Time Will Tell

Genetic influences on ejaculation time

Paddy K.C. Janssen

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Cover:

On the front page a DNA helix is placed in a sand glass referring to modern techniques available to unravel the genetic influences on ejaculation time.

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The back page shows a painting of an old Greek myth describing one of the the first cases of premature ejaculation.

“Athena came to Hephaestus desirous to get arms. He, being forsaken by Aphrodite, fell in love with Athena and began to pursue her; but she fled. When he got near her with much ado (for he was lame) he attempted to embrace her; but she being a chaste virgin, would not submit to him, and he dropped his seed on the leg of the goddess. In disgust she wiped off the seed with wool and threw it on the ground. Erichthonius, a mythical king of Athens was conceived when the seed of Hephaestus fell on Gaea, the goddess of earth (Ehrentheil 1974)”

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Genetische invloeden op ejaculatie tijd

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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door
Paddy Koen Camiel Janssen
geboren op 6 juli 1973 te Roermond

Promotoren**:** Prof. dr. M.D. Waldinger
Prof. dr. B. Olivier

Contents

Chapter 1

General introduction

The history of premature ejaculation (PE) is a history of contrasting hypotheses and sometimes vehement debates among medical specialists and psychologists, but it is also the history of pioneering clinicians and neuroscientists, who all together and throughout the years contributed to a better insight in a syndrome that for a very long time has been neglected in medical sexology and general medicine (Waldinger 2013).

Historical Development of Premature Ejaculation

The phenomenon of premature ejaculation is probably as old as humanity. Writings as early as Greek antiquity made mention of an ejaculatio ante portas (Ehrentheil 1974). But it was not until the late 19th century that the experience was described in the medical literature and conceived as a disorder (Waldinger 1997). In 1887 Gross (Gross 1887) described what is presumably the first case of early ejaculation in the medical literature. A report of the German psychiatrist Krafft-Ebing (Krafft-Ebing 1901) followed in 1901 and referred to an abnormally fast ejaculation but did not yet use the word “praecox”or “premature”.
Waldinger (Waldinger 2004) distinguished four periods in the course of the past century, and three partly contrasting approaches – a somatic (urological or physiological), a psychological (psychoanalytic or behaviouristic) approach, and a neurobiological-genetic approach.

Chronological Classification

1) The first period (1917 to 1950): neurosis and psychosomatic disorder

In 1917 Karl Abraham (Abraham 1917) described early ejaculation which he called ejaculatio praecox. During the first decades of the 20th century, PE was viewed, especially in psychoanalytic theory, as a *neurosis* linked to unconscious conflicts (Abraham 1917, Stekel 1927). Treatment consisted of classical psychoanalysis. The somatic approach in those years was primarily urological. According to this urological view PE is caused by a hyperesthesia of the glans penis,

a too short frenulum of the foreskin and on changes in the posterior section of the urethra. Advocated treatment ranged from prescription of an anaesthetizing ointment to incision of the frenulum. Such urological causes, however, were thought to be present in no more than 5% of the cases (Schapiro 1943).

In 1943 Bernard Schapiro, a German endocrinologist, argued that PE is a *psychosomatic disturbance* caused by a combination of a psychologically overanxious constitution and "an inferior ejaculatory apparatus as a point of least resistance for emotional pressure" (Schapiro 1943). Schapiro described two types of premature ejaculation. Type B representing a continuously present tendency to ejaculate rapidly from the first act of intercourse, and Type A leading to erectile dysfunction. Many years later both types became distinguished as the primary (lifelong) and secondary (acquired) form of premature ejaculation (Godpodinoff 1989). Interestingly, Schapiro noted that male family members of patients with PE Type B were often also troubled by premature ejaculation (Schapiro 1943).

2) The second period (1950 to 1990): learned behaviour

In the second period, PE was considered as *learned behaviour* (Masters and Johnson 1970). An early ejaculation associated with initial rapid intercourse(s) leads to habituation and creates performance anxiety. Support for this behaviouristic view has been sought in physiological experiments in which the phenomenon of anxiety became emphasised. Although behaviour therapy was still predominantly present in the literature, increasingly more publications on psychoactive drugs, such as clomipramine, as a treatment appeared.

3) The third period (1990 to 2005): neurobiology and psychopharmacology

In 1998, Waldinger et al. (Waldinger, Berendsen et al. 1998, Waldinger, Rietschel et al. 1998) postulated that lifelong PE is a neurobiologically and genetically determined dysfunction, which is related to a diminished central serotonergic neurotransmission and activation or inhibition of specific 5-HT receptors. Waldinger thereby rejected the previous pure psychological and behaviouristic views of the etiology and pathogenesis of lifelong PE. The new neurobiological view was based on the outcome data of a number of animal and psychopharmacological treatment studies on PE (Waldinger 2002).

The introduction of the selective serotonin reuptake inhibitors (SSRIs) in the early 1990s, meant a dramatic change in the treatment of premature ejaculation (Waldinger 2002). The efficacy of these drugs to delay ejaculation, combined with the low side effect profile, have made them first choice, yet off-label, agents to treat PE both at a daily as well as on demand base.

4) The fourth period (2005 to present): genetics

Due to new developments in DNA research, investigations of genetic polymorphisms have become easier to perform in the laboratory. It is in this period, that we have started genetic research of investigating the IELT duration in men with lifelong PE. It is also in this period that for the first time a drug, dapoxetine, becomes officially approved by the European Medicines Agency (EMA) for the treatment of PE (Pryor, Althof et al. 2006) and that other companies have become interested in drug treatment of PE as well.

Authority-based versus Evidence-based Research

In contrast to the opinion- or authority based approach of last century, both the third and fourth period (1990-present) are characterised by emphasis on evidence-based animal and human research, which mainly pertains to psychopharmacological, genetic, neurophysiological, research questionnaires, and clinical research (Waldinger 2004).

The Historical Views on Premature Ejaculation

The various and sometimes even conflicting views on the etiology and pathogenesis of PE have throughout the years resulted in a lack of consensus on its definition and classification. However, although the International Society for Sexual Medicine (ISSM) has recently reached a consensus on the definition of lifelong PE and acquired PE and a guideline for an evidence based treatment approach of PE has been formulated (McMahon, Althof et al. 2008, Althof, Abdo et al. 2010, Serefoglu, McMahon et al. 2014), it remains important to understand the various ideas and approaches of PE that have emerged in the last century and which have influenced various generations of medical specialists, psychologists and sexologists.

The views are the psychoanalytic, the psychosomatic, the behaviouristic, the medical, the neurobiological, and the genetic approach.

1)The Psychoanalytic Approach

In 1908, Sandor Ferenczi (Ferenczi 1955), at that time a student of Sigmund Freud, wrote the first psychoanalytic paper on PE. In that paper he paid specific attention to the consequences of PE for the female partner. It was in 1917 that Karl Abraham, an, at the time, renowned psychoanalyst, published a now well-known paper on the presumed unconscious problems of men suffering from PE (Abraham 1917). He also introduced the medical term ejaculatio praecox to denote this phenomenon. Since Abraham was of the opinion that PE was caused by unconscious conflicts he suggested that treatment ought to consist of classical psychoanalysis (Abraham 1917). After Karl Abraham's publication, PE was generally believed to be a psychological disorder, e.g. *a neurosis,* related to unconscious conflicts. For many years psychoanalysis and psychoanalytic psychotherapy became the treatment of first choice. However, only a few publications on psychoanalytic treatment of PE have been published (Abraham 1917, Stekel 1927, Embiricos 1950). Although it may seem rather odd nowadays to focus on merely psychoanalysis to treat PE, one should realize that in the 1920s, hardly anything was known about PE, and that for example a distinction in lifelong and acquired PE had not yet been made. Undoubtedly, the lack of current neurobiological and psychoanalytic knowledge in those days, has, in retrospect, negatively biased the way Karl Abraham interpreted the free associations of his patients who suffered from PE at that time (Waldinger 2006).

2)The Psychosomatic Approach

A purely psychoanalytical explanation was challenged by Bernard Schapiro, a German endocrinologist, who in 1943 postulated that PE was not the expression of a neurosis but a psychosomatic disorder (Schapiro 1943). He argued that both biological and psychological factors contributed to rapid ejaculatory performances. Years ahead of his time, Schapiro advocated drug treatment in the form of anaesthetic ointments to delay ejaculation. In addition, he is credited with distinguishing the two types of PE recognized today as primary (lifelong) and secondary (acquired) PE.

Because he was the first clinician to use a medical approach to PE, Bernhard Schapiro should be regarded as a pioneer in researching this condition. Unfortunately, the accurate differential diagnosis and biological components of Schapiro's arguments were ignored in his time. Psychoanalytic treatment, mainly conducted by psychiatrists, prevailed throughout the 1940s and 1950s.

3)The Behaviouristic Approach

In 1956, James Semans (Semans 1956), a British urologist, described the stop-start technique, a masturbation technique, to delay ejaculation. Although hardly noticed in the following decade, in 1970, William Masters and Virginia Johnson (Masters and Johnson 1970), came up with a modification of Semans technique, the socalled squeeze technique. They argued that PE was the result of self-learned behaviour, as it was assumed that the initial intercourses in these men had been carried out in a hurry. They stated that behavioural treatment in the form of the squeeze technique could cure PE in the majority of cases (Masters and Johnson 1970). However, there still is a paucity of evidence-based studies demonstrating hard data of its efficacy to delay ejaculation in men who for example ejaculate within a few seconds. In the psychological approach pathogenetic biological mechanisms remained unclear, but an increased sensitivity of the glans penis has been suggested. However, penile vibratory studies provided conflicting data about a pathogenetic penile hypersensitivity (Rowland, Haensel et al. 1993, Xin, Chung et al. 1996, Paick, Jeong et al. 1998). Not only the squeeze technique, but all sorts of psychotherapies ranging from thought stopping (Ince 1973, Wish 1975), Gestalt therapy (Mosher 1979), transactional analysis (Waltzlawick, Weakland et al. 1974), group therapy (Kaplan, Kohl et al. 1974, Zeiss, Christensen et al. 1978) and bibliotherapy (Lowe and Mikulas 1996) have been suggested as treatment. Unfortunately, the effectiveness of these therapies has only been suggested in case reports, but have hardly been investigated in well-designed controlled studies using a stopwatch to measure the actual ejaculation time. Of all these treatments, however, the squeeze method is said to give rise to short-term effectiveness. Two (not well-designed) studies did confirm initial effectiveness, but also showed that the ejaculatory control initially attained had virtually been lost after a 3-year follow-up (De Amicis, Goldberg et al. 1985, Hawton and Catalan 1986).

Definition of Premature Ejaculation According to Psychological View

In the psychological approach, consensus about a definition of PE has never been reached due to conflicting ideas about the essence of the syndrome. Masters and Johnson (Masters and Johnson 1970) and Kaplan (Kaplan 1974) suggested qualitative descriptions, e.g. female partner satisfaction or man's voluntary control. Masters and Johnson defined PE as the man's inability to inhibit ejaculation long enough to satisfy his partner in 50% of the time (Masters and Johnson 1970). This definition in terms of a partner's response is rather inadequate, since it implies that any male who is unable to satisfy his partner in 50% of sexual events could be labelled a premature ejaculator and since it would also imply that females "should" be satisfied on 50% of intercourses.

Another way to define PE is by using quantitative measures such as the duration of ejaculatory latency, or the number of thrusts prior to ejaculation. Definitions according to length of time prior to ejaculation, varied from within 1 to 7 minute after vaginal intromission (Obler 1973, LoPiccolo 1978, Zeiss, Christensen et al. 1978, Kilmann and Auerbach 1979, Schover, Friedman et al. 1982, Cooper and Magnus 1984, Spiess, Geer et al. 1984, Strassberg, Kelly et al. 1987, Trudel and Proutx 1987, Strassberg, Mahoney et al. 1990). These cut-off points (1 to 7 minutes) were not derived by objective measurements, but were subjectively chosen by the various authors. PE was a matter of (many) minutes and men who ejaculated within seconds were qualified as serious cases. Equally subjective cut-off points have been proposed for the number of thrusts as a criterion for PE: ejaculation within 8-15 thrusts (Colpi, Fanciullacci et al. 1986, Fanciullacci, Colpi et al. 1988, Segraves, Saran et al. 1993).

Methodology of Psychological Studies

During the many years in which the psychological approach prevailed, the proposed psychological hypotheses and psychotherapeutic treatments failed to be proved in a methodologically adequate scientific study (Assalian 1994). For example, an influential view that prevailed for about two decades was the opinion of Masters and Johnson (Masters and Johnson 1970) who argued that PE was conditioned by having one's first sexual intercourse in a rapid way (e.g. hurried contacts on back seats of cars or in places where detection was possible). However, hard clinical data to support their view have never been reported.

4) The Medical Approach

Pharmacotherapy: Since the nineteen forties, case reports have occasionally been published about various drugs that demonstrated efficacy in delaying ejaculation. Physicians tried to reduce penile sensation and delay ejaculation by applying *local anaesthetics* to the glans penis (Schapiro 1943, Damrau 1963). Others tried to influence the peripheral sympathetic nervous system by prescribing *sympatholytic drugs* like the α_1 and α_2 adrenergic blocker phenoxybenzamine (Homonnai, Shilon et al. 1984, Shilon, Paz et al. 1984, Beretta, Chelo et al. 1986) or the selective α_1 adrenergic blockers alphuzosin and terazosin (Cavallini 1995). In the nineteen sixties, case reports described the ejaculation delaying effects of some *neuroleptics*. For example, thioridazine (Freyhan 1961, Singh 1961) and chlorprothixene (Ditman 1964) delayed ejaculation by blocking central dopamine receptors. In the same period case reports of the delaying effects of nonselective, irreversible *monoamine oxidase inhibitors* (MAOIs), e.g. isocarboxazid (Bennett 1961) and phenelzine (Rapp 1979) were published. The use of these drugs, however, was often contraindicated by their disturbing and sometimes quite serious side-effects.

In 1973, Eaton published the first report on clomipramine as an effective treatment for premature ejaculation (Eaton 1973). Later case reports and double-blind studies (Goodman 1980, Porto 1981, Girgis, El-Haggar et al. 1982, Assalian 1988, Althof 1995, Althof, Levine et al. 1995, Haensel, Rowland et al. 1996) repeatedly demonstrated the effectiveness of clomipramine in low daily doses in delaying ejaculation. In 1993, Segraves and coworkers published a double-blind placebo-controlled study demonstrating that clomipramine 25-50 mg can even be taken on an on-demand basis, approximately six hours prior to coitus (Segraves, Saran et al. 1993). The majority of these pharmacological studies, similar to psychological studies, were designed without a precise definition of PE and without any methodology for quantifying the effects of treatment.

In the 1980s, the efficacy of clomipramine was recognized by some sexologists but never reached international consensus. One may wonder why drug treatment has gone such a long way to become accepted by medical specialists and sexologists as an effective treatment for PE. Indeed, the psychological view and particularly behaviour therapy has predominated the literature and the general view on PE for a number of decades.

On the one hand it may well be that animal research data showing the neurobiological basis of ejaculation has hardly been integrated with clinical experiences regarding drug treatment. This may have been due to clinicians'emphasis on the tremendous successes ascribed to behaviour therapy and/or on the presumed psychogenic nature of PE. This may have been due to the prevailing misconception of the 1970s and 1980s that psychopharmacotherapy only represses symptoms, while the essence of the disorder that had to be treated, e.g. PE, remains psychological (Waldinger 1997). A similar view that treatment with psychoactive drugs does not change the essence of a disorder also prevailed for a long time with respect to psychiatric disorders (Zegerius and Waldinger 1995).

Selective Serotonin Reuptake Inhibitors (SSRIs): In 1994, successful treatment of PE by 40 mg paroxetine was for the first time reported in a placebo-controlled study (Waldinger, Hengeveld et al. 1994). The efficacy of paroxetine in daily doses of 20-40 mg has been replicated in various other studies both at regular daily dose and on an "on-demand" regimen (Ludovico, Corvasce et al. 1996, Waldinger, Hengeveld et al. 1997, McMahon and Touma 1999). In addition, the efficacy of other SSRIs, such as 50-200 mg sertraline and 20 mg fluoxetine, in delaying ejaculation has been demonstrated in various studies (Mendels, Camera et al. 1995, Kara, Aydin et al. 1996, Lee, Song et al. 1996, Haensel, Klem et al. 1998, McMahon 1998, Kim and Paick 1999). The new methodology of these studies contributed to a better comparability of drug treatment study research and encouraged various clinicians to become interested in PE. An important parameter for comparing study results was the intravaginal ejaculation latency time, a measure introduced by Waldinger et al. in 1994 (Waldinger, Hengeveld et al. 1994) and known as the IELT. The IELT was defined as the time between the start of intravaginal intromission and the start of intravaginal ejaculation. The stopwatch, originally introduced in 1973 by Tanner (Tanner 1973) as an accurate tool to measure ejaculation time, has been reintroduced in the 1990s and has since become a standard tool for PE research.

Differential efficacy of SSRIs in delaying ejaculation: By using the IELT, the stopwatch and a 4-week baseline assessment at each intercourse, comparison of placebo-controlled studies have become possible and demonstrated that the various SSRIs differed in the extent in which they delayed ejaculation (Waldinger,

Zwinderman et al. 2004). As such it was demonstrated that paroxetine 20 mg/day exerted the strongest ejaculation delay (Waldinger, Zwinderman et al. 2004).

5) The Neurobiological Approach

The development of accurate measurement of the ejaculation time by using the IELT and a stopwatch together with the availability of the SSRIs have stimulated both human and animal psychopharmacological research of PE. Particularly in the 1990s animal research in rodents using SSRIs contributed much to our understanding of why SSRIs delay ejaculation (Mos, Mollet et al. 1999, Waldinger, van De Plas et al. 2002). The pharmacological knowledge about the mechanism of action of these SSRIs has become the cornerstone of an upcoming neurobiological approach. These animal studies have shown that ejaculation is not only mediated by the central serotonergic system but also by dopaminergic and oxytocinergic pathways (Li, Brownfield et al. 1993, Veening and Coolen 1998, Cantor, Binik et al. 1999, de Jong, Veening et al. 2007, Veening and Olivier 2013, Veening and Coolen 2014).

One of the major unanswered questions in the 1990s was the distribution of the IELT in the general male population. Waldinger et al. (Waldinger, Berendsen et al. 1998) postulated that there is a continuum or biological variability of the IELT in men. This continuum of ejaculation latency was first recognized in male Wistar rats (Pattij, de Jong et al. 2005). By investigation of large samples of male rats it appeared that in a sexual behavioural test of 30 minutes about 10% of rats hardly or do not ejaculate, about 10% of rats have a short ejaculation latency time and the majority have a normal ejaculation latency time. This phenomenon that is present in every large sample of male Wistar rats, has become the basis of a new animal model for the investigation of both lifelong premature and retarded ejaculation (Pattij, Olivier et al. 2005). In 2005, it was the first time that an epidemiological stopwatch study measuring the IELT was performed in the general human male population (Waldinger, Quinn et al. 2005). It confirmed the existence of a variability of the IELT, but is also showed that the IELT has a positive skewed distribution.

In two similar studies, the same skewed distribution was found (Pryor, Althof et al. 2006, Waldinger, McIntosh et al. 2009).

These studies showed that IELT values of less than 1 minute are statistically aberrant compared to the IELT values in the general male population.

Interestingly, both stopwatch and self-reported studies of the IELT in men with lifelong PE show that about 90% of men ejaculate within 1 minute as well, indicating that IELTs of less than 1 minute give rise to bother and complaints and indeed are statistically abnormal (Waldinger, Hengeveld et al. 1998).
The finding of a population based variability of the IELT implicates that rapid ejaculation should be considered a biological phenomenon rather than a behavioural aberration. This biological phenomenon is most probably differently appreciated among individuals, populations and cultures. There are men and women who cope very well with rapid ejaculation and do not find it a major problem. But for other men and their sexual partners rapid ejaculation may become a psychological or emotional problem.

Classification and Definition of Premature Ejaculation

For many years, the various DSM definitions of PE were considered adequate for daily clinical use. However, together with the increasing research into PE of the last two decades, increasing criticism against the DSM definition was uttered by clinicians and neuroscientists (Waldinger and Schweitzer 2006). It soon became well-known that the DSM definition of PE was not the result of evidence-based research but was based on the opinions of a few clinicians and therefore an example of authority-based medicine. The International Society for Sexual Medicine (ISSM) made history in 2007 by organizing a committee of experts in the field, who during a weekend in Amsterdam convened and finally agreed on a first, evidence-based definition of lifelong PE (McMahon, Althof et al. 2008). Another major contribution of the ISSM was the publication in 2009 of the first evidence-based guideline for the treatment of PE (Althof, Abdo et al. 2010). Recently, the ISSM also succeeded in formulating a new definition of acquired PE (Serefoglu, McMahon et al. 2014). Without doubt, both the new definitions of lifelong and acquired PE and the guideline for PE treatment will form a new basis for further evidence-based research of PE. Apart from this new definition of lifelong PE, Waldinger et al. (Waldinger and Schweitzer 2008) proposed a new classification of PE based on the duration of the IELT and the frequency of rapid ejaculations. In this classification, there are four PE subtypes.
First of all, lifelong PE and acquired PE. Both subtypes have become an integral part of PE since their description by Schapiro in 1943 (Schapiro 1943). However, based on recent clinical and epidemiological stopwatch data,

Waldinger postulated the existence of two other PE subtypes: variable PE and subjective PE (Waldinger and Schweitzer 2008, Waldinger 2013). Men with lifelong PE suffer from IELTs consistently shorter than about a minute in most sexual events, since puberty or adolescents. In men with acquired PE, it may be caused by erectile dysfunction, thyroid disorders, prostatitis or relationship problems. In variable PE, men suffer only sometimes of a very short IELT. In subjective PE men have a normal or even high IELT value, but despite these IELT durations still perceive themselves as having PE. Whereas it is postulated that the very short IELT values in men with lifelong PE result from neurobiological processes and genetic factors, it has been postulated that subjective PE is strongly associated with psychological and cultural factors. In these men, IELT is normal but the perception of the IELT is distorted or disturbed. Although there is not yet general consensus on this proposal for a new classification, Serefoglu et al. published two studies confirming the existence of the four PE subtypes in a Turkish population (Serefoglu, Cimen et al. 2010, Serefoglu, Yaman et al. 2011). The existence of these four PE subtypes, with remarkable similar prevalencies, has also been found in a Chinese population (Gao, Zhang et al. 2013, Zhang, Gao et al. 2013).

Prevalence of Lifelong PE in the Netherlands

Among men with PE there is an enormous taboo to talk about PE. For example, in the study of Waldinger et al. (1998) only 14 (12.7%) out of 110 men were willing to talk about PE with first degree male relatives. A similar percentage was found in the study of Porst et al (Waldinger 2007), in which only 9% of men with PE were willing to seek medical treatment.

According to the studies of Serefoglu et al (Serefoglu, Cimen et al. 2010) and Gao et al (Gao, Zhang et al. 2013), 2-3% of men suffer from lifelong PE in Turkey and a Chinese province, respectively. Notably, comparable large scale epidemiological studies on the prevalence of lifelong PE and other PE subtypes in the Netherlands has so far not been conducted. Nevertheless, accepting a prevalence of lifelong PE of 3%, and based on the Centraal Bureau van Statistiek (CBS) data on the ages of all male inhabitants living in the Netherlands in 2013,

a prevalence of lifelong PE can be roughly estimated. The age composition in the Netherlands in 2013 of males, aged between 18 to 70 years, was 5.806.000.

