gene polymorphism rs6295 in responders to citalopram and escitalopram treatment compared with controls.

<table>
<thead>
<tr>
<th></th>
<th>Allele</th>
<th>Genotype</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>C</td>
<td>Total alleles</td>
<td>G/C</td>
<td>G/G</td>
<td>C/C</td>
</tr>
<tr>
<td><strong>Responders</strong></td>
<td>22</td>
<td>18</td>
<td>40 (55%)</td>
<td>14 (70%)</td>
<td>4 (20%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td></td>
<td>(55%)</td>
<td>(45%)</td>
<td></td>
<td>(70%)</td>
<td>(20%)</td>
<td>(10%)</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>128</td>
<td>120</td>
<td>248 (52%)</td>
<td>68 (55%)</td>
<td>30 (24%)</td>
<td>26 (21%)</td>
</tr>
<tr>
<td></td>
<td>(52%)</td>
<td>(48%)</td>
<td></td>
<td>(55%)</td>
<td>(24%)</td>
<td>(21%)</td>
</tr>
</tbody>
</table>


Table 5. Allele and genotype (frequency) distributions of serotonin transporter gene polymorphism 5-HTTLPR in responders to citalopram and escitalopram treatment compared with controls.

<table>
<thead>
<tr>
<th></th>
<th>Allele</th>
<th>Genotype</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>L</td>
<td>Total</td>
<td>S/L</td>
<td>S/S</td>
<td>L/L</td>
<td>Total</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>alleles</td>
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<td></td>
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<tr>
<td>Responders</td>
<td>20</td>
<td>20</td>
<td>40</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>20</td>
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<tr>
<td></td>
<td>(50%)</td>
<td>(50%)</td>
<td></td>
<td>(50%)</td>
<td>(25%)</td>
<td>(25%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>88</td>
<td>94</td>
<td>182</td>
<td>34</td>
<td>25</td>
<td>32</td>
<td>91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(48%)</td>
<td>(52%)</td>
<td></td>
<td>(37%)</td>
<td>(28%)</td>
<td>(35%)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Chapter 5: Discussion

Genotypic or allele frequencies did not significantly differ between patients with depression and anxiety who respond to citalopram or escitalopram treatment and healthy controls. It appears that genotype at the COMT, DRD2, 5-HTR1A or 5-HTTLPR polymorphism is not associated with medication response.

Our non-significant results accord with two studies that also found that the COMT polymorphism was not associated with response to SSRIs, however a few previous studies did find an association. Variation in the COMT gene (SNP rs4680) was recently investigated in patients with MDD and in the treatment response to antidepressants in MDD. No significant difference in the distribution of the COMT SNP was found in the response to SSRIs or between patients with MDD and control subjects (Illi et al., 2010). The COMT polymorphism is also not associated with response to paroxetine (Szegedi et al., 2005). In contrast, patients with MDD with the A/A genotype were found to respond better to the SSRI paroxetine than individuals with the G/A genotype, and G/G homozygotes responded the worst (Benedetti et al., 2008). However, patients with the G/G genotype respond better to both mirtazapine and citalopram (Szegedi et al., 2005; Arias et al., 2006). It is possible that the val158 met polymorphism differs in its association with individual SSRIs. This is the first known study to investigate response to escitalopram and our results suggest that the COMT polymorphism is not associated with response to escitalopram treatment.
This is the first known study to investigate the association of the A1/A2 DRD2 polymorphism with antidepressant treatment response in patients with MDD or GAD. The results of this pilot study suggest that the DRD2 polymorphism does not predict response to citalopram or escitalopram.

The association of the 5-HTR1A polymorphism with treatment outcome is inconsistent. Our finding that 5-HTR1A genotype is not associated with response to citalopram and escitalopram is consistent with a study investigating citalopram. The association of the SNP rs 6295 in 5-HTR1A with response to paroxetine or citalopram was investigated by Illi (2009). The SNP was not found to be associated with MDD or with response to either medication (Illi et al., 2009). Moreover, previous research has suggested that 5-HTR1A genotype is not associated with response to other SSRIs. Levin (2007) also did not find the 5-HT1A polymorphism to be associated with SSRI response.

However, in contrast to our results, previous research has found an association between 5-HTR1A genotype and response to SSRIs. The G allele or G/G genotype has been associated with reduced response to SSRIs in patients with MDD. In Caucasians, the G/G genotype was associated with poor response to flibanserin and fluoxetine (Lemonde et al., 2004). In Chinese populations, the C/C genotype was associated with greater response to SSRI fluoxetine treatment in females, as well as in both males and females (Yu et al., 2006; Hong et al., 2006). Furthermore, the S/S genotype of the serotonin receptor 5-HTT gene polymorphism (5-HTTLPR) and the G/G genotype of the 5-HT1A receptor gene have in combination been associated with reduced response to citalopram
in a Spanish population (Arias et al., 2005). It is possible that the G allele of 5-HTR1A does not alone predict response to citalopram, which is consistent with our results. But, when the interaction of 5-HTR1A genotype with genotypes at other polymorphisms is considered, the G/G genotype at 5-HTR1A can predict treatment response.

A few previous studies have also found that the 5-HTTLPR polymorphism is not associated with antidepressant response. Response to citalopram as well as response to escitalopram in females has not been associated with 5-HTTLPR genotype (Hu et al., 2007; Huezo-Diaz et al., 2009). Gressier (2009) and Kraft (2007) also found that the 5-HTTLPR polymorphism was not associated with response to antidepressant treatment. Furthermore, a recent meta-analysis suggests that 5-HTTLPR is not associated with response to antidepressants (Taylor et al., 2010).

Contrary to previous studies, 5-HTTLPR genotype was not associated with response to citalopram in this study. The S allele is associated with poorer response to SSRIs, including fluvoxetine, citalopram and escitalopram (Zanardi, 2001; Arias et al., 2003; Serretti, 2004; Gressier et al., 2009; Huezo-Diaz et al., 2009). Individuals with MDD homozygous for the S allele had 3 times more risk of not reaching remission after citalopram treatment than patients with any other genotype (Arias et al., 2003). In contrast, the S allele frequency has been found to be significantly higher in Japanese patients with MDD who respond to fluvoxamine compared with non-responders (Yoshida et al., 2002). Chinese patients with MDD with the L/L genotype respond better to fluoxetine compared with those with s alleles (Yu et al., 2002).
It is possible that the association of 5-HTTLPR genotype with response to escitalopram is gender specific. Huezo-Diaz (2009) found that the 5-HTTLPR polymorphism is associated with response to escitalopram. However, when response was analyzed separately for each gender, men with the L/L genotype showed greater response to escitalopram, but genotype was not associated with medication response in women. The sample population in this study was comprised of mainly women, which may partially explain the lack of association found in this study.

The main limitation in this study is the small sample size. Larger samples will have adequate statistical power to detect a smaller genotypic relative risk, which is the ratio of the risk of disease to carriers of a particular genotype versus non-carriers. For example, if genotypic relative risk is 1.2, the risk of not responding to the medication is increased by 20% for individuals with a particular genotype compared to those of a different genotype. To uncover highly significant findings for alleles with genotypic relative risks of 1.1 to 1.4, good or excellent statistical power requires sample sizes of 8,000-20,000 subjects, in addition to a comparison group. To date, no study has collected a sample size this large.

It is possible that rare, as opposed to common variants are associated with treatment response. Rare variants are those with a frequency of less than 1%, meaning they are found on less than 1% of chromosomes in the population. Current technology relies upon the assumption that genetic association with a phenotype is most attributable to common genetic variants- those with a frequency greater than 1%. Most of our
knowledge with respect to genetic variants is largely restricted to common variants because rare variants are difficult to identify.

The variation in ethnicity among participants in this study is a further limitation. While populations have very similar genomes, they have very different sets of genetic variants. The correlation between markers close together in the genome (linkage disequilibrium) differs between populations so a marker highly correlated with a particular phenotype in one population may only be weakly or not at all associated with the phenotype is other populations. Therefore, a sample containing a mixture of different populations can confound the identification of phenotype-associated markers. Most of the knowledge with respect to genetic variations has been obtained from Europeans and very little is known about genetic variations in non-Europeans. This study should be repeated with a sample population of homogeneous ancestry. Future research should aim to uncover genetic variants in non-European populations.

Current technology is unable to accurately detect most copy number variants (CNVs). Copy number variants are deletions and duplications of DNA segments which occur in different sizes and population frequencies. Copy number variations account for a large fraction of genetic variation and are involved in variation in gene expression. It is possible that genetic variants associated with medication response are located in CNVs and were not detected in this study. Copy number variant detection is less accurate, very expensive and CNVs of less than 100,000 base pairs are not reliably detected.
Depression is a heterogeneous disease. There are different types of depressions, most likely with multiple, distinct genetic causes. It is possible that the sample includes patients with different depressions so even though there may be a strong genetic factor associated with treatment response to a particular sub-type of depression, each of these variants will be lost in the noise from the other sub-types of depression. Further research is needed to differentiate between types of depression and categorize patients accordingly.

Future studies should aim to investigate a homogenous population. The severity of depression may be related to the association of genetic variants and treatment response. Previous studies have grouped individuals with severe depression together with individuals with moderate and mild depressions, and possibly those who do not meet the criteria for MDD. It is possible that genetic variation is a better predictor of treatment outcome in those with severe depression versus mild depression. When the severity of depression before treatment is not identified, the significant effect of genetics on treatment outcome in certain depressed populations may be lost.

Furthermore, response to medication should be treated as a continuum, and not as an absolute response or no response. Future studies should avoid the classification of treatment outcome into end-point groups of responders, partial responders and non-responders with an analysis focused on comparing the two categories of response and genotype. Instead, the data should be analyzed using a regression analysis to determine whether genotype is associated with improvement in illness severity.
Finally, future research should focus on the interaction of genetic polymorphisms on treatment response. It is probable that response to medication is not solely related to one genotype, but the combination of particular genotypes at more than one gene.
Chapter 6: Conclusions

This pilot study suggests that genotype at the catechol-\(O\)-methyltransferase, dopamine D2 receptor, serotonin receptor 1A and serotonin transporter genes is not associated with response to citalopram and escitalopram treatment in patients with depression and anxiety. The dopamine D2 receptor polymorphism was associated with medication response at a level which approached significance, indicating that genotype at this site may be related to SSRI response but was not found at a level of statistical significance in this study due to the small sample size. The non-significant results of this study add to the inconsistent finding of previous research, perhaps suggesting that genetic variations are not in fact predictive of response to antidepressants. It is also possible, however, that while variations in candidate genes associated with the pathophysiology of depression are not associated with medication response, variations in other genes unrelated to or unstudied with respect to depression can predict antidepressant response.
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