With a prevalence of 3%, the number of men presumably suffering from lifelong PE is around 175,000. Considering that only 9% of these men is actively seeking medical treatment, around 16,000 males are supposed to have been seeking medical treatment. On the other hand, in 2013, around 1.8 million males were present in the age class till 18 years. When 1.5% or 3% of the general male population are considered to suffer from lifelong PE, it can be calculated that of the current young men under the age of 18 years, around 26.500 men or 53.000 men, resp., will suffer from lifelong PE after they have come to an age of 18 years. Unfortunately, there are no hard data available that our calculated number of around 16,000 males (which is only 9% of the calculated 174.000 men, aged between 18 to 70 years, that is calculated to suffer from lifelong PE), was indeed seeking medical treatment for lifelong PE. Considering the difference between the number of men that is calculated to suffer from lifelong PE, that is supposed to seek treatment, and the ignorance of general physicians about PE, it may well be that either the current prevalence rate of lifelong PE is too high or that even less than 9% of men seek medical treatment in the Netherlands. For example, in contrast to the studies of Serefoglu (Serefoglu, Cimen et al. 2010) and Gao (Gao, Zhang et al. 2013), both of which have been performed with a questionnaire, Waldinger et al (2005; 2009) has performed two epidemiological studies in 5 countries (The Netherlands, UK, Spain, Turkey and the US), using a stopwatch method. In both studies that were performed in a random group of heterosexual men and their sexual partners, the couples were only requested to measure the IELT with a stopwatch during an one month period. In the first and second study, it was shown that 2.5% ejaculated within 1.5 and 1.0 minute, respectively. In both studies, 0.5% ejaculated in 1 min and 10 seconds, respectively. The median IELT in these studies were 5.4 and 6.0 minutes, respectively (Waldinger et al, 2005; 2009). In case that the prevalence of lifelong PE in the general male population is for example 1,5%, still around 87.000 men between 18 and 70 years, would suffer from lifelong PE in 2013, and if 9% would be willing to seek treatment, it is calculated that around 8,000 would have sought treatment for their complaints. However, considering the anecdotal very low percentage of men visiting their GP in the Netherlands for seeking medical treatment for PE, even the number of 8000 seems to be rather high. Therefore, it may be questioned whether in the Netherlands the prevalence of lifelong PE is not even lower than 1.5%. On the other hand it might well be that even a lower number than 9% of men is seeking medical treatment for lifelong PE.

However, considering that lifelong PE is a rather distressing disorder, it is difficult to understand that the majority (=90%) of the calculated 174.000 men (=3% prevalence) or of the 87.000 men (=1.5% prevalence) are not willing to seek medical treatment. The argument of only the taboo or problems to discuss PE with a GP being responsible for not seeking treatment in such a large group of men of different ages, is difficult to accept. Therefore, it is suggested that the prevalence of lifelong PE might be lower than currently estimated. For example, a prevalence of 0.5% for men ejaculating within 1 minute has been found in the first epidemiological stopwatch study by Waldinger et al. (2009). Other epidemiological stopwatch studies are required to investigate the real prevalence of lifelong PE in the general male population.

6)The Genetic Approach

In 1943, Bernard Shapiro (Schapiro 1943) noticed that men with PE Type B seemed to have family members with similar ejaculatory complaints. Remarkably, this interesting observation has never been quoted in the literature until it was mentioned in 1998 in a study performed by Waldinger et al. (Waldinger, Rietschel et al. 1998) who routinely asked 237 men with PE about family occurrence of similar complaints. Due to embarrassment, only 14 of them consented to ask male relatives about their ejaculation. These 14 men were able to point out a total of 11 first degree male relatives with available information for direct personal interview. Indeed, ten of them also ejaculated within one minute or less. The calculated risk in this small selected group of men to have a first relative with PE was 91 % (CI: 59-99%). The odds of family occurrence is therefore much higher compared to a suggested population prevalence rate of 2-39%. Moreover the high odds indicate a familial occurrence of the syndrome far higher than by chance alone. Based on this preliminary observation the influence of genetics as formerly stated by Bernard Schapiro, gains substantial credibility. Notably, in 1998, based on animal sexual psycho-pharmacological research data, Waldinger et al. (Waldinger, Berendsen et al. 1998) postulated that the duration of the IELT in men with lifelong PE, defined in terms of an IELT that occurs consistently within 1 minute, is associated with a diminished 5-HT neurotransmission, a hyperactivity of 5-HT_{1A} receptor functioning and a diminished 5-HT_{2C} receptor functioning.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis was to investigate whether genetic polymorphisms of the central serotonergic (5-hydroxytryptamine; 5-HT) system influence the duration of the intravaginal ejaculation latency time (IELT) in men with lifelong PE. Our main focus was the investigation of the influence of genetic polymorphisms of the 5-HT transporter promoter gene, the 5-HT_{1A} and 5-HT_{2C} receptor gene on the duration of the IELT in heterosexual men both during a drug-free baseline period and during daily paroxetine treatment. The studies have been performed according to a strict method of using a stopwatch, handled by the female partner, at home at each intercourse.

In **Chapter 2** polymorphisms of the 5-HT-transporter (5-HTT) gene were investigated in 89 men with lifelong PE. Their genotype frequencies were compared with the genotype distribution of the 5-HTT gene polymorphism of the general male population in the Netherlands and were also compared with the duration of their IELT, as measured with a stopwatch throughout a one month period. The aim of this study was to investigate whether there is a difference in genotype distribution of this gene polymorphism between men with lifelong PE and the control group and whether and how the duration of the IELT in these men is associated with the genotype of this polymorphism.

In **Chapter 3** the influence of the C(1019)G polymorphism of the 5-HT_{1A} receptor gene on the duration of the IELT was investigated in 54 men with lifelong PE. The aim of this study was to investigate whether and how the duration of the IELT in these men is associated with the genotype of this polymorphism.

In **Chapter 4** the role of Cys23Ser polymorphism of the 5-HT_{2C} receptor on the duration of the IELT was investigated. As this polymorphism is only present at the X chromosome, only homozygote wildtypes and mutants were measured in these men. Similar to both studies reported in chapter 2 and chapter 3, the aim of the study in chapter 43 was also to investigate whether and how the duration of the IELT in these men is associated with the genotype of the 5-HT_{2C} receptor polymorphism.

The study reported in **Chapter 5** had two aims. First of all to investigate the percentage of successful clinically relevant ejaculation delay induced by daily use of paroxetine 20 mg/day by men with lifelong PE. Secondly, the aim was to investigate whether there is an association between paroxetine-induced ejaculation delay in men with lifelong PE, expressed in the fold-increase of the geometric mean IELT compared to baseline values, and polymorphism of the 5-HT transporter gene.

In **Chapter 6,** complete ejaculation delay non-response to paroxetine and another serotonergic antidepressant treatment, is investigated. The participating men were distinguished in paroxetine responders and paroxetine non-responders. The aim of this study was to investigate some parameters, such as paroxetine and prolactine serum concentrations, that might be involved in a rather rare phenomenon, which consists of the absence of ejaculation delay while using different SSRIs.

Chapter 7 describes our investigation of the methodology and design of 6 studies that have recently been published on 5-HTTLPR polymorphism and PE. The aim of our study was to investigate whether these six studies on 5-HTTLPR polymorphism and PE are suitable for inclusion in a meta-analysis. In our investigation we focused on Hardy-Weinberg equilibrium and PCR analysis as reported in these 6 studies.

Finally, in **Chapter 8** all our findings are summarized and discussed in a broader context.

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Chapter 2:

Serotonin Transporter Promoter Region (5-HTTLPR) Polymorphism is Associated with the Intravaginal Ejaculation Latency Time in Dutch Men with Lifelong Premature Ejaculation

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ABSTRACT

Introduction. Lifelong premature ejaculation (LPE) is characterized by persistent intravaginal ejaculation latency times (IELTs) of less than 1 minute, and has been postulated as a neurobiological dysfunction with genetic vulnerability for the short IELTs, related to disturbances of central serotonin (5-hydroxytryptamine [5-HT]) neurotransmission and 5-HT receptor functioning.

Aim. To investigate the relationship between 5-HT transporter gene-linked polymorphism (5-HTTLPR) and short IELTs in men with lifelong PE.

Methods. A prospective study was conducted in 89 Dutch Caucasian men with lifelong PE. IELT during coitus was assessed by stopwatch over a 1-month period. Controls consisted of 92 Dutch Caucasian men. All men with LPE were genotyped for a 5-HTT-promoter polymorphism. Allele frequencies and genotypes of short (S) and long (L) variants of 5-HTTLPR polymorphism were compared between patients and controls. Association between LL, SL, and SS genotypes, and the natural logarithm of the IELT in men with LPE was investigated.

Main Outcome Measures. IELT measured by stopwatch, 5-HTTLPR polymorphism.

Results. In men with lifelong PE, the geometric mean, median, and natural mean IELTs were 21, 26, and 32 seconds, respectively. There were no significant differences in the 5-HTT polymorphism alleles and genotypes between 89 Dutch Caucasian men with LPE (S 47%, L 53%/LL 29%, SL 48%, SS 22%) and 92 Dutch Caucasian controls (S 48%, L 52%/LL 29%, SL 45%, SS 26%). In men with lifelong PE there was a statistically significant difference between LL, SL, and SS genotypes in their geometric mean IELT ($P \leq 0.027$); the LL genotypes had significantly shorter IELTs than the SS and SL genotypes.

Conclusions. The 5-HTTLPR polymorphism is associated with significant effects on the latency to ejaculate in men with lifelong PE. Men with SS and SL genotypes have 100% and 90% longer ejaculation time, respectively than men with LL genotypes.

Introduction

Lifelong premature ejaculation (PE) is defined as a male sexual dysfunction characterized by ejaculation that always or nearly always occurs prior to or within about 1 minute of vaginal penetration, the inability to delay ejaculation on all or nearly all vaginal penetrations, and with negative personal consequences, such as distress, bother, frustration, and/or the avoidance of sexual intimacy (Althof, McMahon et al. 2014).

Based on a persistent short intravaginal ejaculation latency time (IELT) of these men of less than 1 minute (Waldinger, Hengeveld et al. 1998) and a strong ejaculation delaying effects of daily selective serotonin reuptake inhibitor (SSRI) treatment (Waldinger, Hengeveld et al. 1998), Waldinger et al. postulated lifelong PE as a neurobiological dysfunction with a genetic vulnerability for short IELTs related to decreased central serotonin (5-hydroxytryptamine [5-HT]) neurotransmission and/or 5-HT receptor dysfunction, i.e., a hypofunction of 5-HT_{2C} and/or hyperfunction of 5-HT_{1A} receptors (Waldinger, Berendsen et al. 1998, Waldinger, Rietschel et al. 1998, Waldinger 2002).

Indirect clinical support for a genetic vulnerability may be derived from a Dutch study in men with lifelong PE with IELTs of less than 1 minute, showing an increased familial occurrence of lifelong PE with IELTs of less than 1 minute in first-degree male relatives (Waldinger, Rietschel et al. 1998); a Finnish male twin questionnaire study showing a moderate genetic influence on PE (Jern, Santtila et al. 2007); and animal studies showing a subgroup of persistent rapidly ejaculating Wistar rats (Pattij, de Jong et al. 2005, Waldinger and Olivier 2005). Animal research (Ahlenius and Larsson 1991, Ahlenius and Larsson 1998) and SSRI-induced ejaculation delay in both men (Waldinger, Zwinderman et al. 2004, Waldinger 2007) and laboratory rats (de Jong, Pattij et al. 2005, de Jong, Pattij et al. 2005) indicate the involvement of central serotonin (5-HT) neurotransmission, including serotonin transporter (SERT; 5-HTT) and 5-HT receptor functioning, in the regulation of the ejaculation time.

Based on these data we postulate that genetic polymorphism of the 5-HTT, 5-HT receptors involved in ejaculation (e.g., 5-HT_{1A} and 5-HT_{2C} receptors), 5-HT receptors involved in 5-HT synaptic autoregulation (e.g., 5-HT_{1A} and 5-HT_{1B} receptors), and 5-HT regulating enzymes (catechol-O-methyl transferase [COMT] and monoamine oxidase [MAO]) determine the regulation of the intravaginal ejaculation time. More specifically, it is postulated that the persistent occurrence of IELTs of less than 1 minute in men with lifelong PE results from a combination of polymorphisms of the aforementioned serotonergic transporter and receptors, and other neurotransmitters and/or receptors. Between 2005 and 2008 we have investigated the polymorphisms of the aforementioned factors in men with lifelong PE. The current article on polymorphism of the 5-HTT is the first report of these investigations.

For a good understanding of this research it is important to have some basic insight into 5-HTT functioning and 5-HTT polymorphism.

The 5-HTT is a specific protein transporter— localized in the cell membrane—that facilitates serotonin reuptake from the synapse, and it is the target of SSRIs that are known to delay ejaculation (Qian, Melikian et al. 1995). Having a high affinity for serotonin, 5-HTT controls the duration, availability, and signaling capacity of 5-HT in the synapse (Qian, Melikian et al. 1995). If short IELTs in men with lifelong PE—but also in men without lifelong PE—is associated with diminished central 5-HT neurotransmission, it may be argued that an increased function of the 5-HTT is related to the occurrence of PE. Such an increased function may be related to genetic polymorphisms of the 5-HTT. Indeed, it has been shown that the SERT gene is polymorphic (Heils, Teufel et al. 1996). The 5-HTT functioning is moderated by a polymorphism in the 5-HTT promoter region of the SERT gene (SCL6A4), which encodes for the SERT (5-hydroxytryptamine transporter-linked promoter region [5-HTTLPR]) (Kunugi, Hattori et al. 1997, Murphy, Lerner et al. 2004, Smith, Lotrich et al. 2004). The 5-HTTLPR gene has two variant alleles: a short (S) and a long (L) allele. The short allele has 44 base pairs (bps) less than the L allele (Heils, Teufel et al. 1996). The transcriptional activity of the L allele has been reported to be twice as high as the S allele (Lesch 2004). The genotypes composed by these alleles are called LL, SS, and SL. If expressed in cell lines, the short (S) allele of the 5-HTT genotype reduces transcriptional efficiency of the 5-HTT gene promoter, resulting in reduced 5-HTT expression and serotonin uptake compared with the long (L) allele (Lesch, Bengel et al. 1996). Notably, the S allele has been associated with a nearly 50% reduction in expression of the SERT protein, vulnerability for mood disorders, inadequate response to SSRIs, and side-effects (Murphy, Hollander et al. 2004, Hariri and Brown 2006). In Caucasians, the genotype frequencies are approximately 25% SS, 47% SL, and 28% LL (Smits, Smits et al. 2004). Theoretically, men with one or more S alleles for the 5-HTT have fewer functioning transporters and could therefore lead to a higher serotonergic neurotransmission. Consequently, it is postulated that men with SS genotype have longer IELT durations than men with LL genotype. The aim of this study was to investigate whether men with lifelong PE have a relative enrichment of the LL 5-HTT polymorphism.

Methods

Patients and Assessments

Included were men who were actively seeking drug treatment for lifelong PE at the Outpatient Department of Neurosexology. The included men came from all parts of the Netherlands. None of them were recruited by advertisement. None of them used or had ever been using drugs, such as SSRIs or clomipramine, for the treatment of lifelong PE.

IELT was defined as the time between the start of vaginal penetration and the start of intravaginal ejaculation (Waldinger, Hengeveld et al. 1994). Lifelong PE was operationally defined as the lifelong presence of an IELT of 1 minute or less after vaginal penetration occurring on more than 90% of occasions of sexual intercourse with every sexual partner together with complaints of inability to delay ejaculation and feelings of frustration about it (Waldinger, Hengeveld et al. 1998, McMahon, Althof et al. 2008). All patients included were heterosexual men, aged 18 to 65 years. In order not to exclude men with particular psychological difficulties related to PE, a stable relationship with a female partner was not required. However, it was required that during the 1-month period of IELT assessments, intercourse should have taken place with the same woman. Patients were not permitted the use of condoms, topical local anesthetic creams or sprays, or excessive consumption of alcohol within 5 hours prior to intercourse. Exclusion criteria included erectile dysfunction, alcohol or substance abuse, mental disorders, physical illnesses affecting ejaculatory functioning, concomitant medications, a history of sexual abuse reported by the patient and/or his partner, serious relationship problems, pregnancy of the partner, or the desire to become pregnant in the near future. Erectile dysfunction was determined by the abbreviated version of the International Index of Erectile Function-5(Rosen, Cappelleri et al. 1999). Patients attended the Outpatient Department approximately 1 month before the start of daily SSRI treatment (first baseline assessment), on the day before treatment (second baseline assessment), and at the end of two consecutive series of 5 weeks of daily SSRI treatment. The partners accompanied the patients on the first and last visit. At the first visit, patients and partners were interviewed individually by the last author (M.D. Waldinger) and asked for an independent estimation of the IELT. A stopwatch and instructions on how to measure the IELT were provided. The couples measured the IELT at home over the following 4 weeks. The female partners had to handle the stopwatch. Couples were instructed not to have interrupted intromission or to change their usual way or frequency of intercourse. If intercourse took place more than once at the time of IELT measurement, only the first occurrence was included. Patients were not recruited by advertisement and were not reimbursed for their participation. All laboratory testing, including blood sampling and genetic testing, were conducted by the first author (P.K.C. Janssen). The study was conducted without any involvement of a pharmaceutical industry. All laboratory facilities and test materials were granted by the two participating laboratories. Informed consent was obtained from all patients after explaining the purpose of the study. The female partner also had to agree to participate in the study. The study was approved by the Hospital Medical Ethical Committee and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983.

The control group consisted of 92 physically and mentally healthy male individuals recruited in another study conducted by the Department of Psychiatry of the Utrecht Medical Center, Utrecht, the Netherlands (Jungerius, Hoogendoorn et al. 2007). All of these control participants had been previously genotyped for the 5-HTTLPR polymorphism. In addition, all male controls had at least three grandparents who were born in the Netherlands. The control group was randomly sampled and is considered representative of the general Dutch population (Jungerius, Hoogendoorn et al. 2007). Neither occurrence of complaints of PE nor stopwatch assessments of IELT has been investigated in the control group.

Genotyping (DNA isolation and Polymerase Chain Reaction [PCR] analysis)

DNA Isolation

Genomic DNA was extracted from 10 mL of EDTA anticoagulated whole blood using a standard salting-out method protocol.

PCR Analysis

The 44-bp insertion/deletion polymorphism within the promoter region of the SERT (SLC6A4) gene was amplified by PCR. The insertion/deletion in the SERT gene-linked polymorphic region (5-HTTLPR) was amplified using the following oligonucleotide primers: forward 5′-GGCGTT GCCGCTCTGAATC-3′, and reverse; 5′-GAG GGACTGAGCTGGACAACCAC-3′, flanking the 5-HTT gene-linked polymorphic region (5-HTTLPR). Corresponding to the nucleotide positions ranging from −1,416 to −1,397 and from −910 to −889 of the 5-HTT gene regulatory region, a 484-bp or a 528-bp fragment was generated.

Reagents and conditions for the PCR were: 1 µL of 10 times polymerase buffer; 0.2 mmol/L deoxyribonucleotide triphosphates; 2.0 mmol/L $MgCl_2$, 0.4 µM mol/L of each primer (Biolegio BV, Nijmegen, the Netherlands); 0.5 U AccuPrime Pfx DNA polymerase (Invitrogen Life Technologies, Strathclyde, UK); and 50 ng of genomic DNA, in a total reaction volume of 10 µL.

The PCR program on a thermal cycler (GeneAMP type 9700; Perkin Elmer, Waltham, MA, USA) was as follows: Reactions were cycled with initial denaturation at 94°C for 4 minutes, followed by 33 PCR cycles of 94°C for 30 seconds, 61°C for 60 seconds, 68°C for 60 seconds, and a final extension step of 4 minutes at 72°C.

The amplification products were electrophoresed on 2% agarose gels at 100 V for 120 minutes. The gel and running buffers were 1× TBE (0.89 m Tris-Base, 0.89 m boric acid, 20 mM Na2EDTA). The fragments were visualized by ethidium bromide under ultraviolet transillumination.

Statistics

The mean, median, and geometric mean IELT was calculated of stopwatch-determined IELTs. Hardy–Weinberg equilibrium to check laboratory efficacy of PCR analysis was determined in the control group and the patient group using a chi-square test. Allele and genotype frequencies between patients and controls were compared using SPSS 15.0 for Windows (Chicago, IL, USA). $P < 0.05$ was considered statistically significant. Statistical power calculations were performed using the Genetic Power Calculator package. Assuming that the risk allele has a frequency of 0.44 in the population, our sample has 83% power to detect a locus with relative risk of 1.5 ($P = 0.05$) (Purcell, Cherny et al. 2003). Analysis of variance (anova) was performed to determine an association between the genotype in the patient group and their IELTs.

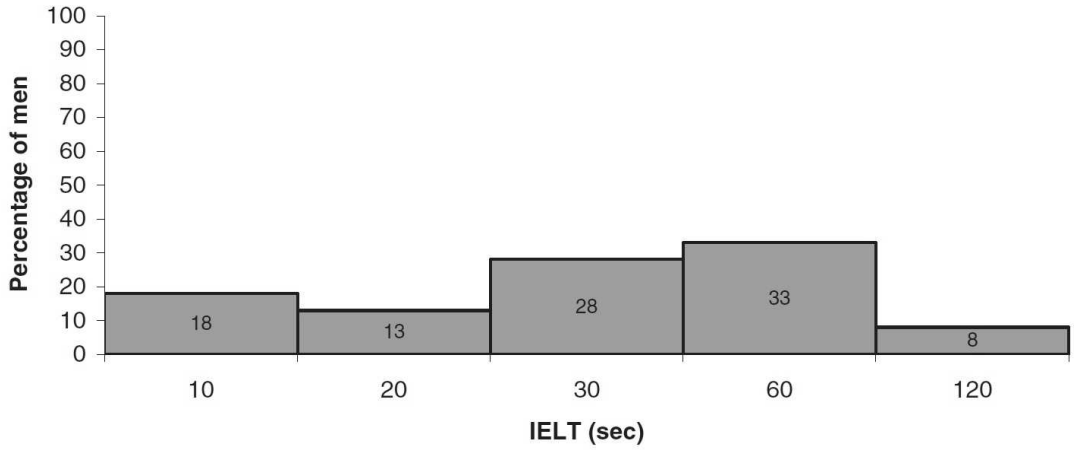
Results

The study included 89 patients and 92 controls. Table 1 shows the characteristics of the men with lifelong PE and the control group. The mean ± standard deviation frequency of intercourse per month was 3.4 (±1.4) ranging from two to eight intercourses. The lifelong PE and control groups differed significantly in age and marital status ($P < 0.05$). Of men with lifelong PE, the majority (92%) ejaculated within 1 minute after vaginal penetration; of all these men, 18% ejaculated within 10 seconds, 13% within 20 seconds, 28% within 30 seconds, and 33% within 60 seconds after vaginal penetration (Figure 1). As seen before, the IELT distribution in this study was skewed with geometric mean, median, and natural mean IELTs 21, 26, and 32 seconds, respectively (Waldinger, Zwinderman et al. 2008). We therefore decided to perform statistical analysis of IELT after logarithmic transformation.

Table 1. Patient and control characteristics

Characteristics	**Patients (N)**	**Controls (N)**	***P***
Population	89	92	
Age (years)			<0.05
Mean	36.0	53.6	
Range	20–61	27–78	
Standard deviation (SD)	9.0	15.3	
Age partner (years)			
Mean	34.3		
Range	21–63		
SD	8.3		
Nationality			
Dutch (Caucasian)	95%	100%	
Marital status			<0.05
Married	43.5%	70.0%	
Relationship but not married	51.9%	30.0%	
No relationship	4.6%	0.0%	
Duration of relation (years)	10.6		
Range	0.1–30		
SD	7.9		
Education			0.36
Low	13.0%	13.0%	
Medium	30.6%	24.6%	
High	56.4%	62.3%	

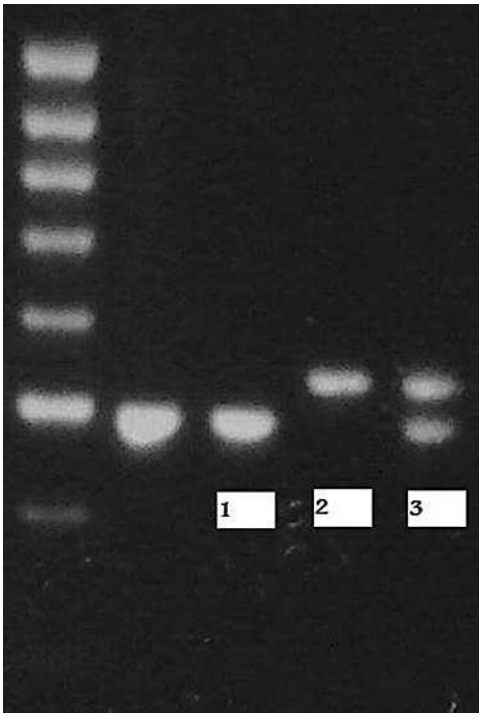
Figure 1.



Intravaginal ejaculation latency time (IELT) distribution in Dutch men with lifelong PE.

The PCR reaction resulted, as expected, in a 528- and a 484-bps long fragments for the “L” and “S” alleles, respectively, resulting in LL, SL, or SS genotypes (Figure 2). Genotyping completeness was 98% in patients and 97% in controls.

Figure 2.



Photograph of illuminating DNA fragments on gel under ultraviolet light. Lane 1: homozygous patient for LL alleles. Lane 2: homozygous patient for SS alleles. Lane 3: heterozygous patient for LS alleles. L = long; S = short.

Hardy–Weinberg equilibrium was not rejected for genotype distributions of the polymorphisms investigated in patients ($P = 0.99$) and controls ($P = 0.59$). Of the 89 men with lifelong PE, 26 (29%) had LL genotype, 43 (48%) had SL genotype, and 20 (22%) had SS genotype. Of the 92 controls, LL, SL, and SS genotype were present in 27 (29%), 41 (45%), and 24 (26%), respectively. No statistically significant differences were found in 5-HTTLPR allelic variations. In addition, no statistically significant differences were found in 5-HTTLPR gene variations. Genotyping and association testing are represented in Table 2.

Table 2. Results of genotyping and association testing

Allele/genotype	Patients		Controls		*P* value
	Count	Frequency (%)	Count	Frequency (%)	
S	97	54.5	89	48.4	0.24
L	81	45.5	95	51.6	
Sum	178	100	184	100	
SS	19	21.3	24	26.1	0.46
SL	43	48.3	41	44.6	
LL	27	30.3	27	29.3	
Sum	89	99.9	92	100	

Anova of the natural logarithm (ln) of IELT showed a statistically significant difference in men with lifelong PE and with LL, SL, and SS genotypes ($P = 0.027$), indicating that men with LL genotypes have a shorter IELT than men with SS and SL genotypes (Table 3).

The geometric mean IELT in the LL, SL, and SS genotypes were 13.2, 25.3, and 26.04 seconds, respectively. The fold increase of the geometric mean IELT in the SS and SL genotype groups compared with the LL genotype group were 2.0 and 1.9, respectively, indicating that men with SS genotypes and SL genotypes, on average, show a 100% and 90% stronger ejaculation delay than men with LL genotypes in this group of men with lifelong PE.

Table 3. Natural logarithm of intravaginal ejaculation latency time (IELT) per genotype in men with lifelong premature ejaculation

Genotype	N	Mean ln IELT(SD)	Geometric mean IELT	95% CI of the geometric
LL	27	2.6 (1.3)	13.2	8.2–22.2
SL/LS	43	3.2 (0.9)	25.3	18.6–32.3
SS	19	3.2 (1.1)	26.0	14.8–40.6
Total	89	3.0 (1.1)	20.1	15.9–25.4

CI = confidence interval; L = long; SD = standard deviation; S = short.

Discussion

The current study showed a strong similarity in 5-HTTLPR polymorphism in men with lifelong PE and male controls. Because Hardy–Weinberg equilibrium was valid in both patient and control group outcome data, it is unlikely that laboratory biases or other disturbances have affected the outcomes. Given the indications for involvement of central serotonergic neurotransmission in regulating the duration of IELT in men with lifelong PE, we have compared functional polymorphisms in the 5-HT transporter gene between men with lifelong PE and mentally and physically healthy controls that were representative for the Dutch male population. The current sample of men with lifelong PE has a similar IELT distribution as has been found in two other IELT studies in Dutch men with lifelong PE, and seems therefore representative for this group of patients: about 60% ejaculates within 30 seconds, and about 90% ejaculates within 1 minute after vaginal penetration (Waldinger, Hengeveld et al. 1998, Waldinger, Zwinderman et al. 2007). In the current study only 8% of men ejaculated between 1 and 2 minutes after vaginal penetration. This low percentage is also similar to the two previous studies in Dutch men in which 10% and 8% ejaculated between 1 and 2 minutes (Waldinger, Hengeveld et al. 1998, Waldinger, Zwinderman et al. 2007). As only 8% of the current cohort of men ejaculated between 1 and 2 minutes, and it is known from previous studies that about 10% of men with lifelong PE report IELTs between 1 and 2 minutes (Waldinger, Hengeveld et al. 1998, Waldinger, Zwinderman et al. 2007, McMahon, Althof et al. 2008), it was decided to also include the 8% of men in the current study in order to avoid investigating a diagnostic criterion rather than a genuine cohort. The healthy controls differed significantly in age and marital status from the patient group. However, for the purpose of the current study of genetic research in men with lifelong PE, this difference in patient characteristics is not regarded as an impediment, as lifelong PE is a chronic ejaculatory dysfunction with similar prevalence among different age groups.

The current study did not show any significant differences between patients and controls regarding 5-HTTLPR polymorphism. This suggests that the current sample of men with lifelong PE is representative of the Dutch male population. However, the control group was not investigated on the existence of lifelong PE and it is likely that the control group includes about 2.5% of men with lifelong PE and IELTs of less than 1 minute (Waldinger, Quinn et al. 2005, Waldinger, Zwinderman et al. 2005). Interestingly, the current study showed that the IELT in men with lifelong PE is associated with 5-HTT polymorphism, i.e., that men with LL genotype have a significantly shorter IELT than men with SS and SL genotypes. This finding is in line with psychopharmacological knowledge on central serotonin neurotransmission. Theoretically, men with LL genotype have more (or better) functioning 5-HT transporters that would correspond with lower synaptic serotonin and consequently lower stimulation of any 5-HT receptor. Animal research has shown that decreased 5-HT neurotransmission is associated with facilitated ejaculation latencies (Ahlenius, Eriksson et al. 1971, Gessa and Tagliamonte 1974, Dahlof and Larsson 1979, Qureshi, Forsberg et al. 1989, Lesch, Balling et al. 1994). It is remarkable that in a group of men with extremely short ejaculation times, a serotonin neurotransmission influencing polymorphism still has such a strong effect. The current study has shown that the geometric mean IELT in the LL, SL, and SS genotypes were 13.2, 25.3, and 26.04 seconds, respectively. The fold increase of the geometric mean IELT in the SS and SL genotype groups compared with the LL genotype group was 2.0 and 1.9, respectively, indicating that men with SS genotype and SL genotype, on average show a 100% and 90% longer ejaculation time than men with lifelong PE and with LL genotype. In the current group of men with lifelong PE, the median IELT was 26 seconds. In contrast, stopwatch assessment of the IELT in the general male population yielded a median IELT of 5.4 minutes (Waldinger, Quinn et al. 2005), indicating a difference of 5 minutes compared with the median IELT in men with lifelong PE. Although we have found that the IELT in men with lifelong PE is associated with 5-HTTLPR polymorphism—indicating a 100% shorter IELT in men with LL genotype compared with the IELT in men with SS and SL genotypes—it is assumed that, based on the difference of 5 minutes with the median IELT in the general male population, apart from 5-HTTLPR polymorphism, also other genetic and possibly nongenetic factors may be involved in the regulation of the IELT. This is also more in line with our hypothesis that the very short IELTs in men with lifelong PE result from a combination of different polymorphisms in central serotonergic neurotransmission, enzymes involved in serotonergic metabolism, serotonergic receptors related to ejaculation functioning, and serotonergic receptors involved in synaptic autoregulation. For example, it may well be that the regulation of the IELT is also regulated by the second polymorphism in the 5-HTT gene, the variable tandem of repeat numbers, located in the second intron of the 5-HTT gene (Lesch, Balling et al. 1994).

But it may also be that polymorphism of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} receptors, and polymorphism of COMT and MAO play a role in regulating the IELT. These factors are currently investigated by our group. The current study shows evidence that polymorphism of the 5-HTTLPR plays a role in the regulation of the duration of the IELT in men with lifelong PE. However, there currently is no evidence that polymorphism of the 5-HTTLPR forms the genetic basis of lifelong PE. The current study shows that within the group of men with lifelong PE with IELTs of less than 1 minute, one can distinguish men with persistently rapid and ultrarapid ejaculations dependent on the 5-HTTLPR polymorphism genotype. A limitation of the current study is the absence of stopwatch measurement of the IELT in the control group. However, an interesting option derived from the current findings is whether all human males show a 5-HTTLPR polymorphism-dependent IELT that is superimposed upon a basic ejaculation time. The latter seems independent from serotonin neurotransmission and, although nothing is known about its underlying mechanisms, it could be determined or modulated by genetic and/or nongenetic factors. Twin studies could help in unraveling these extremely interesting questions.

Conclusion

This is the first study investigating 5-HTTLPR genotypes in relation to the IELT in men with lifelong PE. The study shows evidence that 5-HTTLPR polymorphism is associated with the IELT in men with lifelong PE. Men with LL genotypes have statistically shorter IELTs than men with SS and SL genotypes. The current study shows, for the first time, the clinical notion and our hypothesis of genetic influences on the IELT in men with lifelong PE. We postulate that apart from 5-HTTLPR polymorphisms other genetic factors are also involved in the regulation of the IELT. Further genetic research in this group of men is warranted. In this respect, genetic research on 5-HT receptors associated with ejaculation and synaptic autoregulation, and enzymes involved in 5-HT metabolism is currently being further investigated by our group.

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Chapter 3:

The 5-HT_{1A} Receptor C(1019)G Polymorphism influences the Intravaginal Ejaculation Latency Time in Dutch Caucasian Men with Lifelong Premature Ejaculation

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ABSTRACT

Introduction. Lifelong premature ejaculation (LPE) is characterized by persistent intravaginal ejaculation latency times (IELTs) of less than 1 minute, and has been postulated as a neurobiological dysfunction related to diminished serotonergic neurotransmission with 5-HT_{1A} receptor hyperfunction and 5-HT_{2C} hypofunction.

Aim. To investigate the relationship between 5-HT_{1A} receptor gene (HTR1A)-C(1019)G promoter polymorphism and IELT in men with LPE. This polymorphism is known to increase 5-HT_{1A} receptor expression.

Methods. A prospective study was conducted in 54 Dutch Caucasian men with LPE. Baseline IELT during coitus was assessed by stopwatch over a 1-month period. All men were genotyped for HTR1A gene polymorphism. Allele frequencies and genotypes of C and G variants of HTR1A polymorphism were determined. Association between CC, CG, and GG genotypes, and the IELT in men with LPE were investigated.

Main Outcome Measures. IELT measured by stopwatch, HTR1A polymorphism

Results. In this cohort of men with LPE, the geometric mean IELT was 23.8 seconds. Of the 54 men, the CC, CG and GG genotype frequency for the C(1019)G polymorphism of the 5-HT1A gene was 33%, 43% and 24%, respectively. The geometric mean IELT for the CC, CG and GG genotypes were 14.5, 27.7 and 36.0 seconds, respectively (p=0.019). Compared to GG and CG genotypes, men with CC genotype had a 250% and 190% shorter ejaculation time, respectively.

Conclusions. HTR1A gene polymorphism is associated with the IELT in men with LPE. Men with CC genotype have shorter IELTs than men with GG and CG genotypes.

Keywords. Lifelong premature ejaculation; 5-HT_{1A} receptor gene C(-1019)G polymorphism; Genetics; Genotype; IELT

Introduction

Lifelong premature ejaculation (PE) is defined as a male sexual dysfunction characterized by ejaculation that always or nearly always occurs prior to or within about 1 minute of vaginal penetration, the inability to delay ejaculation on all or nearly all vaginal penetrations, and with negative personal consequences, such as distress, bother, frustration, and/or the avoidance of sexual intimacy (McMahon, Althof et al. 2008). The prevalence of lifelong PE in the general male population is 2.5-3% (Serefoglu, Yaman et al. 2011, Gao, Zhang et al. 2013). Daily treatment by selective serotonin reuptake inhibitors (SSRIs) has shown that SSRIs exert differing ejaculation delaying effects (Waldinger, Zwinderman et al. 2004). Of all SSRIs, daily use of 20 mg paroxetine exerts the strongest ejaculation delay (Waldinger, Hengeveld et al. 1998, Waldinger, Zwinderman et al. 2004). In 1998, Waldinger et al. postulated that lifelong PE in terms of an intravaginal ejaculation latency time (IELT) of less than 1 minute is genetically determined and associated with disturbed central serotonin (5-hydroxytryptamine: 5-HT) neurotransmission, a hypersensitivity of 5-HT_{1A} receptors and/or hypofunction of 5-HT_{2C} receptors (Waldinger, Berendsen et al. 1998, Waldinger, Rietschel et al. 1998, Waldinger 2002). Notably, due to an absence of selective 5-HT_{1A} and 5-HT_{2C} receptor ligands for safe human usage, this hypothesis has sofar not been confirmed in humans. However, as ejaculation is facilitated by selective 5-HT_{1A} receptor agonists in rats (Haensel and Slob 1997), we postulate that functional polymorphisms of the 5-HT_{1A} receptor gene may be associated with the IELT. Various polymorphisms of the 5-HT_{1A} receptor are known. The most well-known polymorphism that has been investigated is the mutation C(-1019)G, indicating that at position 1019 a cytosine base is replaced by a guanine base (Wu and Comings 1999). The gene for the 5-HT_{1A} receptor, HTR1A, is located on chromosome 5q11.2-q13 (Parks and Shenk 1996). The C(-1019)G polymorphism (dbSNP database identification number, rs6295) is a common SNP in the promoter region of the 5-HTR_{1A} gene (Parks and Shenk 1996, Huang, Battistuzzi et al. 2004). This polymorphism is located in the regulatory region of the 5-HT_{1A} receptor promoter (Parks and Shenk 1996, Huang, Battistuzzi et al. 2004), and is part of a 26 basepair imperfect palindrome (Lemonde, Turecki et al. 2003). This palindromic region is recognized by two transcription factors, that operate in an allele-specific manner; the transcription factors bind to the C-allele but not the G-allele. The G-allele is associated with higher expression of 5-HT_{1A} receptors in the raphe nuclei (autoreceptors) and decreased 5-HT release. Several studies have suggested an association between GG genotype and major depression (Parsey, Oquendo et al. 2006, Molina, Cervilla et al. 2011) and anxiety (Molina, Cervilla et al. 2011) and a lower response to SSRIs (Villafuerte, Vallabhaneni et al. 2009). The C(-1019)G 5-HT_{1A} receptor promoter polymorphism is apparently influencing 5-HT neurotransmission,

probably in concord with that induced by the serotonin transporter gene linked promoter region. However, sofar an association between lifelong PE and depression or anxiety disorder has not been found. In addition, it remains to be investigated whether men with lifelong PE and a GG-genotype have a lower ejaculation delaying response than men lifelong PE and CC and CG genotype. The vulnerability of the IELT to modulation of serotonergic neurotransmission as evident in the 5-HTTLPR polymorphismn (Janssen, Bakker et al. 2009), makes it interesting to investigate the polymorphism C(-1019)G of the 5-HT_{1A} receptor gene in the IELT in men with lifelong PE. The aim of the current study is to investigate the relationship between 5-HT_{1A} receptor gene (HTR1A)-C(1019)G promoter polymorphism and IELT in men with LPE, as this polymorphism is known to increase 5-HT_{1A} receptor expression.

Methods

Patients and Assessments

Included were men who were actively seeking drug treatment for lifelong PE. The included men came from all parts of the Netherlands. They were not recruited by advertisement and none of them were reimbursed for their participation. All of them visited the outpatient department of the last author. IELT was defined as the time between the start of vaginal penetration and the start of intravaginal ejaculation (Waldinger, Hengeveld et al. 1994). Lifelong PE was defined according to the ISSM definition (McMahon, Althof et al. 2008). All patients included were heterosexual men, aged 20 to 60 years. They suffered from PE since their first sexual encounters in puberty. In order not to exclude men with particular psychological difficulties related to PE, a stable relationship with a female partner was not required. However, it was required that during the 1-month period of IELT assessments, intercourse should have taken place with the same woman. Patients were not permitted the use of condoms, topical local anesthetic creams or sprays, or excessive consumption of alcohol within 5 hours prior to intercourse. Exclusion criteria included erectile dysfunction, alcohol or substance abuse, mental disorders, physical illnesses affecting ejaculatory functioning, concomitant medications, a history of sexual abuse reported by the patient and/or his partner, serious relationship problems, pregnancy of the partner, or the desire to become pregnant in the near future, a history of very low intercourse frequency, and a history of 100% anteportal ejaculation.

Patients attended the Outpatient Department of Neurosexology, HagaZiekenhuis, The Netherlands and after explanation of the purpose of the study they agreed with a baseline IELT measurement at home during each intercourse throughout 1 month. At the first visit, patients were interviewed individually by the last author and asked for an independent estimation of the IELT.

A stopwatch and instructions on how to measure the IELT were provided. The IELT was measured at home over the following 4 weeks. If intercourse took place more than once at the time of IELT measurement, only the first incident was included. With regard to the overall good health of the participants a physical examination was not required at baseline. All the data presented in the current study pertain to the baseline period in which the patients did not use any medication.

All laboratory testing, including blood sampling and genetic testing, were conducted by the first author. The study was conducted without any involvement of a pharmaceutical industry. All laboratory facilities and test materials were granted by the participating laboratory. Informed consent was obtained from all patients after explaining the purpose of the study. The study was approved by the Hospital Medical Ethical Committee and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Genotyping (DNA isolation and Polymerase Chain Reaction [PCR] analysis)

DNA Isolation.

Genomic DNA was extracted from 10 mL of EDTA-anticoagulated venous blood samples using a standard salting out method protocol (Miller, Dykes et al. 1988).

PCR Analysis

The PCR protocol to determine the 5-HT_{1A} −1019C/G polymorphism was: P1 (5'-GGC TGG ACT GTT AGA TGA TAA CG-3') and P2 (5'-GGA AGA AGA CCG AGT GTG TC**A** T-3''). The underlined nucleotide is a mismatch with the 5HT sequence, creating a restriction site in the PCR product. PCR conditions were as follows: 7 min at 94 ^{o}C; 35 cycles of 1 min at 94 ^{o}C, 1 min at 59 ^{o}C and 1 min at 72 ^{o}C; and finally 7 min at 72 ^{o}C. The size of the amplified product was 163 bp. Then the PCR product (10 µl) was digested with BseGII (Fermentas) in a total volume of 15 µl for 1 h at 55 C and subsequently analyzed on a 3% agarose/Tris-borate-EDTA gel with ethidium bromide staining. The fragments obtained for the wild-type allele was 163bp, for the variant allele the fragments were 146 and 17 bp. (Strobel et al, 2003).

For genotyping quality control about 10% positive controls were additionally genotyped according to the same protocol which resulted in concordance rates of 100%.

Statistical Analysis

The mean, median, and geometric mean IELT were calculated from stopwatch-determined IELTs (Waldinger, Zwinderman et al. 2008). The Chi-square test was used for Hardy-Weinberg equilibrium.

Allele and genotype frequencies were assessed using SPSS 19.0 for Windows (Chicago, IL, USA). P≤0.05 was considered statistically significant. Analysis of variance (ANOVA) was performed to determine an association between the genotypes and the IELTs.

Results

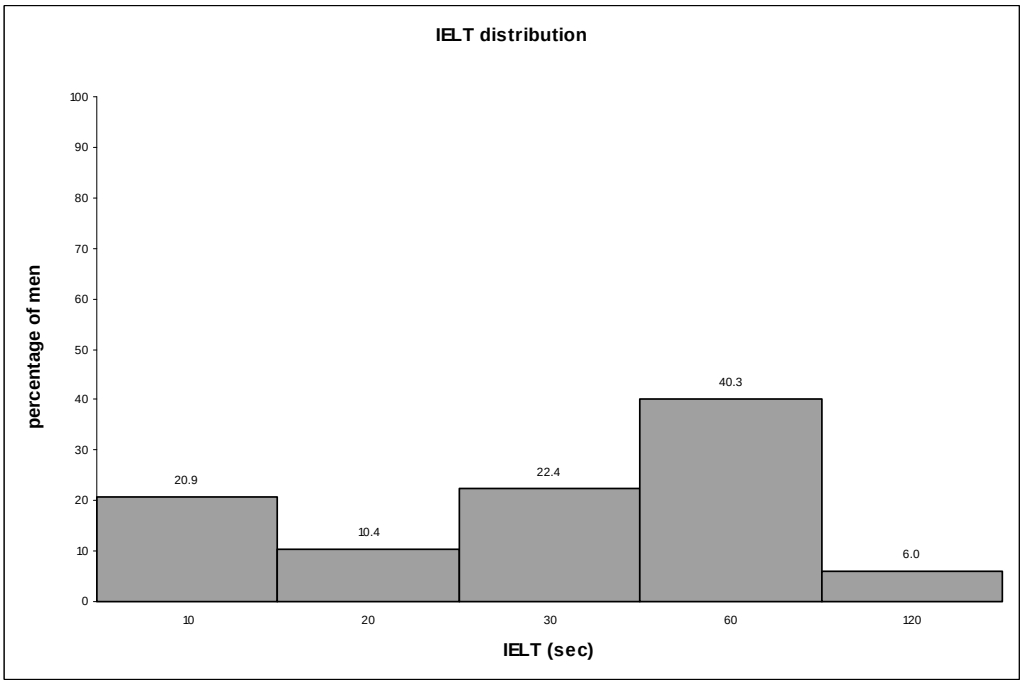
The study included 54 patients. Table 1 shows the characteristics of the men with lifelong PE.

Table 1. Patient Characteristics

Characteristics	**(N)**
Population	54
Age (years)	
Mean	39
Range	20 - 60
std dev	9.0
Age Partner (years)	
Mean	34.0
Range	21 – 57
std dev	8.5
Nationality	
Dutch (Caucasian)	94 %
Marital Status	
Married	46 %
Relationship, not married	54 %
No relationship	-
Duration of relationship **(years)** ((((years(years)	10
Range	0.2 – 29
std dev	7.1
Education	
Low	16 %
medium	49 %
High	35 %

Of all men the mean ± standard deviation frequency of intercourse in the baseline period was 3.3 (±1.3) ranging from 1 to 12 intercourses. Of all men, the majority (94%) ejaculated within 1 minute after vaginal penetration, e.g., 20.9% ejaculated within 10 seconds, 10.4% within 10-20 seconds, 22.4% within 20-30 seconds, and 40.3% between 30-60 seconds after vaginal penetration (Figure 1). In the current cohort, age and duration of the relationship have not been associated with the duration of the IELT. Men with a relationship of 0-2 yrs, 2-5 yrs and 5-10 yrs and more than 11 yrs, had a mean (±SD) IELT duration of 39,7 (± 25,6), 36,1 (±19,0), 26,8 (±15,9), and 31,6 (±25,7) seconds, respectively. In addition, men aged 20-30, 31-40, 41-50, and older than 51 years had a mean (±SD) IELT duration of 36,4 (±18,2), 23,6 (±17,5), 31,3 (±24,1), and 33,0 (±16,2) seconds, respectively.

Figure 1 : IELT Distribution in Dutch Men with Lifelong PE



As seen before, the IELT distribution in this study was skewed with geometric mean, median, and natural mean IELTs 23.8, 28.0, 34.4 seconds, respectively (Janssen, Bakker et al. 2009). Therefore, statistical analysis of IELT was performed after logarithmic transformation (Waldinger, Zwinderman et al. 2008).

The PCR reaction resulted in 45% and 55% C and G alleles, respectively. Genotyping completeness was 100%. Hardy-Weinberg equilibrium was not rejected for genotype distribution of the polymorphisms (p=0.972). Of the 54 men, the CC, CG and GG genotype frequency for the C(1019)G polymorphism of the 5-HT1A gene was 33%, 43% and 24%, respectively. Genotyping and association testing is represented in Table 2.

Table 2: Genotype Frequencies in Patients

Allele/Genotype	**Count**	**Frequency (%)**			**p-value**
C	59	55			0.860
G	49	45			
Sum	108	100			
CC	18	33			0.972
CG	23	43			
GG	13	24			
Sum	54	100			

Analysis of variance (ANOVA) of the natural logarithm (ln) of IELT showed a statistically significant difference in men with lifelong PE with CC, CG and GG genotypes (p=0.019) (Table 3).

Table 3: Natural logarithm of IELT per Genotype in Men with lifelong PE

Genotype	**N**	**Mean** Ln IELT(SD)	**Geometric** Mean IELT(sec)	**95% CI of the** geometric mean
CC	18	2.7 (1.0)	14.5	**8.7 - 24.2**
CG	23	3.3 (0,8)	27.7	**19.5 - 39.5**
GG	13	3.6 (0.9)	36.0	**20.8 - 62.3**
Total	54	3.2 (1.0)	23.8	**18.2 - 31.0**

The geometric mean IELT in the CC, CG and GG genotypes were 14.5, 27.7, and 36.0 seconds, respectively. The fold-increase of the geometric mean IELT in the GG genotype compared to CC genotype was 2.5. In addition, the fold-increase of the geometric mean IELT in the CG genotype compared to the CC genotype was 1.9. These data indicate that men with CC genotype compared to GG and CG genotypes on average show a 250% and 190% shorter intravaginal ejaculation time in this group of men with lifelong PE.

Discussion

This is the first study to investigate the effects of a functional 5-HT_{1A} receptor promoter polymorphism on the IELT in men with lifelong PE. The current study showed the presence of an association between 5-HT_{1A} polymorphism and the IELT duration in men with lifelong PE.

The study showed that men with CC genotype had a significantly shorter (p=0.007) IELT (14.5 seconds) compared to men with CG and GG-genotype (30,5 seconds). The population was in Hardy-Weinberg equilibrium (p=0.972).

The current sample of men with lifelong PE has a comparable IELT distribution as has been found in three other IELT studies in Dutch men with lifelong PE, and seems therefore representative for this group of patients: about 60% ejaculates within 30 seconds, and about 90% ejaculates within 1 minute after vaginal penetration (Waldinger, Hengeveld et al. 1998, Waldinger 2002, Janssen, Bakker et al. 2009).

In the current study only 6% of men ejaculated between 1-2 minutes after vaginal penetration. This low percentage is also similar to the three previous studies in Dutch men in which 10%, 8% and 8% ejaculated between 1-2 minutes (Waldinger, Hengeveld et al. 1998, Waldinger, Zwinderman et al. 2007, Janssen, Bakker et al. 2009). As only 6% of the current cohort of men ejaculated between 1 and 2 minutes, and it is known from previous studies that about 10% of men with lifelong PE report IELTs between 1 and 2 minutes (Waldinger, Hengeveld et al. 1998, Waldinger, Zwinderman et al. 2007), it was decided to also include the 6% of men in the current study in order to avoid investigating a diagnostic criterion rather than a genuine cohort.

Interestingly, the current study showed that the IELT in men with lifelong PE is associated with the 5-HT_{1A} C(-1019)G polymorphism, e.g., that men with CC genotype have a shorter IELT than men with either CG or GG genotype.

It is difficult to immediately connect this rather large difference to functional differences in 5-HT_{1A} receptor functioning. 5-HT_{1A} receptors exist as auto-receptors on serotonergic cell bodies and dendrites in the raphe nuclei and as heteroreceptors in a large range of brain structures. Chronic elevated serotonin levels, as induced by SSRI treatment or genetic ablation of the SERT (Snoeren, Chan et al. 2010, Chan, Snoeren et al. 2011) leads to functional adaptation of 5-HT_{1A} autoreceptors (desensitization). Accordingly, the functional 5-HT_{1A} (1019)CG polymorphism has been associated with functional changes in 5-HT neurotransmission (Le Francois, Czesak et al. 2008). Accordingly, CC genotypes might have more (or better) functioning 5-HT_{1A} receptors that would correspond to faster ejaculation. It is not clear however whether pre- or postsynaptic 5-HT_{1A} receptors are involved and in what brain or spinal cord area. Animal research has shown that selective 5-HT_{1A} receptor agonists facilitate ejaculation latency times (Waldinger 2002). Similarly as we previously found in 5-HTTLPR polymorphism (Janssen, Bakker et al. 2009), where men with LL SERT genotype had shorter IELTs than men with SL and SS genotype, it is remarkable that in a group of men with already extremely short ejaculation times, a 5-HT_{1A} receptor influencing polymorphism still can exert such a strong effect.

In the current group of men with lifelong PE, the median IELT was 28 seconds. In contrast, two stopwatch assessment surveys of the IELT in the general male population yielded a median IELT of 5.4 and 6.0 minutes, respectively (Waldinger, Quinn et al. 2005, Waldinger, McIntosh et al. 2009), indicating a difference of about 5.5 minutes compared with the median IELT in men with lifelong PE. As postulated in our previous study, it is assumed that, based on the difference of 5.5 minutes with the median IELT in the general male population, apart from 5-HTTLPR polymorphism and 5-HT_{1A} receptor gene polymorphism, also other genetic and possibly nongenetic factors may be involved in the regulation of the IELT. This is also more in line with our hypothesis that the variation in very short IELTs in men with lifelong PE results from a combination of different polymorphisms in central serotonergic neurotransmission, enzymes involved in serotonergic metabolism, serotonergic receptors related to ejaculation functioning, and serotonergic receptors involved in synaptic autoregulation. As in our previous study on 5-HTTLPR polymorphism, also the current study shows that within the group of men with lifelong PE with IELTs of less than 1 minute, one can distinguish men with persistently rapid and ultrarapid ejaculations dependent on 5-HT_{1A} receptor gene polymorphism genotype. The data of both studies support the hypothesis of Waldinger et al. (Waldinger, Berendsen et al. 1998, Waldinger 2002) that lifelong PE in terms of an IELT of less than 1 minute is related to genetic vulnerability,

disturbed central 5-HT neurotransmission and functionality of the 5-HT_{1A} receptor. With reference to our previous study (Janssen, Bakker et al. 2009) on 5-HTTLPR polymorphism, it may be speculated that men with lifelong PE and LL-genotype combined with CC-genotype, have a more sever PE than males with other allele combinations. However, this should be further investigated.

Notably, it is of relevance to know, that although we investigated the influence of the C(-1019)G polymorphism of the gene, expressing both the functionality of the pre-(=autoreceptor) and postsynaptic 5-HT_{1A} receptor, variations of other genes influencing the expression of the 5-HT_{1A} receptor gene have not been investigated in the current study. Interestingly, the 5-HT_{1A} receptor promoter region at the C(-1019) allele is known to become repressed by Hes and Deaf1 proteins (Albert, Le Francois et al. 2011). On the other hand, apart from the C(-1019)G polymorphism, there are other gene variations that influence the gene expression of the human 5-HT_{1A} receptor (Albert, Le Francois et al. 2011). For example, the repressor element-1 (RE-1) and particularly the dual repressor element (DRE), mediate the strongest repression of the 5-HT_{1A} receptor (Ou, Jafar-Nejad et al. 2000). Both the RE-1 and DRE, located upstream of the promoter region, are influenced by inhibiting proteins, such as Freud-1 and Freud-2 (DRE) and REST (RE-1), whereas Pet-1 exerts a strong enhancing activity of the 5-HT_{1A} receptor gene expression (Hendricks, Francis et al. 1999). Notably, mutation in the DRE may de-repress 5-HT_{1A} transcription. In the current study, we only investigated the C(-1019) polymorphism and found that it influences the duration of the IELT in men with lifelong PE. However, it may well be that activity of various proteins influencing the DRE, RE-1 and C(-1019) G allele or mutation of the DRE part of the gene is in some way associated with the IELT in men with lifelong PE. However, as the proteins of these gene elements are located in particular parts of the brain, it is unlikely that we will be able to investigate their influence in men, although by in vivo animal research this may perhaps be possible.

Conclusion

The current study shows evidence that 5-HT_{1A} receptor gene C (-1019)G polymorphism is associated with the IELT in men with lifelong PE. Men with CC genotypes have statistically shorter IELTs than men with CG and GG genotypes. The current study adds to our previously stated hypothesis that apart from 5-HTTLPR polymorphism, the IELT in men with lifelong PE is also influenced by other genetic polymorphism of the serotonergic system. Further genetic research in this group of men is warranted.

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Chapter 4:

The 5-HT_{2C} Receptor Gene Cys23Ser Polymorphism influences the Intravaginal Ejaculation Latency Time in Dutch Caucasian Men with Lifelong Premature Ejaculation

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ABSTRACT

Introduction. It has been postulated that the persistent short intravaginal ejaculation time (IELT) of men with lifelong PE (LPE) is related to 5-HT_{2C} receptor functioning.

Aim. The aim of this study was to investigate the relationship of Cys23Ser 5-HT_{2C} receptor gene polymorphism and the duration of IELT in men with LPE.

Methods. Therefore a prospective study was conducted in 64 Dutch Caucasian men with LPE. Baseline IELT during coitus was assessed by stopwatch over a 1-month period. All men were genotyped for Cys23Ser 5-HT_{2C} receptor gene polymorphism. Allele frequencies and genotypes of Cys and Ser variants of 5-HT_{2C} receptor gene polymorphism were determined. Association between Cys/Cys and Ser/Ser genotypes, and the natural logarithm of the IELT in men with LPE were investigated.

Main Outcome Measures. IELT measured by stopwatch, 5-HT_{2C} receptor gene Cys23Ser polymorphism

Results. As result the geometric mean, median, and natural mean IELT were 25.2, 27.0, 33.9 seconds, respectively. Of all men, 20.0%, 10.8%, 23.1% and 41.5% ejaculated within 10 seconds, 10-20 seconds, 20-30 seconds, and 30-60 seconds after vaginal penetration. Of the 64 men, the Cys/Cys and Ser/Ser genotype frequency for the Cys23Ser polymorphism of the 5-HT_{2C} receptor gene was 81% and 19%, respectively. The geometric mean IELT of the wildtypes (Cys/Cys) is significantly lower (22.6 sec; CI 18.3-27.8 sec) than in male homozygous mutants (Ser/Ser) (40.4%; CI 20.3-80.4) ($p=0.03$).

Conclusion. It is concluded that Cys23Ser 5-HT_{2C} receptor gene polymorphism is associated with the IELT in men with LPE. Men with Cys/Cys genotype have shorter IELTs than men with Ser/Ser genotypes.

Keywords. Lifelong premature ejaculation; 5-HT_{2C} receptor gene Cys23Ser polymorphism; IELT

Introduction

According to the International Society for Sexual Medicine (ISSM) lifelong premature ejaculation (LPE) is defined as an ejaculation that occurs within about 1 minute after penetration in the majority of sexual encounters, with an inability to delay ejaculation and with associated negative personal consequences such as bother and avoidance of sexual activity (McMahon, Althof et al. 2008). In 1998, Waldinger (Waldinger, Berendsen et al. 1998) postulated that the IELT of less than 1 minute in men with lifelong PE is influenced by genetic factors and associated with disturbed central serotonin (5-hydroxytryptamine: 5-HT) neurotransmission, hypersensitivity of 5-HT_{1A} receptors and/or hypofunction of 5-HT_{2C} receptors. Notably, due to an absence of selective 5-HT_{1A} and 5-HT_{2C} receptor ligands for safe human usage, this hypothesis has sofar not been confirmed (Waldinger 2013). However, recent stopwatch mediated genetic research of the IELT in men with lifelong PE has provided indications that gene polymorphisms of the 5-HT transporter (5HTT) and the 5-HT_{1A} receptor are associated with the duration of the IELT. For example, by measuring the IELT with a stopwatch in 89 Dutch men with lifelong PE, Janssen (Janssen, Bakker et al. 2009) have shown that 5-HTTLPR polymorphism is associated with the IELT duration. Of these men who ejaculated within 1 minute after vaginal penetration, men with LL genotype had a 100% and 90% shorter IELT than men with SS and SL genotypes, respectively ($p=0.027$) (Janssen, Bakker et al. 2009). However, there were no significant differences between these men and a control group of 92 Dutch Caucasian men with regard to 5-HTT polymorphism alleles and genotypes (Janssen, Bakker et al. 2009). Using the same stopwatch method of associating the IELT duration with gene polymorphism, Janssen *et al.*(Janssen, van Schaik et al. 2014) have recently shown that men with lifelong PE and with the CC genotype of the C(1019)G polymorphism of the 5-HT_{1A} receptor, also ejaculate statistically significantly faster than men with GC and GG genotype. Up to now the stopwatch method for genetic research as applied by Janssen (Janssen, Bakker et al. 2009, Janssen, van Schaik et al. 2014) has not been used by other researchers. Instead, other research groups have attempted to investigate the question whether the frequency of certain genotypes of genetic polymorphism in men with lifelong PE differs from those in a control group. For example, two questionnaire studies by Jern (Jern, Eriksson et al. 2013) and Zuccarello (Zuccarello, Ghezzi et al. 2012) confirmed the finding of Janssen (Janssen, Bakker et al. 2009) that there is no association in 5-HTTLPR polymorphism between men with lifelong PE and a control group. In contrast, three other studies (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011) reported a higher SS genotype frequency in men with lifelong PE compared with a control group.

But as the latter three studies were not in Hardy Weinberg equilibrium (HWE) - most probably due to technical laboratory insufficiencies of polymerase chain reaction (PCR) interpretation- their results are not considered to be reliable (Waldinger, Janssen et al. 2009, Waldinger, Janssen et al. 2009, Janssen, Olivier et al. 2014). In a previous questionnaire study in a large Finish cohort of twins, Jern (Jern, Westberg et al. 2012) did not find an association of 5-HT_{1A} receptor gene polymorphism C(-1019)G and the ELT. With regard to the 5-HT_{2C} receptor in relation to PE, two studies have previously been performed (Luo, Lu et al. 2010, Jern, Westberg et al. 2012). Luo (Luo, Lu et al. 2010) reported that compared to a control group, men with lifelong PE and with G-697C polymorphism of the 5-HT_{2C} receptor gene, have an increased Odds for PE. On the other hand, based on a retrospective self-reported measure of the ejaculation time in 1399 male twins, Jern (Jern, Westberg et al. 2012) reported no association of G-697C polymorphism of the 5-HT_{2C} receptor and the self-reported measure of the ejaculation time.

In the current study in men with lifelong PE, we investigated whether the 5-HT_{2C} receptor gene Cys23Ser polymorphism is associated with the duration of the IELT by using a stopwatch to measure the IELT.

Methods

Patients and Assessments

Included were 64 men who were actively seeking drug treatment for lifelong PE at the Outpatient Department of Neurosexology of HagaZiekenhuis in the Netherlands. The included men came from all parts of the Netherlands. They were not recruited by advertisement and none of them was reimbursed for their participation. None of them used or had ever been using drugs, such as SSRIs or clomipramine, for the treatment of lifelong PE. IELT was defined as the time between the start of vaginal penetration and the start of intravaginal ejaculation (Waldinger, Hengeveld et al. 1994). Lifelong PE was defined according to the ISSM definition [1].

All patients included were heterosexual men, aged 20 to 60 years. In order not to exclude men with particular psychological difficulties related to PE, a stable relationship with a female partner was not required. However, it was required that during the 1-month period of IELT assessments, intercourse should have taken place with the same woman. Patients were not permitted the use of condoms, topical local anesthetic creams or sprays, or excessive consumption of alcohol within 5 hours prior to intercourse. Exclusion criteria included erectile dysfunction, alcohol or substance abuse, mental disorders, physical illnesses affecting ejaculatory functioning, concomitant medications, a history of sexual abuse reported by the patient and/or his partner, serious relationship problems, pregnancy of the partner, or the desire to become pregnant in the near future, a history of very low intercourse frequency,

a history of 100% anteportal ejaculation, and the possibility of dangerous situations arising at work in the case of paroxetine induced side effects.

Patients attended the Outpatient Department approximately 1 month before the start of daily SSRI treatment (first baseline assessment), on the day before treatment (second baseline assessment), and at the end of 10 weeks of daily SSRI treatment.

At the first visit, patients were interviewed individually by the last author and asked for an independent estimation of the IELT. A stopwatch and instructions on how to measure the IELT with a stopwatch were provided. The patients measured the IELT at home over the following 4 weeks. The female partners had to handle the stopwatch. It was advised not to have interrupted intromission or to change the usual way of frequency of intercourse. If intercourse took place more than once at the time of IELT measurement, only the first incident was included.

All laboratory testing, including blood sampling and genetic testing, were conducted by the first author. The study was conducted without any involvement of a pharmaceutical industry. All laboratory facilities and test materials were granted by the participating laboratory. Written informed consent was obtained from all patients. The study was approved by the Hospital Medical Ethical Committee and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Genotyping (DNA isolation and Polymerase Chain Reaction [PCR] analysis)

DNA Isolation.

Genomic DNA was extracted from 10 mL of EDTA-anticoagulated venous blood samples using a standard salting out method protocol.

PCR Analysis:

We investigated the single nuclear polymorphism (SNP) Cys23Ser.

PCR Analysis of the Cys23Ser polymorphism

5HT2C(Cys23Ser): P7 (5'-TTG GCC TAT TGG TTT GGG AAT-3') and P8 (5'-GTC TGG GAA TTT GAA GCG TCC-3''). The underlined nucleotide is a mismatch with the 5HT sequence, creating a restriction site in the PCR product. PCR conditions were as follows: 7 min at 94 ^{o}C; 35 cycles of 1 min at 94 ^{o}C, 1 min at 50 ^{o}C and 1 min at 72 ^{o}C; and finally 7 min at 72 ^{o}C.The size of the amplified product was 104 bp. Then the PCR product (10 μl) was digested with HinfI (Roche) in a total volume of 15 μl for 1 h at 37 C and subsequently analyzed on a 3% agarose/Tris-borate-EDTA gel with ethidium bromide staining. The fragments obtained for the wild-type allele was 104 bp, for the variant allele the fragments were 86 and 18 bp (Lappalainen, Zhang et al. 1995).

Statistical Analysis

The mean, median, and geometric mean IELT was calculated from stopwatch-determined IELTs. SPSS 19.0 for Windows (Chicago, IL, USA) was used. $P<0.05$ was considered statistically significant. Analysis of variance (ANOVA) was performed to determine an association between the genotypes and their IELTs.

Results

The study included 64 patients. Table 1 shows the characteristics of the men with lifelong PE.

Characteristics	**(N)**
Population	64
Age (years)	
Mean	39
Range	20 – 60
std dev	9.0
Age Partner (years)	
Mean	34.0
Range	21 – 57
std dev	8.5
Nationality	
Dutch (Caucasian)	94 %
Marital Status	
Married	46 %
Relationship but not married	54 %
No relationship	-
Duration of relation (years)	10
Range	0.2 – 29
std dev	7.1
Education	
Low	16 %
Medium	49 %
High	35 %

Table 1. Patient Characteristics

Of all men the mean ± standard deviation frequency of intercourse during one month in the baseline period was 3.3 (±1.3) ranging from 1 to 12 intercourses. Of all men, the majority (96%) ejaculated within 1 minute after vaginal penetration. Of all men, 20.0% ejaculated within 10 seconds, 10.8% within 10-20 seconds, 23.1% within 20-30 seconds, and 41.5% between 30-60 seconds after vaginal penetration (Figure 1).

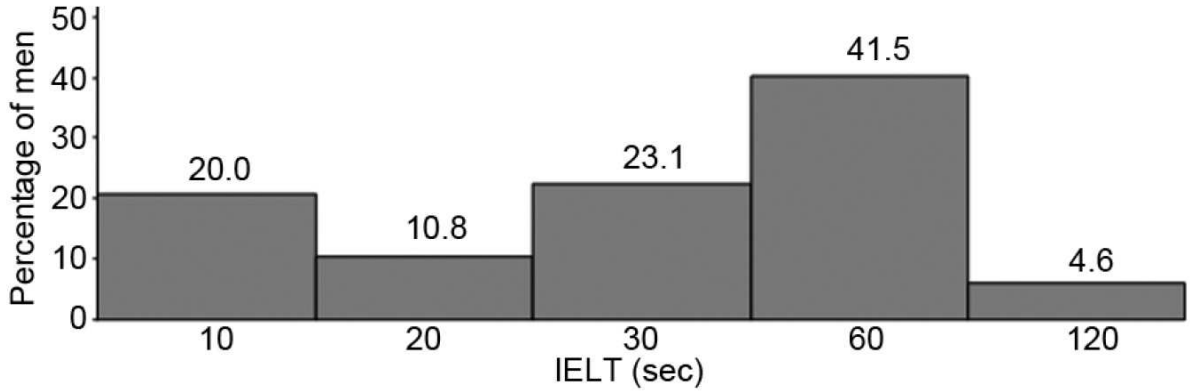

Figure 1: IELT Distribution in Dutch Men with Lifelong PE

As seen in our previous studies (Waldinger, Hengeveld et al. 1998, Janssen, Bakker et al. 2009), the IELT distribution in the current study was skewed with geometric mean, median, and natural mean IELTs of 25.2, 27.0, 33.9 seconds, respectively. Therefore, statistical analysis of IELT was performed after logarithmic transformation (Waldinger, Zwinderman et al. 2008).

Table 2 shows the results of the genotyping of the Cys23Ser 5-HT_{2C} receptor polymorphism. As the gene is only located at the X-chromosome, no heterozygotes of the Cys23Ser 5-HT_{2C} receptor polymorphism exist in males. Accordingly, although the number of alleles is twice as high as the genotypes, the distribution of the genotypes is similar to the distribution in alleles, as the alleles do not distribute on heterozygotes in this case.

Allele/Genotype	**Count**	Frequency (%)
W	52	81
M	12	19
Sum	64	100

Table 2: Results of Genotyping Testing of the Cys23Ser 5-HT_{2C} receptor polymorphism: W = wildtype (Cys/Cys), M = mutant (Ser/Ser)

The geometric mean IELT of the wildtypes (Cys/Cys) in the current group of men is significantly lower than the geometric mean IELT in male homozygous mutants (Ser/Ser) (p=0.03) (Table 3).

Genotype	N	Mean Ln IELT(SD)	**Geometric Mean IELT(sec)**	**95% CI of the geometric mean**
W	52	3.1 (0.7)	22.6	**18.3 - 27.8**
M	12	3.7 (1,1)	40.4	**20.3 - 80.4**
Total	64	3.2 (0.8)	25.2	**20.4 - 31.1**

Table 3: Natural logarithm of IELT per Genotype in Men with Lifelong PE

Discussion

The results of the current study show that wildtypes Cys/Cys of the 5-HT_{2C} receptor gene, which is exclusively located at the X-chromosome, have a statistically significant faster (p=0.03) IELT than men with a mutant genotype (Ser/Ser). In the current study, we did not use a control group, but compared the genotype frequencies of the 5-HT_{2C} receptor gene polymorphism with the European HapMap-CEU population (rs=6318. 2014), consisting of 120 men and women, in which the allele distribution was 15.8% Ser/Ser and 84.2% Cys/Cys. Although this is not a Dutch reference population, but a European population, the genotype frequency of the current cohort of men does not deviate from the European population. Notably, although, Luo (Luo, Lu et al. 2010) did not use our method of comparing the IELT duration values of each single patient with 5-HT_{2C} receptor gene polymorphism, he did find an association of Cys23Ser 5-HT_{2C} receptor polymorphism with lifelong PE by comparing the frequency of genotypes with the frequency of these genotypes in a Han Chinese population. However, as noted, we did not find such an association with the European HapMap-CEU population.

Although the current study shows an association of 5-HT_{2C} receptor Cys23Ser polymorphism and the IELT duration in men with lifelong PE, it remains unknown whether 5-HT_{2C} receptor Cys23Ser polymorphism is in the same way associated with the IELT duration in the general male population. For that purpose, very large population based stopwatch studies or male twin studies are recommended. However, in a retrospective questionnaire study in Finnish twins, Jern (Jern, Westberg et al. 2012) did not find an association of Cys23Ser 5-HT_{2C} receptor polymorphism with the ejaculation latency time (ELT) duration, as measured by a questionnaire. By using our method of stopwatch measurement of the IELT in men with lifelong PE, and comparing gene polymorphisms with the IELT duration in the same group of men, the current study shows that there is an association of the IELT duration and Cys23Ser 5-HT_{2C} receptor gene polymorphism.

By using the same method, we have previously also found an association of the IELT duration in men with lifelong PE with polymorphism of the 5-HTTLPR gene and the C(1019)G polymorphism of the 5-HT_{1A} receptor (Janssen, Bakker et al. 2009, Janssen, van Schaik et al. 2014). However, our findings of three genetic polymorphism associations with the IELT duration in an exclusive group of men with lifelong PE by using a stopwatch to prospectively and exactly measure the IELT duration have previously not been found in a large cohort of Finnish twins in which the ELT was retrospectively assessed by the use of a questionnaire (Jern, Westberg et al. 2012, Jern, Eriksson et al. 2013).

More studies are needed in a large cohort of men with lifelong PE and a control group with well controlled PCR analysis in order to replicate and confirm the robustness of the current outcome data indicating an association of Cys23Ser 5-HT_{2C} receptor gene polymorphism and the IELT duration in men with lifelong PE. Nevertheless, it is intriguing to note that the studies of Janssen (Janssen, Bakker et al. 2009, Janssen, van Schaik et al. 2014) including the current study and the animal studies (Berendsen and Broekkamp 1987, Foreman, Love et al. 1988) that formed the basis for the hypothesis of Waldinger (Waldinger, Berendsen et al. 1998) provide (preliminary) indications that the persistent short IELTs of men with lifelong PE may be associated with central 5-HT neurotransmission, and central 5-HT_{1A} and 5-HT_{2C} receptor functioning, as mirrored by the associations of their gene polymorphisms with the IELT duration.

A limitation of the current study is that not all polymorphisms of the 5-HT_{2C} receptor gene have been investigated. For that purpose, genotyping of the whole 5-HT_{2C} receptor gene is required. Such a study in men with lifelong PE has so far not been published. Another limitation of the current study is the relatively low number of participants. However, our number of 64 males does not differ from the average number of male and female patients investigated in other studies on 5-HT_{2C} receptor polymorphism. For example, in 5 studies (Gutierrez, Fananas et al. 1996, Oruc, Verheyen et al. 1997, Frisch, Postilnick et al. 1999, Vincent, Masellis et al. 1999, Lerer, Macciardi et al. 2001) on major depression and bipolar disorder the average number of male and female participants was 67 (range 42-98). Notably, of these 5 studies, 3 studies reported an association between Cys23Ser polymorphism and major depression or bipolar disorder (Gutierrez, Fananas et al. 1996, Oruc, Verheyen et al. 1997, Frisch, Postilnick et al. 1999, Vincent, Masellis et al. 1999, Lerer, Macciardi et al. 2001). However, in order to avoid any misunderstanding such association does not mean that lifelong PE is in any way associated with major depression or bipolar disorder. Moreover, at the moment genetic research of lifelong and acquired PE is only meant for scientific purposes and therefore is not part of daily practice to evaluate and diagnose PE (Jannini, Maggi et al. 2011).

Conclusion

The current study shows evidence that 5-HT_{2C} receptor gene Cys23Ser polymorphism is associated with the IELT duration in men with lifelong PE. Men with Cys/Cys genotype have statistically shorter IELTs than men with Ser/Ser genotype. The current study adds to our previously stated hypothesis that apart from 5-HTTLPR polymorphism, the IELT in men with lifelong PE is also influenced by other genetic polymorphism of the serotonergic system.

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Chapter 5:

Serotonin transporter promotor region (5-HTTLPR) polymorphism is not associated with paroxetine induced ejaculation delay in dutch men with lifelong premature ejaculation

Janssen, P.K., Zwinderman A.H., Olivier B., Waldinger M.D. (2014).
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ABSTRACT

Introduction. Daily paroxetine treatment induces a clinically relevant ejaculation delay in men with lifelong premature ejaculation (LPE).

Aim. To investigate the relationship between 5-HT transporter gene-linked polymorphism (5-HTTLPR) and paroxetine-induced ejaculation delay in men with lifelong PE.

Methods. A prospective study was conducted in 54 men with LPE. IELT was measured by stopwatch. Controls consisted of 92 Caucasian men. All men with LPE were genotyped for a 5-HTT-promoter polymorphism. Allele frequencies and genotypes of short (S) and long (L) variants of 5-HTTLPR polymorphism were compared between patients and controls. Association between LL, SL, and SS genotypes, and fold increase of paroxetine-induced geometric mean IELT in men with LPE was investigated.

Main outcome measures. Fold increase of geometric mean IELT, 5-HTTLPR polymorphism.

Results. Of all 54 patients, 43 (79,6%) responded to paroxetine treatment with an ejaculation delay, whereas 11 (20,4%) patients did not respond to paroxetine treatment; 44%, 18% and 18% had a fold increase of 2-10, 10-20, and of more than 20. Of the 54 men, 14 (25,9%) had LL genotype, 29 (53,7%) had SL genotype and 11 (20,4%) had SS genotype. Of the 92 controls, LL, SL and SS genotype were present in 27 (29,3%), 41 (44,6%) and 24 (26,1%), respectively. No statistically significant differences were found in 5-HTTLPR allelic variations. No statistically significant differences were found in 5-HTTLPR gene variations. In all men treated with 20 mg paroxetine, including the 80% with ejaculation delay and the 20% who did not respond with ejaculation delay, analysis of variance (ANOVA) of the natural logarithm (ln) of fold increase showed no statistically significant difference in men with LL, SL and SS genotypes (p=0.83) with regard to the fold-increase of the IELT

Conclusions. 5-HTTLPR polymorphism is not associated with daily 20 mg paroxetine treatment induced ejaculation delay in men with lifelong PE.

Introduction

Since 2009, a number of studies with different methodologies and designs (for example, stopwatch vs. questionnaire) have investigated the relationship between genetic polymorphisms and premature ejaculation (PE) (Waldinger 2011). In a stopwatch study, Janssen (Janssen, Bakker et al. 2009) found that in men with lifelong PE, 5-HTTLPR polymorphism is associated with statistically significant effects on the latency to ejaculate; men with LL genotype ejaculate 100% faster than men with SS genotype (Janssen, Bakker et al. 2009). In addition, men with lifelong PE and with CC genotype of the C(1019)G polymorphism of the 5-HT_{1A} receptor, also ejaculate statistically significantly faster than men with GC and GG genotype (Janssen, van Schaik et al. 2014).

It is a well-established clinical fact that daily use of SSRIs in men with lifelong PE, clinically very relevantly delays ejaculation (Janssen, Schaik et al. 2014). Compared to other SSRIs, daily treatment with 20 mg paroxetine exerts the strongest ejaculation delay (Waldinger, Zwinderman et al. 2004, Janssen, Schaik et al. 2014). However, this is not always the case. In some men daily use of SSRIs only moderately delay ejaculation, whereas in others there is no ejaculation delay at all (Waldinger, Hengeveld et al. 1998). Animal studies have shown that pharmacodynamic factors, such as the amount of serotonin neurotransmission, 5-HT_{1A} receptor sensitivity and/or 5-HT_{2C} receptor sensitivity are associated with the duration of the ejaculation latency time (Ahlenius, Larsson et al. 1981) (Foreman, Love et al. 1988).

In the current study in men with lifelong PE who responded to daily paroxetine 20 mg treatment, we investigated the role of 5-HTTLPR polymorphism on paroxetine induced ejaculation delay.

Materials and methods

Patients and Assessments.

Included were men who were actively seeking drug treatment for lifelong PE at the Outpatient Department of Neurosexology. The included men came from all parts of the Netherlands. None of them used or had ever been using drugs, such as SSRIs or clomipramine, for the treatment of lifelong PE. IELT was defined as the time between the start of vaginal penetration and the start of intravaginal ejaculation (Waldinger, Hengeveld et al. 1994). Lifelong PE was operationally defined according to the ISSM definition as the lifelong presence of an IELT of 1 minute or less after vaginal penetration occurring on more than 90% of occasions of sexual intercourse with every sexual partner together with complaints of inability to delay ejaculation and feelings of frustration about it (McMahon, Althof et al. 2008). All patients included were heterosexual men, aged 18 to 65 years.

In order not to exclude men with particular psychological difficulties related to PE, a stable relationship with a female partner was not required. However, it was required that during the 1-month period of IELT assessments, intercourse should have taken place with the same woman. Patients were not permitted the use of condoms, topical local anesthetic creams or sprays, or excessive consumption of alcohol within 5 hours prior to intercourse. Exclusion criteria included erectile dysfunction, alcohol or substance abuse, mental disorders, physical illnesses affecting ejaculatory functioning, concomitant medications, a history of sexual abuse reported by the patient and/or his partner, serious relationship problems, pregnancy of the partner, or the desire to become pregnant in the near future. Patients attended the the Outpatient Department approximately one month before the start of daily SSRI treatment (first baseline assessment), on the day before treatment (second baseline assessment), and at the end of two consecutive series of 5 weeks of daily SSRI treatment. At the first visit, patients were interviewed by the last author and asked for an independent estimation of their IELT. A stopwatch and instructions on how to measure the IELT were provided. The female partners measured the IELT and handled the stopwatch at home at every intercourse over the following 4 weeks. Patients were instructed not to have interrupted intromission or to change their usual way or frequency of intercourse. If intercourse took place more than once at the time of IELT measurement, only the first occurrence was included. Patients were not recruited by advertisement and were not reimbursed for their participation. All laboratory testing, including blood sampling and genetic testing, were conducted by the first author. The study was conducted without any involvement of a pharmaceutical industry. All laboratory facilities and test materials were granted by the participating laboratory. Informed consent was obtained from all patients after explaining the purpose of the study. The study was approved by the Hospital Medical Ethical Committee and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983. The control group consisted of 92 physically and mentally healthy male individuals recruited in another study conducted by the Department of Psychiatry of the Utrecht Medical Center, Utrecht, the Netherlands (Jungerius, Hoogendoorn et al. 2007). All of these control participants had been previously genotyped for the 5-HTTLPR polymorphism. In addition, all male controls had at least 3 grandparents who were born in the Netherlands. The control group was randomly sampled and is onsidered representative of the general Dutch population (Jungerius, Hoogendoorn et al. 2007). Neither occurrence of complaints of PE nor stopwatch assessments of IELT has been investigated in the control group.

A Responder was operationally defined as an individual who on daily paroxetine 20 mg treatment had a fold-increase of the geometric mean IELT of 2 and more, e.g. more than 100% increase of the baseline IELT value. A Non-responder was defined as an individual who had a fold-increase of the geometric mean IELT of less than 2.

The cut-off point of 2 was based on the outcome data of a meta-analysis of daily SSRI treatment for PE, in which placebo response was consistently lower than a 2 fold-increase of the geometric mean IELT compared to baseline values (Waldinger, Zwinderman et al. 2004).

Genotyping

DNA Isolation.

Genomic DNA was extracted from 10 mL of EDTA anticoagulated whole blood using a standard salting-out method protocol.

Polymerase Chain Reaction (PCR Analysis).

The 44-bp insertion/deletion polymorphism within the promoter region of the SERT(SLC6A4) gene was amplified by PCR. The insertion/deletion in the SERT gene-linked polymorphic region (5-HTTLPR) was amplified using the following oligonucleotide primers: forward 5′-GGCGTTGCCGCTCTGAATC-3′, and reverse; 5′-GAG GGACTGAGCTGGACAACCAC-3′, flanking the 5-HTT gene-linked polymorphic region (5-HTTLPR). Corresponding to the nucleotide positions ranging from -1,416 to -1,397 and from -910 to -889 of the 5-HTT gene regulatory region, a 484-bp or a 528-bp fragment was generated.

Reagents and conditions for the PCR were: 1 mL of 10 times polymerase buffer; 0.2 mmol/L deoxyribonucleotide triphosphates; 2.0 mmol/L MgCl2, 0.4 mMmol/L of each primer (Biolegio BV, Nijmegen, the Netherlands); 0.5 U AccuPrime Pfx DNA polymerase (Invitrogen Life Technologies, Strathclyde, UK); and 50 ng of genomic DNA, in a total reaction volume of 10 mL. The PCR program on a thermal cycler (GeneAMP type 9700; Perkin Elmer, Waltham, MA, USA) was as follows: Reactions were cycled with initial denaturation at 94°C for 4 minutes, followed by 33 PCR cycles of 94°C for 30 seconds, 61°C for 60 seconds, 68°C for 60 seconds, and a final extension step of 4 minutes at 72°C. The amplification products were electrophoresed on 2% agarose gels at 100 V for 120 minutes. The gel and running buffers were 1¥ TBE (0.89 m Tris-Base, 0.89 m boric acid, 20 mM Na2EDTA). The fragments were visualized by ethidium bromide under ultraviolet transillumination.

Statistics.

The mean, median, and geometric mean IELT was calculated of stopwatch-determined IELTs. Hardy–Weinberg equilibrium to check laboratory efficacy of PCR analysis was determined in the control group and the patient group using a chisquare test. Allele and genotype frequencies between patients and controls were compared using SPSS 19.0 forWindows (Chicago, IL, USA). $P < 0.05$ was considered statistically significant. Analysis of variance (anova) was performed to determine an association between the genotype in the patient group and their fold increases.

Results

The characteristics of patients and controls are shown in Table 1. The mean age of the controls was significantly higher than that of the patients. However, as lifelong PE affects all age categories, this difference does not affect the purpose of the study.

Characteristics	**Patients (N)**	**Controls (N)**	**P**
Population	54	92	
Age (years)			<0.05
Mean	36.1	53.6	
Range	25 – 58	27 - 78	
std dev	8.6	15.3	
Age Partner (years)			
Mean	34.6		
Range	22 – 57		
std dev	9.6		
Nationality			
Dutch (Caucasian)	95%	100%	
Marital Status			<0.05
Married	33.3%	70.0%	
Relationship but not married	64.8%	30.0%	
No relationship	1.9%	0.0%	
Duration of relation (years)	12		
Range	0.1 – 34		
std dev	9.5		
Education			0,40
low	11.1%	13.0%	
medium	33.3%	24.6%	
high	55.6%	62.3%	

Table 1. Patient and Control Characteristics

The paroxetine induced ejaculation delay, expressed in percentage Fold Increase of the geometric mean IELT compared to baseline IELT value, is shown in Figure 1. Notably, paroxetine was only used by the patients with lifelong PE and not by the controls.
Of all 54 patients, 43 (79,6%) responded to paroxetine treatment with an ejaculation delay, whereas 11 (20,4%) patients did not respond to paroxetine treatment; 50%, 12% and 18% had a fold increase of 2-10, 10-20, and of more than 20.

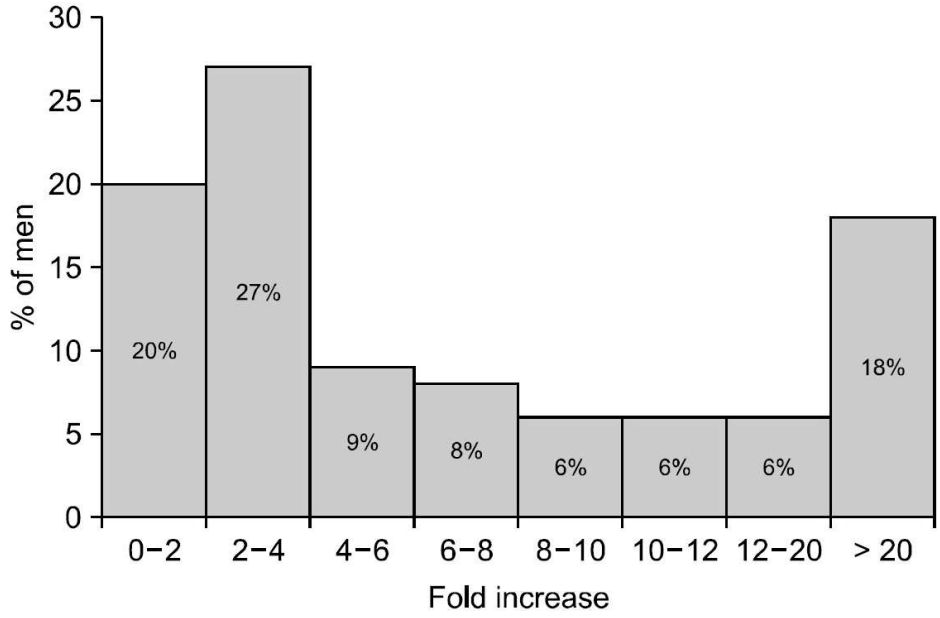

Figure 1: Distribution of Fold Increase (FI) of the Geometric mean IELT on daily Paroxetine 20 mg Treatment in Men with Lifelong PE; 20% had no paroxetine induced ejaculation delay (FI 0-2), whereas 80% had an ejaculation delay (FI >2)

A photograph of illuminating DNA fragments on gel under UV light is shown in Figure 2.

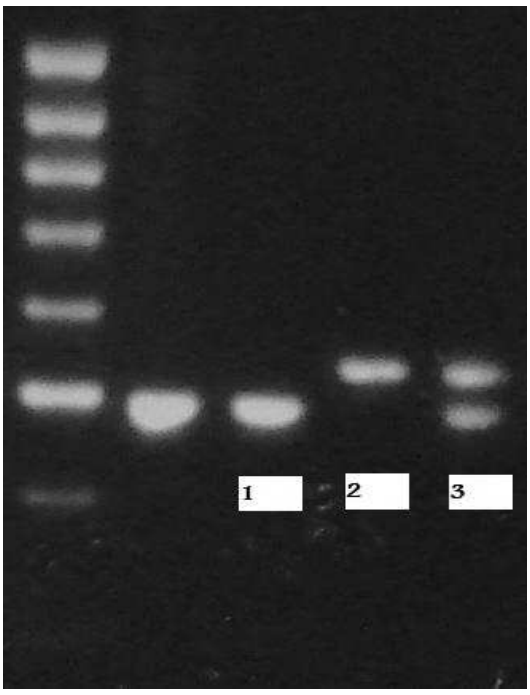

Figure 2: Photograph of illuminating DNA fragments on gel under UV light. Lane 1: homozygous patient for LL alleles / Lane 2: homozygous patient for SS alleles / Lane 3: heterozygous patient for LS alleles

Hardy-Weinberg equilibrium was not rejected for genotype distributions of the polymorphisms investigated in patients (p=0.83) and controls (p=0.59). Of the 54 men with lifelong PE, 14 (25,9%) had LL genotype, 29 (53,7%) had SL genotype and 11 (20,4%) had SS genotype. Of the 92 controls, LL, SL and SS genotype were present in 27 (29,3%), 41 (44,6%) and 24 (26,1%), respectively. No statistically significant differences were found in 5-HTTLPR allelic variations. In addition, no statistically significant differences were found in 5-HTTLPR gene variations. Genotyping and association testing are represented in Table 2.

	Patients		Controls		
Allele/	Count	Frequency(%)	Count	Frequency (%)	
S	57	52,8	89	48,4	0,47
L	51	47,2	95	51,6	
Sum	108	100	184	100	
SS	11	20,4	24	26,1	0,43
SL	29	53,7	41	44,6	
LL	14	25,9	27	29,3	
Sum	54	100	92	100	

Table 2. Results of Genotyping and Association Testing in Patients and Controls.

In all men treated with 20 mg paroxetine, including the 80% of men with ejaculation delay and the 20% who did not respond with an ejaculation delay, analysis of variance (ANOVA) of the natural logarithm (ln) of FI showed no statistically significant difference in men with LL, SL and SS genotypes (p=0.83) with regard to the fold-increase of the IELT (Table 3).

Genotype	N	Mean	**Geometric**	**95% CI of the**
LL	14	1,92	**6,80**	**3,58 – 12,93**
SL / LS	29	1,67	**5,30**	**3,35 – 8,41**
SS	11	1,73	**5,65**	**2,02 – 15,86**
Total	54	1,75	**5,73**	**4,09 – 8,04**

Table 3. Natural logarithm of Fold Increase (FI) per genotype in men with lifelong PE

Discussion

Owing to the lack of genome-wide studies in men with lifelong PE, we selectively chose to investigate the 5-HTTLPR polymorphism because the results of *in vivo* animal and clinical human research with SSRIs indiate that the serotonin content in the neuronal synapse is involved in ejaculation delay. Moreover, daily use of 20 mg paroxetine, which inhibits the reuptake of 5-HT by inhibiting the activity of the 5-HT transporter, exerts the strongest ejaculation delay of the SSRIs (Waldinger, Hengeveld et al. 1998, Waldinger 2003).

The difference in age between the patients and controls in the current study did not influence the comparison of men with lifelong PE and controls, because lifelong PE is present "lifelong," that is, both at a young age and in older aged men. Why the number of married men was higher in the control group is unclear but, speculatively, may be explained by the difference in age, as younger men may be more inclined to live together with a female partner instead of being married.

In the current study of a cohort of 54 men with lifelong PE it was shown that on daily treatment with 20 mg paroxetine, 80% of men responded with ejaculation delay expressed in a fold increase of more than 2. About 18% of these men had a clinically very relevant ejaculation delay, as mirrored by a fold increase of 10-20. Another 18% of these men even had a clinically very strong ejaculation delay (FI > 20). However, 20% of all men did not respond with an ejaculation delay.

The patient and control group were in Hardy Weinberg equilibrium with regard to the 5-HTTLPR genotype polymorphism (Janssen, Olivier et al. 2014). In addition, with regard to 5-HTTLPR polymorphism both the patient and control group did not differ in their genotype frequencies.

Importantly, although the majority of men responded with a clinically relevant ejaculation delay, in the current study it was found that 5-HTTLPR genotype polymorphism is not associated with the paroxetine induced ejaculation delay.

Interestingly, in a previous study in 89 men with lifelong PE (who at the moment of investigation did not use medication) it was found that 5-HTTLPR polymorphism was associated with the duration of the IELT (Janssen, Bakker et al. 2009). Men with LL genotype, had a 100% shorter IELT than men with SS and SL genotype (Janssen, Bakker et al. 2009). In the current study, in which a part of the previous group of men were included, the paroxetine-induced ejaculation delay was not associated with the 5-HTTLPR polymorphism. It might be argued that this negative outcome is due to the low number of participants of the study. This is indeed a limitation of the current study. However, it should be noted that the data of the current study did not show any tendency toward a paroxetine-induced ejaculation delay that was associated with 5-HTTLPR polymorphism, as mirrored by a p-value of 0.83. For a definite answer, a stopwatch study in a much larger cohort of men with lifelong PE and treated by daily 20 mg paroxetine is required. Notably, as various genetic polymorphisms may interact, future research of paroxetine induced ejaculation delay in men with lifelong PE should also focus on interaction of various genetic polymorphisms.

Conclusion

In the current study of 54 men with lifelong PE, 80% of men reported an clinically relevant ejaculation delay and 20% did not report any paroxetine induced ejaculation delay. In addition, 5-HTTLPR polymorphism in these men was not associated with daily 20 mg paroxetine induced ejaculation delay.

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Chapter 6:

Non-responders to daily paroxetine and another SSRI in men with lifelong premature ejaculation: a pharmacokinetic dose-escalation study for a rare phenomenon

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ABSTRACT

Introduction. Non-response on any SSRI treatment is a rare phenomenon.

Aims. To investigate ejaculation delay non-response on paroxetine treatment in men with lifelong premature ejaculation (LPE), who are also known with non-response on other SSRIs.

Methods. Five males with LPE, known with paroxetine and other serotonergic antidepressant non-response, and eight males with LPE, specifically recruited, were included. Blood sampling occurred one month and on the day before start of treatment and at the end of three consecutive series of 4 weeks of daily treatment with 10mg, 20mg and 30mg paroxetine, respectively. Leptin and paroxetine were taken at 08.30, 09.20, 10.00 and 11.30h, respectively. At 09.00h one tablet of 10, 20 or 30 mg paroxetine were taken after the first, second and third month, respectively. IELT was measured with stopwatch

Main Outcome Measures. Fold-increase of geometric-mean IELT, serum leptin and paroxetine concentration, body-mass-index (BMI), 5-HT_{1A} receptor C-1019G polymorphism, CYP2D6 mutations.

Results. Between 7 paroxetine responders and 6 non-responders the fold- increase of geometric-mean IELT was significantly different after daily 10mg ($p=0.003$), 20mg ($p=0.002$), and 30mg paroxetine ($p=0.026$), and ranged from 2.0-8.8, and 1.1-1.7, respectively. BMI at baseline and at end of study was not significantly different in responders and non-responders. Serum leptin levels at baseline were similar in responders and non-responders and did not change during each month of treatment. Serum paroxetine concentration increased with increasing dosages and was not significantly different between responders and non-responders. There was no association between fold-increase of geometric mean IELT and serum paroxetine levels during three treatment periods, and between leptin levels during treatment periods and paroxetine serum levels. For the 5-HT_{1A} receptor C-1019G variation, all responders and all non-responders had the CC genotype and GC genotype, respectively.

Conclusion. Complete absence of paroxetine-induced ejaculation delay is presumably related to pharmacodynamic factors and perhaps to 5-HT_{1A} receptor gene polymorphism.

Introduction

Daily use of selective serotonin reuptake inhibitors (SSRIs) very effectively delays ejaculation in men with lifelong premature ejaculation (PE) (Waldinger, Zwinderman et al. 2004). Compared to the other SSRIs, daily use of 20 mg paroxetine hemihydrate exerts the strongest ejaculation delay (Waldinger, Zwinderman et al. 2004). However, this is not always the case. The extent of ejaculation delay differs between men. For example, in a stopwatch study of 54 men with lifelong PE investigating the association between paroxetine-induced ejaculation delay and 5-HTTLPR polymorphism (Janssen, Zwinderman et al. 2014), 43 (80%) men responded with an ejaculation delay whereas 11 (20%) did not experienced an ejaculation delay. A similar result was found by Salonia et al (Salonia, Rocchini et al. 2009) in a group of 65 men with lifelong PE with an estimated IELT of less than 1 minute. Of these patients, 15 (23%) males discontinued daily paroxetine treatment within 3 months as the ejaculation delay was below expectation, e.g. had minimal ejaculation delaying effects (Salonia, Rocchini et al. 2009).

In rather rare cases men with lifelong PE do not respond on any SSRI treatment with an ejaculation delay. This phenomenon has sofar not been mentioned in the literature of PE and has never been investigated. Theoretically, ultra extensive metabolizers of paroxetine may not respond with an ejaculation delay as an adequate paroxetine concentration may not be established (Søren, Sindrup et al. 1992). However, apart from this potential pharmacokinetic factor, animal studies have shown that also pharmacodynamic factors, such as the amount of serotonin neurotransmission and/or 5-HT_{1A} receptor activation are associated with the extent of ejaculation delay (de Jong, Pattij et al. 2005, Waldinger, Schweitzer et al. 2005). However, whether pharmacokinetic and/or pharmacodynamic factors may explain the (nearly) complete absence of SSRI-induced ejaculation delay in some men with lifelong PE remains unknown.

It is of note that SSRI-induced ejaculation delay has been suggested to be related to decreased leptin serum levels (Atmaca, Kuloglu et al. 2003) and that there is a relationship between serum plasma testosterone levels and the serum leptin level (Behre, Simoni et al. 1997).

The aim of the current study was to investigate the role of various pharmacokinetic factors (e.g., paroxetine dosage, serum paroxetine level, Cyp2D6 activity) and serum leptin, gonadotrophin and prolactin levels, all of which had previously been suggested to be related to PE and SSRI-induced ejaculation delay (Atmaca, Kuloglu et al. 2002, Atmaca, Kuloglu et al. 2003, Nikoobakht, Tajik et al. 2008). In addition, based on the hypothesis of Waldinger et al. that the IELT in men with lifelong PE is related to 5-HT_{1A} and/or 5-HT_{2C} receptor functioning (Waldinger, Berendsen et al. 1998), we investigated the role of 5-HT_{1A} receptor gene C(-1019)G polymorphism.

Due to the rarity of the phenomenon, the small number of patients and controls was inevitable and necessitated a strict protocol of procedures and interventions.

Methods

Patients

Included were patients with complaints of lifelong PE. As (nearly) complete absence of any paroxetine-induced ejaculation delay together with absence of ejaculation delay after another serotonergic antidepressant is an - sofar known - infrequent clinical phenomenon, we informed 5 males, which have been (unsuccessfully) treated by the last author before the start of the current study about the purpose of the study. All of them agreed with participation to the current study, which meant that they could not take an SSRI or clomipramine for 6 months prior to the start of the study. On the other hand, we recruited 8 new patients with lifelong PE, who after telephone screening, were seen at the Outpatient Department of Neurosexology. All men were informed about the purpose of the current study which was to investigate factors that contribute and do not contribute to paroxetine-induced ejaculation delay.

All patients included were heterosexual men, aged 18 to 65 years, and suffering from lifelong PE. They had a stable relationship with a female partner. Exclusion criteria included erectile dysfunction, alcohol or substance abuse, mental disorders, physical illnesses, concomitant medications, a history of sexual abuse, serious relationship problems, a history of very low intercourse frequency, pregnancy of the partner, or pregnancy wish in the near future, and possible unacceptable risks for occupational hazards due to side effects of the study drugs. Informed consent was obtained from all patients after explaining the study and possible side effects. The study was approved by the hospital medical ethical committee and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Assessment and Treatment

Lifelong PE was defined according to the ISSM definition of lifelong PE, e.g., a male sexual dysfunction characterized by ejaculation which always or nearly always occurs prior to or within about one minute of vaginal penetration, and the inability to delay ejaculation on all or nearly all vaginal penetrations, and negative personal consequences, such as distress, bother, frustration and/or the avoidance of sexual intimacy (McMahon, Althof et al. 2008).

The intravaginal ejaculation latency time (IELT) was defined as the time between the start of vaginal intromission and the start of intravaginal ejaculation (Waldinger, Hengeveld et al. 1994).

As placebo response to SSRI-induced ejaculation delay has been shown to be less than 2 fold increase of the geometric mean IELT (Waldinger, Zwinderman et al. 2004), response to treatment in the current study was defined as a fold increase of the geometric mean IELT higher than 2 (Fold-increase= IELT value at the end of treatment period / IELT value at baseline).

Patients attended the outpatient department approximately 1 month before the start of treatment (1^{th} baseline assessment), on the day before treatment (2^{nd} baseline assessment), and at the end of three consecutive series of 4 weeks of daily treatment with 10 mg, 20 mg and 30 mg paroxetine, respectively.

At the first visit, patients were interviewed individually by the last author and asked for an independent estimation of the IELT. A stopwatch and instructions on how to measure the IELT with the stopwatch were provided. The IELT was measured at home over the following 16 weeks. The female partners had to handle the stopwatch. Patients were instructed not to have interrupted intromission or to increase their speed of intercourse. This instruction was checked during later visits to the clinic. If intercourse took place more than once at the time of IELT measurement, only the first episode of vaginal penetration with ejaculation was included. Patients were not permitted to use condoms or topical anaesthetics during the study, and no psychotherapeutic interventions were made.

After a 1-month baseline period, patients received half tablets of paroxetine hydrochloride hemihydrate 20 mg for 4 weeks. The study medication was provided by the hospital pharmacy department. The patients were requested to take half a tablet (10 mg) once a day in the morning in the first month of treatment and one tablet (20 mg)/day in the morning in the following 4 weeks. In the last month, the patients took 1 tablet of paroxetine of 30 mg per day in the morning. After each period of 4 weeks, patients returned to the hospital. On that day blood samples of leptin and paroxetine were taken at 4 fixed times in the morning e.g., at 08.30, 09.20, 10.00 and 11.30 h, respectively. At 09.00 h one tablet of 10, 20 mg or 30 mg paroxetine were taken after the first, second and third month, respectively. At the time of blood sampling and paroxetine intake the patients were sober after 12 hours of fasting. Peripheral venous blood samples were taken by the first author.

Laboratory Analysis

***Leptin Concentration*.**

The leptin serum concentrations were assessed with a validated method of analysis [Human Leptin RIA kit (250 tubes) (Cat.#HL-81K) (Human Linco Research, Inc. St. Charles, USA, MO 63304-uSA)] with a lower limit of quantification of 0,5 ng/ml and a variation coëfficiënt which was dependent on the leptin serum concentrations (Behre, Simoni et al. 1997);

8.3% at 4.9 ng/ml, 4.6% at 7.2 ng/ml and 3.6% at 25.6 ng/ml. After 12 hours of fasting, at 8.30 AM, and at 9.30, 10.30 and 11.30 AM, peripheral venous blood was taken.

***Paroxetine Concentration*.**

The paroxetine serum concentrations were assessed with a validated method of analysis with a lower limit of quantification of 3,7 ug/l and a variation coëfficiënt of 2,9%.

As the concentration of paroxetine is dependent on its metabolism, it should be noted that the metabolism of paroxetine has a linear and a non-linear component (Ma, Gingerich et al. 1996). The non-linear component is caused by transversion of paroxetine by CYP2D6. CYP2D6 has been investigated on the presence of mutations *3, *4 and *6. The CYP2D6 *3, *4, and *6 mutations are coding for enzymes which are less active than the (wildtype) enzymes (Ma, Gingerich et al. 1996). Genotyping of the CYP2D6 enzymes was performed by Taqman analysis. Of the CYP2D6 mutations, the *3,*4, and *6 mutations are most frequently seen in Caucasians (Ma, Gingerich et al. 1996). With these mutations more than 95% of slow metabolisers in a Caucasian population can be determined (Søren, Sindrup et al. 1992).

5-HT$_{1A}$ Receptor Gene Polymorphism (C-1019G).

For a 50-µl polymerase chain reaction (PCR), we used about 10 ng of genomic DNA. The primers of the 5-HT$_{1A}$ receptor gene polymorphism (C-1019G) were: P1 (5'-GGC TGG ACT GTT AGA TGA TAA CG-3') and P2 (5'-GGA AGA AGA CCG AGT GTG TCA T-3''). The underlined nucleotide is a mismatch with the 5HT sequence, creating a restriction site in the PCR product. PCR conditions were as follows: 7 min at 94 ^{o}C; 35 cycles of 1 min at 94 ^{o}C, 1 min at 59 ^{o}C and 1 min at 72 ^{o}C; and finally 7 min at 72 ^{o}C. The size of the amplified product was 163 bp. Then the PCR product (10 µl) was digested with BseGII (Fermentas) in a total volume of 15 µl for 1 h at 55 C and subsequently analyzed on a 3% agarose/Tris-borate-EDTA gel with ethidium bromide staining. The fragments obtained for the wild-type allele was 163bp, for the variant allele the fragments were 146 and 17 bp.

Statistical analysis.

Data analysis was performed with SPSS19. Student's t-test was performed for comparing the various concentrations of leptin and paroxetine. Regression analysis was performed to assess the alterations of leptin concentrations during the day and throughout the 16 weeks duration of the current study. Differences were considered significant at $P< 0.05$.

Results

The characteristics of the patients are shown in Table 1. The study included 6 paroxetine non-responders and 7 responders. Notably, one of these non-responders was among the newly recruited men. As this individual only appeared to not respond to daily paroxetine treatment, this man differed from the 5 previously known men who did not respond to paroxetine and another SSRI or clomipramine. Both groups showed no significant differences in age, marital status and duration of relationship.

	Age Pat	**Age Prt**	**Marital Status (N)**		**Duration**	**Education**	
	year	year			year		
Responders	41 ± 8,1	35 ± 10,1	Married	57 %	12,7	University	-
(N=7)			Divorced	-		Higher Edu	57 %
			Girlfriend	43 %		Lower Educ	29 %
			Single	-		etc	14 %
Non-responders	44 ± 6,1	39 ± 7,4	Married	60 %	13,0	University	-
(N=6)			Divorced	-		Higher Educ	-
			Girlfriend	40 %		Lower Educ	50 %
			Single	-		etc	50 %

Table 1. Patient Characteristics

Table 2 shows the baseline IELT values, the fold-increases of the geometric mean IELT after the three paroxetine treatment periods and the BMI at baseline and at the end of the study. The IELTs in both the responders and non-responders were not significantly different at baseline. All 6 paroxetine non-responders had a fold-increase of less than 2, ranging from 1.1 to 1.7. In contrast, the 7 paroxetine responders had a fold-increase of more than 2, ranging from 2 to 8.8 in relation to the dosage of paroxetine treatment. The number of intercourses at baseline in the responder and non-responder group were 7 and 5, respectively. In the three drug treatment periods, the number of intercourses was 5, 4, and 4 in the responders and 6, 5, and 5 in the non-responder group, respectively.

In the responders, the fold-increase of the geometric mean IELT during the three paroxetine treatment periods was significantly different between the 10 mg, 20 mg and 30 mg dosage ($p=0.004$) and between the 20 mg and 30 mg dosage ($p=0.049$). In the non-responders, the fold-increase of the geometric mean IELT during the three paroxetine treatments periods was not significantly different, e.g., 10 mg versus 20 mg ($p=0.43$) and 20 mg versus 30 mg ($p=0.11$). Between responders and non-responders the fold increase of the geometric mean IELT was significantly different after both 10 mg paroxetine ($p=0.003$), 20 mg paroxetine ($p=0.002$), and 30 mg paroxetine ($p= 0.026$), respectively.

Body mass index (BMI) at baseline and at the end of study, was not significantly different in the responders and non-responders (p=0.49).

	Baseline IELT		**Paroxetine induced**			**BMI**	
Responders	Mean	25.9				27.0 (4.0)	27.0 (4,5)
(N=7)	Geom. Mean	16.9	2.0	6.6	8.8		
	Median	20.0					
Non-responders	Mean	38.4				27.0 (0.8)	26.5 (1.0)
(N=6)	Geom Mean	21.7	1.1	1.5	1.7		
	Median	18.2					

Table 2: Baseline Intravaginal Ejaculation Latency Time (IELT) values, the paroxetine-induced fold-increase of the geometric mean IELT during the 10, 20 and 30 mg paroxetine treatments, and the Body Mass Index (BMI) values at baseline and after the 3 treatment periods.

The serum leptin levels at baseline at 8.30 AM and at 9.30, 10.30 and 11.30 AM and after each month of treatment with 10, 20 and 30 mg paroxetine, are shown in Table 3A. Serum leptin levels at baseline were similar in the responders and non-responders, and during the three paroxetine treatment periods they were not significantly different compared to their baseline values: serum leptin level compared to baseline during the paroxetine 10 mg period was not significantly different (p=0.20), neither was this during paroxetine 20 mg period (p=0.30) and during the 30 mg period (p=0.31). Moreover, serum leptin levels were not significantly different between responders and non-responders (p=0.42).

	Serum Level Leptin	**Serum Level Leptin ug/L 10 mg**				**Serum Level Leptin ug/L 20 mg**				**Serum Level Leptin ug/L 30 mg**			
	baseline	08.30	09.30	10.30	11.30	08.30	09.30	10.30	11.30	08.30	09.30	10.30	11.30
Responders	4,4	4,3	4,2	4	4,1	4,4	4,1	4,1	4	4,1	3,8	3,8	3,8
Non-Responders	4,3	4,5	4,1	4,4	4,3	5,1	5	4,9	5	4,6	4,8	4,6	4,6
		Serum Level Paroxetine ug/L 10 mg				**Serum Level Paroxetine ug/L 20 mg**				**Serum Level Paroxetine ug/L 30 mg**			
		08.30	09.30	10.30	11.30	08.30	09.30	10.30	11.30	08.30	09.30	10.30	11.30
Responders		3,7	4,1	4,4	7,5	12,9	12,6	18,1	25,4	26,6	27,2	34,8	42,7
Non-Responders		3	3	3,1	3,9	10,5	10,5	12,5	21,4	24,5	23,3	28,2	45,6

Table 3. A. Serum Leptin Levels at Baseline and during the three Paroxetine Treatment Periods. B. Serum Paroxetine Concentrations during the three Paroxetine Treatment Periods. Intake of paroxetine hemihydrate at 09.00 AM.

The serum paroxetine concentrations at 8.30, 9.30, 10.30 and 11.30 AM after each month of treatment with 10, 20 and 30 mg of paroxetine are shown in table 3B. In the responders, serum paroxetine concentrations increased between the 10 mg and 20 mg dosage (p=0.023), between 20 mg and 30 mg (p=0.095) and between the 10 mg and 30 mg dosages (p=0.014). In the non-responders, serum paroxetine concentrations increased between the 10 mg and 20 mg dosage (p=0.064), between the 20 mg and 30 mg dosage (p=0.034), and between 10 mg and 30 mg (p= 0.025). Between the responders and non-responders there was no significant difference between the 10 mg dosage (p=0.45), the 20 mg dosage (p=0.33), and the 30 mg dosage (p=0.17).

Between the fold-increase of the geometric mean IELT and serum paroxetine levels during the three treatment periods, no association was found ($y = 0{,}1035x + 2{,}9411 / R^2 = 0{,}066$). (Figure 1). Moreover, also between leptin levels during the treatment periods and paroxetine serum levels no association was found.

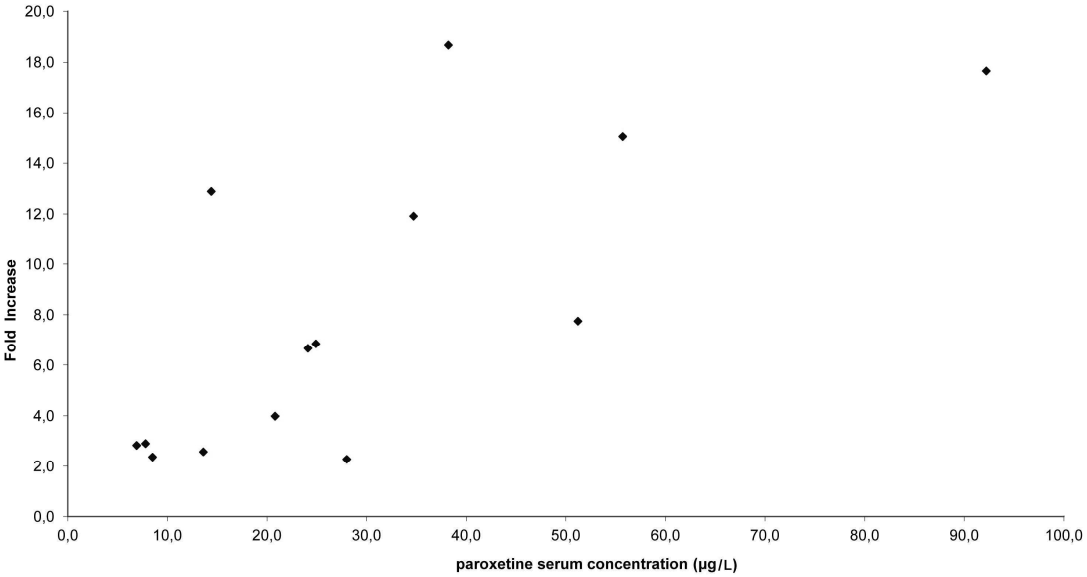

Figure 1. The fold-increase of the geometric mean IELT as function of the paroxetine plasma level (Fold Increase at Y axis; paroxetine serum concentration [µgram/liter] at X axis).

Genetic research demonstrated that all non-responders to paroxetine treatment were heterozygote (GC genotype) for the 5-HT_{1A} receptor C-1019G variation. In contrast, all paroxetine responders had the wildtype (CC) genotype for the 5-HT_{1A} receptor C-1019 G variation (Figure 2).

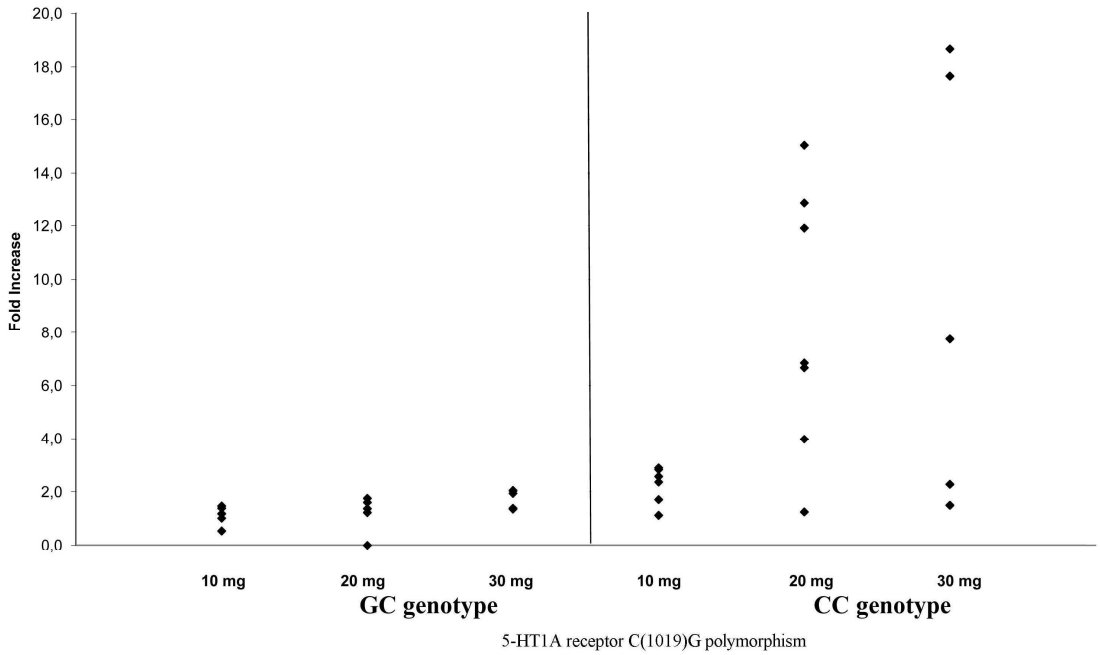

Figure 2. Fold-increase of the geometric mean IELT (Y-Axis) as a function of the serum paroxetine dosages (X-Axis). The second horizontal line represents a Fold-increase of 2. In the current study non-response is defined as a FI of less than 2, and response is defined as a FI of more than 2. At the left side are the FIs of the paroxetine non-responders (GC genotype for the 5-HT_{1A} receptor C-1019 G). At the right side, are the FIs of the paroxetine responders (CC genotype for the 5-HT_{1A} receptor C-1019 G).

Table 4 shows the serum concentrations of testosterone, LH, FSH, SHBG, prolactine, TSH and T4-free at baseline and at the end of 8 weeks of paroxetine treatment in both the paroxetine responders and non-responders. The table shows that within both groups treatment did not result in significant changes of serum concentrations. However, it is remarkable that prolactin serum levels increase during treatment in the responder group, whereas prolactin serum levels tend to decrease during treatment in the non-responder group. This change in prolactin serum concentrations is represented in Figure 3. The change in the prolactin serum level in the responder group compared to its change in the non-responder group was statistically significant different (p=0.044). However, although this phenomenon suggests an association between prolactin release and paroxetine treatment response and perhaps with central 5-HT metabolism, it should not be considered in terms of a causal relationship as this remains unclear.

	Baseline	**Baseline**	**Week 8**	**Week 8**	**Baseline vs week 8**
NONRESPONDERS	$\bar{X}$	**SD**	**gem**	**SD**	**P**
FSH (U/L)	2,2	0,4	2,3	0,5	0,63
LH (U/L)	3,4	1,3	3,7	1,0	0,57
Prolactin (mU/L)	136,8	56,1	112,0	32,8	0,42
SHBG (nmol/L)	36,8	4,6	31,0	12,8	0,52
T4 free (pmol/L)	17,2	1,9	16,3	4,0	0,65
Testosterone (nmol/L)	16,6	3,1	15,3	5,6	0,64
TSH (mU/L)	0,8	0,2	0,5	0,2	0,04
	Baseline	**Baseline**	**Week 8**	**Week 8**	**Baseline vs week 8**
RESPONDERS	$\bar{X}$	**SD**	**gem**	**SD**	**P**
FSH (U/L)	2,9	2,2	3,2	2,3	0,79
LH (U/L)	3,2	1,5	4,0	1,1	0,20
Prolactin (mU/L)	109,4	46,2	147,0	30,8	0,09
SHBG (nmol/L)	25,8	8,9	17,5	2,1	0,11
T4 free (pmol/L)	16,3	2,4	16,7	3,4	0,77
Testosterone (nmol/L)	16,5	6,2	15,9	6,9	0,86
TSH (mU/L)	1,0	0,6	0,7	0,2	0,24

Table 4. Endocrinological Parameters in Responders and Nonresponders

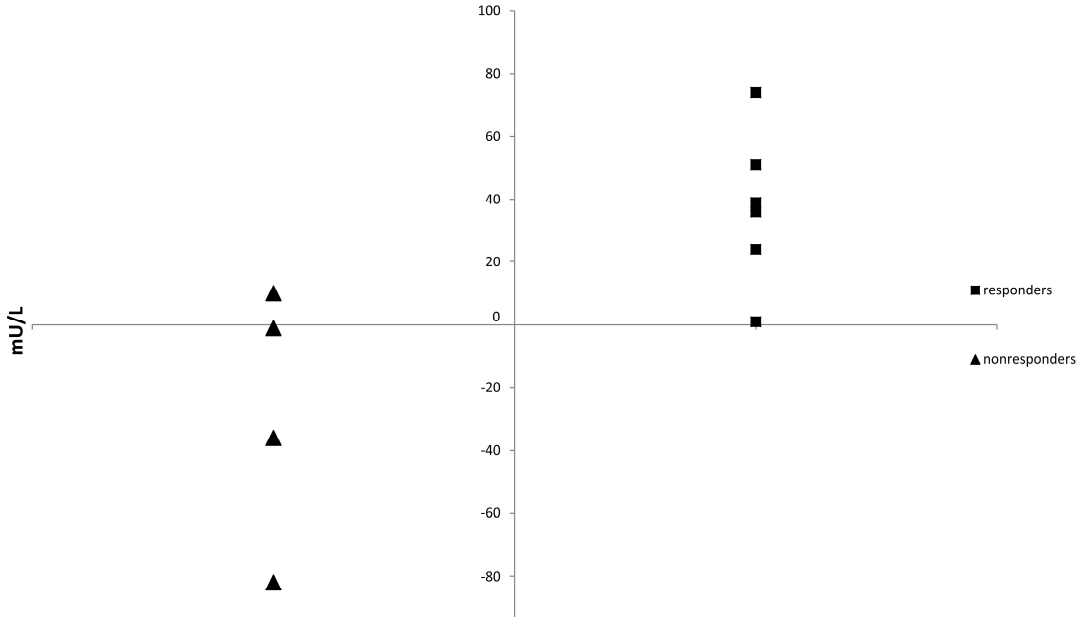

Figure 3. Paroxetine induced serum prolactin concentration changes in men with lifelong PE. In the paroxetine non-responders prolactin concentrations tend to become reduced, whereas in paroxetine responders prolactin concentrations increase.

Discussion

As complete non-response to paroxetine and another SSRI or clomipramine is rare, we were only able to include 5 non-responders, who had been unsucccesfully treated at our outpatient department in the previous 2.6 (SD ±3.6) years with paroxetine 20-30 mg/day, sertraline (n=3), on-demand use of clomipramine (N=2), EMLA crème (n=3), and Tramadol (n=1). As one of the newly recruited men did also not have any response on paroxetine treatment, we decided after the end of the study to include this man into the non-responder group.

Therefore, the current study involved 7 responders and 6 non-responders to daily paroxetine treatment. Five of the 6 non-responders to daily paroxetine treatment in the current study, did also not respond to daily paroxetine treatment and another SSRI or clomipramine treatment in the previous 2.6 (±3.5) years. A clear dose-effect relationship was found in the responder group, with the highest fold increase (FI=8.8) of the geometric mean IELT in men using 30 mg paroxetine compared to those using 10 mg paroxetine (FI=2.0).

At baseline (e.g., 08.30 AM) the serum concentrations of paroxetine in both the responders as non-responders were similar at the start of paroxetine treatment. In both the responders and non-responders the serum concentration of paroxetine increased with increasing dosage. Between the responders and non-responders there was no significant difference between the serum levels at the different dosages of paroxetine throughout the whole period of the study. Therefore, it may be concluded that in the non-responders, increasing serum levels of paroxetine do not result in a clinically relevant ejaculation delay despite the fact that the serum paroxetine levels were similar between the responders and non-responders.

Notably, as serum levels of leptin were not associated with the three different dosages of paroxetine, and remained similar in the responders and non-responders throughout the whole study period, it may also be concluded that the paroxetine-induced ejaculation delay is not associated with the serum leptin level. In this respect, it should be emphasized that at baseline, in both the responders and non-responders, serum leptin levels were similar and within the normal range (3.8 ± 1.8 ng/ml) of males with a BMI of 18-25 (Inc). Notably, the BMI of both the responders and non-responders was similar at baseline and at the end of the study. Importantly, in both the responder and non-responder men, the serum leptin level was not associated with the serum paroxetine levels and did not show any association with the fold increase of the geometric mean IELT.

In the current study, investigation of Cyp2D6 genotypes was investigated by measuring *3, *4, *6 and ultra extensive metabolism (UEM). There were no patients with UEM. The majority of men had Cyp2D6 *1 (homozygote wildtype) but there were two men with Cyp2D6 *3 and *4 mutations, respectively. In other words, in these men there is less Cyp2D6 activity which may result in higher paroxetine plasma levels. Indeed, it was found that while using 30 mg of paroxetine per day, these men had a higher serum paroxetine level than men without these Cyp2D6 mutations. However, as the number of patients was low, one can not make any conclusion about the significance of this higher paroxetine level in relation to these mutations. Moreover, the IELTs of these men were not significantly different, and the BMI and the leptin serum level in these men were also not significantly different compared to men without the Cyp2D6 mutation. In other words, the IELT in these men was not dependent on the serum paroxetine level, the serum leptin level and their BMI.

Interestingly, in the current study we found that the 6 paroxetine non-responders were heterozygous (GC-genotype) for the 5-HT_{1A} receptor C-1019G variation, whereas the 7 responders had the wildtype (CC) genotype.

However, it should be noted that these findings could be the result of a selection bias, as before the onset of the study, we purposely selected 5 non-responders from previous unsuccessful paroxetine and other SSRI and clomipramine treatment.
Intriguingly, although the number of patients in both groups are very low, a similar finding in responders and non responders was not present in our previous study investigating 5-HTTLPR polymorphism and paroxetine-induced ejaculation delay in 54 men with lifelong PE, in which non-response to paroxetine-induced ejaculation delay was present in 20% of men (Janssen, Zwinderman et al. 2014). As the 5-HT_{1A} receptor is important in mediating ejaculation, the current finding warrants further research of this phenomenon in larger group of men with lifelong PE.
Apart from the aforementioned difference in 5-HT_{1A} receptor gene polymorphism in paroxetine responders and non-responders, the current study shows another interesting difference in both groups. The prolactin serum level increases in the responders, whereas it tends to decrease in the nonresponders. Although the prolactin increase in the responders is not significantly different compared to the baseline values in the responder group, and the prolactin decrease in the nonresponders is not significantly different compared to the baseline values in the non-responder group, the change (+delta) of the prolactin levels in the responder group is significantly different ($p=0.044$) compared to the change (-delta) of the prolactin levels in the nonresponder group. Although caution is warranted to interpret this finding in the light of the small number of patients, the phenomenon is intriguing and warrants further research in a larger group of men.
Based on the aforementioned results, it may be derived that in the current group of men with lifelong PE, (i) both lifelong PE and the duration of the IELT are not associated to serum leptin levels, (ii) the serum leptin level in the current group of men with lifelong PE is within the range of the normal population (iii) paroxetine treatment-induced ejaculation delay is not associated with serum leptin levels, (iv) paroxetine treatment induced ejaculation delay is not solely related to serum paroxetine levels, (v) paroxetine treatment-induced ejaculation delay is not associated to serum leptin levels, and (vi) and non-response to paroxetine treatment is not related to paroxetine serum levels, not related to serum leptin levels, and not related to the patients BMI. Instead, it may well be that paroxetine induced ejaculation delay and non-response to paroxetine treatment is associated with perhaps 5-HT_{1A} receptor C-1019 G polymorphism and/or prolactin metabolism or underlying mechanisms of action.

The strength of the current study is its strict sampling protocol, IELT measurement with a stopwatch during a baseline period and during three consecutive 4 weeks daily paroxetine treatment periods, the inclusion of SSRI (including paroxetine) non-responders and paroxetine responders,

blood sampling at 4 different fixed moments of time during the morning after 12 hours of fasting, at the onset of and after 4 weeks of the baseline period, and after three consecutive daily paroxetine treatment periods with 10, 20 and 30 mg paroxetine hemihydrate, paroxetine serum level assessment and leptin, prolactin and gonadotrophin serum level assessments in relation to three different dosages of paroxetine, Cyp2D6 assessment and 5-HT_{1A} receptor polymorphism genotyping. However, a limitation of the current study is the small number of patients.

Although this is the first study investigating SSRI-non response in men with lifelong PE, our findings regarding leptin serum levels are not in line with three previous studies on leptin in relation to PE (See Table 4). For example, in 2002, Atmaca et al. reported a high serum leptin levels of 25.7±3.9 ng/mL in 15 Turkish men with PE compared to a level of 7.9 ±2.1 ng/mL in 15 healthy controls, after adjusting for BMI and age (Atmaca, Kuloglu et al. 2002). In a second study, Atmaca et al (Atmaca, Kuloglu et al. 2003) reported significantly decreased leptin levels (8.3±2.8 ng/mL) in 15 men with PE after 8 weeks of daily 30.7 ± 9.3 mg citalopram treatment, compared to baseline values (23.9 ± 5.3 ng/mL). Atmaca et al. therefore suggested that PE is associated with increased serum leptin levels and that citalopram treatment induces a decrease of leptin (Atmaca, Kuloglu et al. 2003). However, although it was mentioned in the latter study that the patients had the same weight at the end of the study, the BMI at the end of the study has not been reported. It therefore remains rather unclear whether the decreased leptin level after citalopram treatment was related to a change in the BMI of the PE patients. Notably, in contrast to their first study, the second study of Atmaca (Atmaca, Kuloglu et al. 2003) reports a high serum leptin concentration in their control males (24.2 ± 3.8 ng/mL). This finding of Atmaca et al also contrasts the findings of Nikoobakht (Nikoobakht, Tajik et al. 2008) who in 46 Iranian men with PE found a normal but higher baseline serum leptin level (8.3 ± 3 ng/mL) than in a control group of 44 men with nepholithiasis (3.3 ± 1 ng/mL).

In our current study, all patients had leptin levels which had a normal value compared to a standard control group. Moreover, our patients, both the responders and non-responders, had the same leptin levels, both at baseline and at the end of the three paroxetine treatment periods. Indeed, in our study both the responders and non-responders also had an identical BMI both at the start and at the end of the study. Based on the aforementioned findings, there is no reason to believe that lifelong PE or the IELT is related to weight and/or serum leptin concentration. Neither there is a reason to believe that paroxetine treatment influences or is influenced by the serum leptin levels.

In other words, based on our study, in which serum leptin and testosterone levels, other gonadotrophic parameters, TSH, T4-free and BMI remained homogenous throughout paroxetine treatment in both responders and nonresponders, serum leptin levels can not be considered a biological marker of PE as has been suggested by Atmaca et al (Atmaca, Kuloglu et al. 2002, Atmaca, Kuloglu et al. 2003) and Nikoobakht (Nikoobakht, Tajik et al. 2008). On the contrary, there are still many questions regarding leptin that have to be investigated in the context of lifelong PE. As is summarized in Table 5, it is of note that the four studies on leptin, including the current study, have used different leptin kits.
In the current study, a radio immune assay (RIA) method, specific for human leptin, was used, whereas Nikoobakht (Nikoobakht, Tajik et al. 2008) used an enzyme linked immune assay (ELISA) method, specific for human leptin. In contrast, Atmaca (Atmaca, Kuloglu et al. 2002, Atmaca, Kuloglu et al. 2003) used a RIA method, specific for rat leptin to measure human serum leptin concentrations. Although our human kit has a cross reactivity of 100% with human leptin, the cross-reactivity of Atmaca (Atmaca, Kuloglu et al. 2002, Atmaca, Kuloglu et al. 2003) is only 40% (Inc). However, whether the use of different leptin kits contributes to the different findings of Atmaca (Atmaca, Kuloglu et al. 2002, Atmaca, Kuloglu et al. 2003), Nikoobakht (Nikoobakht, Tajik et al. 2008) and the current study remains unknown. Another explanation for the different findings, may perhaps be related to different testosterone levels of the included patients. It has been reported that high testosterone in males is associated with low serum leptin level (Behre, Simoni et al. 1997). As serum testosterone has not been measured in any of the aforementioned studies, it can't be excluded that the differences between the four studies are related to differences in serum testosterone levels. It is therefore recommended that for future studies on PE and leptin, serum testosterone will be measured.

Study	PE men at baseline	controls at baseline	after SSRI treatment PE men	after SSRI treatment controls	BMI PE Men at baseline	BMI PE Men end study	BMI controls baseline	BMI controls end study	Assay Type / leptin kit *3	Time of Blood Sampling	serum concentration medication	Genetic variations
	leptin	**leptin**	**leptin**	**leptin**								
	ng/ml	**ng/ml**	**ng/ml**	**ng/ml**								
Atmaca, 2002	25.7 ± 3.9	7.9 ± 2.1			24.0 ± 2.8		24.4 ± 3.3		RIA rat *1	08:00	N	-
Atmaca, 2003	23.9 ± 5.3	24.2 ± 3.8	8.3 ± 2.8	24.9 ± 3.5	23.7 ± 1.9	-	23.3 ± 2.3	-	RIA rat *1	08:00	N	-
Nikoobakth 2008	8.3 ± 3.0	3.3 ± 1.0			28.7 ± 2.3		24.7 ± 3.6		ELISA DRG *2	08:00	N	-
Janssen, 2013									RIA human *1	08:30 09:30 10:30 11:30	paroxetin	5HT1A (C-1019 G) CYP2D6 (*3 *4 *6)
responder	4.4 ± 0.6	-	4.1 ± 0.3	-	27.0 ± 4.0	27.0 (4.5)	-	-				5HT1A CC
nonresponder	4.3 ± 0.5	-	4.6 ± 0.3	-	27.0 ± 0.8	26.5 ± 1.0	-	-				5HT1A CG

*1 Linco research (St. Charles, MO)
*2 DRG diagnostics Marburg diagnostics
*3 sample material = serum

Table 5. Summary of four Studies on Premature Ejaculation and Leptin

Conclusion

Using a very strict and elaborate sampling protocol at different dosages of daily use of paroxetine, we have found no explanation for the absence of paroxetine-induced ejaculation delay in six men with lifelong PE.

Moreover, we have found not any indication that the level of leptin is associated with either the presence of lifelong PE, the dosage or duration of daily paroxetine treatment, the serum level of paroxetine, or the duration of the IELT at baseline or at paroxetine treatment. In addition, we have found no evidence that daily use of paroxetine influences plasma leptin levels in both the responders and non-responders to daily paroxetine treatment.

Although it was found that increasing daily dosages of paroxetine was associated with a stronger ejaculation delay, we have found no association between paroxetine serum concentration and paroxetine-induced fold increase of the geometric mean IELT. However, in the current study it was found that serum concentrations of paroxetine are higher in two men with Cyp2D6 *3 and*4 variations, respectively, but as there is no serum paroxetine concentration and IELT effect relationship, this genotype for paroxetine metabolism is not relevant for paroxetine-induced ejaculation delay in the current study. Interestingly, in the current group of men, we have found two intriguing phenomenons. Firstly, all paroxetine non-responders were heterozygous (GC-genotype) for the 5-HT_{1A} receptor C-1019G variation, whereas all responders had the wildtype (CC) genotype. However, although we have argued that these results may be related to a selection bias, further research in larger group of men with lifelong PE is warranted to investigate whether this polymorphism plays a role in paroxetine treatment non-response. Secondly, serum prolactine levels increased in the paroxetine responders, whereas they tended to decrease in the paroxetine non-responders. The change in prolactin serum level after paroxetine treatment in the responders was significantly different compared to the prolactin serum level change in the nonresponders. Also this phenomenon deserves further investigation in future studies.

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Chapter 7:

Measurement errors in polymerase chain reaction are a confounding factor for a correct interpretation of 5-HTTLPR polymorphism effects on lifelong premature ejaculation: a critical analysis of a previously published meta-analysis of six studies.

Janssen, P.K., Olivier B., Zwinderman A.H., Waldinger M.D. (2014).
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ABSTRACT

Introduction. It will be argued that a reliable comparison of 6 previously published articles on 5-HTTLPR polymorphism and premature ejaculation can not be performed.

Aim. To analyze a recent meta-analysis of 6 studies on 5-HTTLPR polymorphism and lifelong premature ejaculation (PE).

Methods. Calculation of the fraction of observed and expected genotype frequencies and Hardy Weinberg equilibrium (HWE) of cases and controls. LL, SL and SS genotype frequencies of patients were subtracted from genotype frequencies of an ideal population (LL 25%, SL 50%, SS 25%, p=1 for HWE). Analysis of PCRs of 6 studies and re-analysis of Odds ratios (ORs) reported in the recent meta-analysis.

Results. Three studies deviated from HWE in patients and one study deviated from HWE in controls. In 3 studies in-HWE the mean deviation of genotype frequencies from ideal population was small. LL (1.7%), SL (-2.3%), SS (0.6%). In 3 studies not-in-HWE the mean deviation of genotype frequencies was high: LL (-3.3%), SL (-18.5%) and SS (21.8%) with a very low percentage SL genotype concurrent with a very high percentage SS genotype. The most serious PCR deviations were reported in the 3 studies not-in-HWE. The 3 studies in-HWE had normal OR. In contrast, the 3 studies not-in-HWE had a low OR.

Conclusions. In 3 studies not-in-HWE and with very low OR, inadequate PCR analysis and/or inadequate interpretation of its gel electrophoresis resulted in very low SL and a resulting shift to a very high SS genotype frequency outcome. Consequently, PCR of these 3 studies are not reliable. Unawareness of inadequate PCR tests makes such PCRs a confounding factor in the clinical interpretation of genetic studies. Based on 3 studies in-HWE and with OR of about 1 there is not any indication that in men with lifelong PE the frequency of LL, SL and SS genotype deviates from the general male population and/or that the SL or SS genotype is in any way associated with lifelong PE.

Keywords. genetics, lifelong premature ejaculation, 5-HTTLPR polymorphism, meta-analysis, Hardy Weinberg Equilibrium, polymerase chain reaction, IELT

Introduction

Lifelong premature ejaculation (PE) is defined as a male sexual dysfunction characterized by ejaculation that always or nearly always occurs prior to or within about 1 minute of vaginal penetration, the inability to delay ejaculation on all or nearly all vaginal penetrations, and with negative personal consequences, such as distress, bother, frustration, and/or the avoidance of sexual intimacy (McMahon, Althof et al. 2008). In contrast, men with acquired PE have never suffered from complaints of PE but experience a reduction of the ejaculation time later in life, often to an estimated intravaginal ejaculation latency time (IELT) of less than about 3 minutes (Serefoglu, Cimen et al. 2010, Serefoglu, Yaman et al. 2011, Zhang, Gao et al. 2013). In 1998, Waldinger et al postulated that lifelong PE in terms of an IELT of less than 1 minute is related to genetic factors and to diminished central 5-HT neurotransmission and/or dysfunctional 5-HT1A and 5-HT2C receptors (Waldinger, Berendsen et al. 1998). Although lifelong PE is not regarded a genetic hereditary disorder, Waldinger (Waldinger, Rietschel et al. 1998) reported a familial occurrence of lifelong PE in first degree relatives of some male patients with lifelong PE. After the publication of the first study on the influence of 5-HTTLPR polymorphism and IELT duration in Dutch men with lifelong PE by Janssen (Janssen, Bakker et al. 2009), five similar studies have been published (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013).

Recently, Zhu (Zhu, Mi et al. 2013) published a meta-analysis on these 6 studies and concluded that L-alleles of 5-HTLPR polymorphism might protect men against lifelong PE risk (Zhu, Mi et al. 2013). However, with regard to potential laboratory insufficiencies and differences in design and methodology, it is questioned whether it is allowed to combine these 6 studies for meta-analysis. In this context, disturbances of Hardy-Weinberg equilibrium (HWE) as indicator of a laboratory insufficiency in genetic studies on lifelong PE has previously already been emphasized (Waldinger, Janssen et al. 2009, Waldinger, Janssen et al. 2009, Waldinger 2011).

Similarly, Yonan (Yonan, Palmer et al. 2006) have shown that lowering the magnesium concentration of the mixture of the polymerase chain reaction (PCR) resulted in a shift of the relative allele frequencies. As a result, the initial outcome ($p=0.06$) of the HWE, suggestive of an association with autism spectrum disorder, was restorated. In other words, the initially found linkage of 5-HTTLPR polymorphism and autism spectrum disorder, disappeared by correction of the magnesium content of the PCR. Therefore, Yonan et al, correctly concluded that higher magnesium concentrations of the PCR caused allele-dependent, non-random genotyping errors.

In the current article, we show that out of the 6 previously published articles on 5-HTTLPR polymorphism and premature ejaculation used for the meta-analysis, laboratory data show that 3 studies were not in HWE and that in those 3 studies the deviation of HWE is due to technical insufficiencies and/or measurement errors of the PCR. As the 6 studies also differed in clinically relevant factors of design and methodology, it will be argued that a reliable comparison of the 6 studies by a meta-analysis can not be performed.

Material and Methods

We analyzed the 6 articles that were used for the meta-analysis performed by Zhu (Zhu, Mi et al. 2013), and also analyzed the statistical calculations as described in the meta-analysis of Zhu (Zhu, Mi et al. 2013). For this analysis, we only used the data that were mentioned in the 6 articles. Based on the absolute genotype frequencies we calculated the fraction of observed and expected genotype frequencies. With these data we calculated the HWE of cases and controls. For comparison with an ideal population (characterized by LL 25%, SL 50%, SS 25% and therefore p=1 for HWE) we subtracted the LL, SL and SS genotype frequencies of the patients and the controls from the genotype frequencies of the ideal population. In other words, we calculated the difference between the observed genotype percentages and the percentages of the ideal population.

An analysis was also performed on the polymerase chain reaction (PCR) of the 6 studies, as far as the details of the PCR were provided by the authors. The details pertained (i) to the content of the reaction mixture, (ii) the PCR-program and (iii) the gel-electrophoresis.

Ad (i).The content of the reaction mixture included forward and reverse primers, polymerase buffer (PB), dNTPs, magnesiumchloride, concentration of the primers, polymerase concentration, amount of genomic DNA and its total volume.

Ad (ii). The PCR-program included the first step of temperature and duration of preheating followed by cycles of duration and temperature of denaturation, annealing, extension and final hold at the end of the cyclus.

Ad (iii).The gel-electrophoresis included the concentration of the gel, the applied voltage and the duration of the procedure.

For analysis of the methods and design of the six studies we summarized whether the studies were performed with a stopwatch or questionnaire, whether men reported lifelong, acquired and or both PE subtypes, and whether the IELT was within or longer than 1 minute.

Statistics: Hardy–Weinberg equilibrium to check laboratory efficacy of PCR analysis was determined using a Chi-square test. Allele and genotype frequencies were compared using SPSS 19.0 forWindows (Chicago, IL, USA). $P \leq 0.05$ was considered statistically significant.

Results

Hardy-Weinberg Equilibrium.

Table 1 shows the 6 studies on 5-HTTLPR polymorphism and premature ejaculation. It shows the genotype frequencies (LL, SL, and SS) of both the patients and the control individuals. Three (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011)of these studies showed deviation of HWE in the patients, as reflected by their p values of ≤ 0.05, and one study (Luo, Wang et al. 2011) also showed a deviation of HWE in the controls.

Table 1: 5-HTTLPR genotype frequencies in patients and controls as reported by the authors of 6 studies

Author	Year of	Cases	Cases				Controls	Controls				
	publication	N	PWeinberg	%	%	%	N	PWeinberg	%	%	%	P case
				LL	SL	SS			LL	SL	SS	control
Janssen	2009	89	0,9707	30,3	48,3	21,4	92	0,5862	29,3	44,6	26,1	0,5657
Safarinejad	2009	82	0,0318	29,2	35,4	35,4	82	0,1116	42,7	36,6	20,7	0,0025
Luo	2011	119	0,0003	20,1	28,6	51,3	90	0,0156	27,8	34,4	37,8	0,0002
Ozbek	2009	69	0,0543	15,9	30,4	53,7	69	0,7698	17,4	53,6	29,0	0,0002
Zuccarello	2012	89	0,6217	24,7	55,1	20,2	100	0,9174	33,0	51,0	16,0	0,0787
Jern	2012	33	0,9809	25,1	39,7	35,2	33	0,9961	30,9	44,2	24,9	0,9297

Table 2. Difference of the genotype frequencies of the patients and controls of 6 studies with an ideal genotype frequency.

Author	Year of	Cases	Cases				Controls	Controls			
	publication	N	P Weinberg	25	50	25	N	P Weinberg	25	50	25
				LL	SL	SS			LL	SL	SS
Janssen (ref 7)	2009	89	0,9707	5,3	-1,7	-3,6	92	0,5862	4,3	-5,4	1,1
Safarinejad (ref 8)	2009	82	0,0318	4,2	-14,6	10,4	82	0,1116	17,7	-13,4	-4,3
Luo (ref 10)	2011	119	0,0003	-4,9	-21,4	26,3	90	0,0156	2,8	-15,6	12,8
Ozbek (ref 9)	2009	69	0,0543	-9,1	-19,6	28,7	69	0,7698	-7,6	3,6	4,0
Zuccarello (ref 11)	2012	89	0,6217	-0,3	5,1	-4,8	100	0,9174	8,0	1,0	-9,0
Jern (ref 12)	2012	33	0,9809	0,1	-10,3	10,2	33	0,9961	5,9	-5,8	-0,1

Table 2 shows the frequency difference of the genotype frequencies of the 6 studies with the ideal population, characterized by (the ideal) genotype frequencies of LL (25%), SL (50%) and SS (25%). For example, in the study of Safarinejad (Safarinejad 2009), SL frequency is 35.4%, which is 14.6% lower than the 50% SL frequency in the ideal population. Similarly, in the study of Luo (Luo, Wang et al. 2011), the SL frequency was 28.6%, which is 21.4% lower than the 50% SL frequency in the ideal population.

Interestingly, in the 3 studies which do not deviate from HWE (Janssen, Bakker et al. 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013), the mean deviation of the genotype frequencies from the ideal population is rather low: LL (1.7%), SL (-2.3%) and SS (0.6%).

In contrast, in the 3 studies which do deviate from HWE (Safarinejad 2009), the mean deviation of the genotype frequencies from the ideal population is very high: LL (-3.3%), SL (-18.5%) and SS (21.8%). Importantly, in the 3 studies that are not-in-HWE (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011), the direction of the deviation is similar: a vey low percentage of SL genotype concurrent with a very high percentage of SS genotype.

PCR-analysis.

Table 3 shows the differences of the PCR test of the 6 studies. It shows that the PCRs of the 6 studies differed from one another. Apart from the fact that 5 authors did not report all the relevant information of a PCR reaction mixture, it was found that there was a difference in both the forward and reversed primers, with only 2 studies (Janssen, Bakker et al. 2009, Ozbek, Tasci et al. 2009) using identical primers. Moreover, the 6 studies differed in the polymerase buffer, the concentration of the dNTPs, the magnesium chloride concentration, the absolute concentration of the primers, the concentration of polymerase, and the concentration of genomic DNA. Moreover, the total volume of the reaction mix differed from 10 to 50 µliter.

Table 3. Differences of the PCR test of the 6 studies with regard to primers and PCR reaction mixture; FP=forward primer, RP=reversed primer, PB=polymerase buffer, dNTPs=oligonucleotides, MgCl=magnesiumchloride.

Author	Year of	FP 5'-3'	RP 5'-3'	PB	dNTPs	MgCl2	primers	Polymerase	genomic DNA	Total
	publication						each			Volume
								U	ng	ul
Janssen	2009	GGCGTTGCCGCTCTGAATC	GAGGGACTGAGCTGGACAACCAC	1 ul 10 times	0,2 mmol/L	2.0 mmol/L	0.4 uM/L	0.5	50	10
Safarinejad	2009	GGCGTTGCCGCTCTGAATGC	AGGGGACTGAGCTGGACAAC	-	10mM	1.5 mM	2 uM	-	20	50
Luo	2011	CTGGCGTTGCCGCTCTGAAT	GAGGGACTGAGGTGGACAACCAC	-	0,25 mmol/L			1.0	100	20
Ozbek	2009	GGCGTTGCCGCTCTGAATC	GAGGGACTGAGCTGGACAACCAC	-	0,2 mmol/L	2,0 mmol/L	0,4 uM/L	1.0	100	25
Zuccarello	2012	TGAATGCCAGCACCTAACCC	TTCTGGTGCCACCTAGACGC	2.5 uL 10 times	-	-	10 uM	-	100	23
Jern	2012	ATGCCAGCACCTAACCCCTAATGT	GGACCGCAAGGTGGGCGGGA	-	-	1.5 mM	0,3 uM	1.0	50	15

Table 4 shows the specification of the polymerase used in the various studies. Five of the six studies provided the specification of the polymerase that was used in the reaction mixture.

Author	Year of	Polymerase used	Firm
	Publicati		
Janssen	2009	AccuPrime Pfx DNA polymerase	Invitrogen Life Technologies, Strathclyde, UK
Safarinej	2009	Polymerase in: GC-Rich PCR System	Roche Molecular Biochemicals, Basel, Switzerland
Luo	2011	-	-
Ozbek	2009	Taq Polymerase	MBI Fermentas, Hanover, MD, USA
Zuccarell	2012	Taq DNA Polymerase	Roche Diagnostics, Milano, Italy
Jern	2012	Hotstar Taq Polymerase	Qiagen

Table 5 shows the PCR program. All the 6 studies differed in the various parameters of the PCR-program. There was a significant difference in the duration of the preheating period. In addition, 2 studies (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011) differed in the duration of the denaturation period from the 4 other studies. The duration of the annealing differed in 2 studies (Janssen, Bakker et al. 2009, Safarinejad 2009) from the 4 other studies. The duration of extension was aberrant in one study (Ozbek, Tasci et al. 2009). The duration of the final hold differed significantly from 4 to 10 minutes among 5 studies. The number of cycles differed from 33 to 37 among 5 studies.

Table 5. Differences of the PCR test of the 6 studies with regard to PCR program

Author	Year of	preheating	preheating	denaturation	denaturation	annealing	annealing	extension	extension	final	final	Cycles
	publication	Min	C	min	C	min	C	min	C	min	C	
Janssen	2009	4	94,0	0,5	94,0	0	60	1	68	4	72	33
Safarinejad	2009	3	95.5	1	95,5	1	60	1	72	7	72	35
Luo	2011	5	94,0	1	94,0	0,5	61	1	72	10	72	-
Ozbek	2009	4	94,0	0,5	94,0	0,5	60	0,75	72	8	72	33
Zuccarello	2012	4	94,0	0,5	94,0	0,5	61	1	72	4	72	37
Jern	2012	15	95,0	0,5	95,0	0,5	66	1	72	-	-	35

Table 6: Differences of the PCR test of the 6 studies with regard to the gel electrophoresis

Author	Year of	agarose gel	agarose gel	agarose gel
	Publication	%	min	V
Janssen	2009	2,0	120	100
Safarinejad	2009	2,0	-	-
Luo	2011	-	60	100
Ozbek	2009	2,0	30	100
Zuccarello	2012	2,5	45	150
Jern	2012	2,0	-	-

Table 6 shows the gel-electrophoresis. It was found that only 4 studies (Janssen, Bakker et al. 2009, Ozbek, Tasci et al. 2009, Luo, Wang et al. 2011, Zuccarello, Ghezzi et al. 2012) provided information of the gel-electrophoresis. In these 4 studies, the duration of the gel-electrophoresis differed significantly from 30 to 120 minutes.

Table 7 shows the differences in study design and methodology of the 6 studies. Only two studies(Janssen, Bakker et al. 2009, Safarinejad 2009) have used a stopwatch to measure the IELT, whereas the other four studies relied on the information on a questionnaire. Of all studies, most authors used an inclusion criterion of an IELT $\leq$ 60 sec in more than 90% of sexual events. However, the study of Ozbeck (Ozbek, Tasci et al. 2009) also included men who only in 50% of sexual events ejaculated within 1 minute. Moreover, three studies (Janssen, Bakker et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011) reported the characteristics of the investigated cohort of men,

whereas two studies (Ozbek, Tasci et al. 2009, Zuccarello, Ghezzi et al. 2012) only partially reported the characteristics and one study (Jern, Eriksson et al. 2013) failed to do so.

Table 7. Differences of the study design and methodology of the 6 studies (Y=yes, N=no)

	publication	Stopwatch	IELT ≤ 60 sec	population
				description
Janssen	2009	Y	Y	Y
Safarinejad	2009	Y	Y	Y
Luo	2011	N	Y	Y
Ozbek	2009	N	Partially	Partially
Zuccarello	2012	N	Y	Partially
Jern	2012	N	Y	N

Discussion

In the current study we have shown that from the 6 studies, used in the meta-analysis of Zhu (Zhu, Mi et al. 2013), 3 studies (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011) were not in HWE, as represented by a $p \leq 0.05$. By analysing the data of the 6 studies and comparison of these data with the calculated genotype frequencies of an ideal population, we have found that the SL and SS genotype frequencies were normally distributed in the 3 studies that were in-HWE (Janssen, Bakker et al. 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013). However, they were abnormally distributed in the 3 remaining studies that were not in Hardy Weinberg equilibrium (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011). Most importantly, we found that the direction of this abnormal distribution was similar in all the three studies, e.g., very low SL and very high SS genotype frequencies (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011).

Our findings concur with the study of Yonan (Yonan, Palmer et al. 2006), who initially also found a low percentage of SL genotype concurrent with a high percentage of SS genotype in a study of 5-HTTLPR polymorphism and autism disorders. However, correction of the PCR reaction mixture by increasing its magnesium concentration resulted in a change of the genotype frequency distribution.

The remarkable similarity of the deviation in the 3 studies (e.g., very low SL genotype frequency concurrent with very high SS genotype frequency) only becomes clear by understanding the procedure of a polymerase chain reaction (PCR), and the consequences of technical insufficiencies and/or inadequate interpretation of its gel electrophoresis.

The PCR is a biochemical technique in a biological research lab to amplify a single or a few copies of a piece of DNA towards thousands to millions of copies of a particular DNA sequence (Mullis 1990). The method relies on thermal cycling, i.e., alternately heating and cooling of the reaction to induce melting of the DNA and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target DNA region along with a heat-stable DNA polymerase (after which the method is named) are key components to enable selective and repeated DNA amplification. As PCR progresses, the

DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.
A basic PCR set up requires several components and reagents (Sambrook and Russel 2001). These components include: DNA template containing the DNA target region, two primers, Taq polymerase, deoxynucleotide triphosphates, a buffer solution providing a suitable chemical environment for optimum activity and stability of the DNA polymerase, divalent cations, magnesium or manganese ions, and monovalent cation potassium ions.
To check whether the PCR generated the anticipated DNA fragment (the amplimer or amplicon) “agarose gel electrophoresis” is employed for size separation of the PCR products. With this technique the amplification products are electrophoresed on 2% agarose gels at 100 Volt for 120 minutes. For this purpose the gel and running buffers need to contain the right content. In order to see the DNA fragments they need to be visualized by ethidium bromide under UV transillumination. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products (see Figure 1).

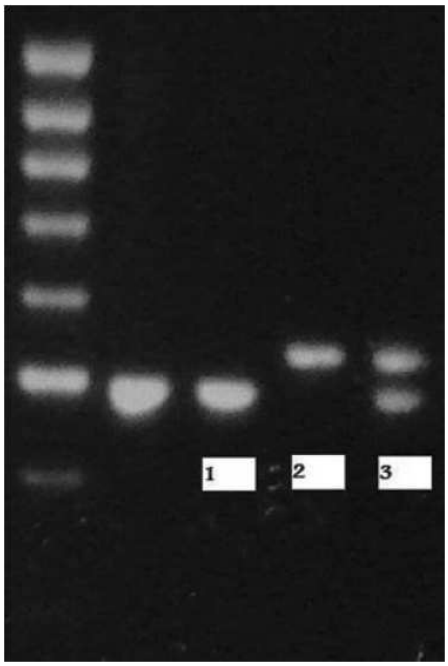

Figure 1. Photograph of illuminating DNA fragments on gel under ultraviolet light after electrophoresis. DNA bands in lane 1, 2 and 3 indicate successful amplification of the target sequence. The gel also shows a positive control, and a DNA ladder containing DNA fragments of defined length for sizing the bands in the experimental PCRs. Lane 1: homozygous patient for LL alleles, Lane 2: homozygous patient for SS alleles; Lane 3: heterozygous patient for LS alleles (L=long, S=short).

Figure 1 shows a PCR product (e.g., DNA of a patient) after gel electrophoresis. For a good interpretation of this test, clear distinction of the short and the long allele is essential. However, clear distinction can be obscured by insufficiencies of the test itself. For example, a lower concentration of magnesium in the gel (or an allele specific reaction in the gel) may diminish the visibility of the long allele. As a result, the investigator will count less long alleles(L) and more short alleles (S), although these long alleles are present in the DNA content. In other words, in case of a heterozygote SL (SL in lane 3 in Figure 1) the short allele S will be visible, whereas the long allele L will be less visible. This induces the risk that the (wrong) conclusion will be made that the SL genotype frequency is low, whereas the SS genotype frequency will be high.

Our finding that in the 3 studies that were not in HWE, the SL genotype frequencies are strongly decreased concurrent with a strongly increased frequency of SS genotype, fits perfectly well with the aforementioned wrong interpretation of gel electrophoresis in case of an insufficient gel mixture of the PCR. However, it may also be due to inexperience of the laboratory investigator with this type of lab research.

Indeed, our additional analysis of the PCRs of the 6 studies, shows essential differences in the PCRs which may have influenced the outcome of these PCRs. An additional finding was that of the 6 articles, 5 authors did not provide all the required information of the PCR analysis (See Figure 2).

	Data shown In table 2	Janssen	Safarinejad	Luo	Ozbeck	Zuccarello	Jern
reaction mix	lack of information	0	2	3	1	3	2
	major differences	0	1	0	0	1	0
program	Data shown In table 3						
	lack of information	0	0	2	0	0	2
	major differences	0	1	1	1	0	0
product separation and reading	Data shown In table 4						
	lack of information	0	2	1	0	0	2
	major differences	0	0	1	1	1	
	sum total	0	6	8	3	5	6
	sum major differences	0	2	2	2	2	0

Figure 2. Aberrations in PCR Analysis in Six Studies. Details are represented in Tables 2-4; 0=no aberration, 1=one aberration; 2=two aberrations; 3= three aberrations.

Notably, of the information that has been published, it appears that there are important aberrations in the PCR reaction mixture, the PCR program and the gel-electrophoresis. In these 3 phases of the PCR the most relevant aberrations are found in the studies of Safarinejad, Luo and Ozbeck (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011). For example, Luo and Ozbeck (Ozbek, Tasci et al. 2009, Luo, Wang et al. 2011) used a very short electrophoresis time, which may result in inadequate separation of the PCR products. Also the duration of denaturation and extension was different in the studies of Safarinejad, Luo and Ozbeck (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011). This may have resulted in inadequate extension of the DNA. By lack of provided information, it remains unknown what the amount of polymerase buffer has been in the studies of Safarinejad, Luo and Ozbeck (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011).

In summary, our analysis of genotype frequencies has shown that of the 6 studies the studies of Safarinejad, Luo and Ozbeck (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011) are not-in-HWE. Additional analysis of the PCRs of the 6 studies shows that the major differences in the PCRs are found in the studies of Safarinejad, Luo and Ozbeck (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011).

Therefore we suggest that in the 3 studies that were not-in-HWE and had the most significant aberrations in PCR (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011), the PCR test had a preference for the short allele to become visible for the laboratory investigator. As a result, part of the heterozygotes (SL) are erratically interpreted as homozygote mutant (SS). Indeed, in the 3 studies not-in-HWE (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011), there is a very low frequency of SL genotype and a very high frequency of SS genotype, compared to the studies of Janssen, Zuccarello and Jern (Janssen, Bakker et al. 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013) who are in-HWE, whereas the percentage of the homozygote LL genotype does not appear to be affected.

It should be noted that the study of Ozbeck (Ozbek, Tasci et al. 2009) shows a marginally significant effect ($p \leq 0.0543$). Moreover, in this study the SS genotype deviation from the ideal population is 28.7%, which is the highest of all studies.

Apart from the aforementioned technical insufficiencies of the PCR analysis and/or its interpretation, it has been found that the 6 studies significantly differed in clinical design and methodology. Most importantly, only two studies investigated the IELT values measured by stopwatch (Janssen, Bakker et al. 2009, Safarinejad 2009).

The findings of our study show that apart from an good clinical design and methodology, a correct laboratory performance and interpretation of the PCR is an essential requirement for an evidence based study of 5-HTTLPR polymorphism and lifelong PE.

An inadequate PCR test is a serious confounder as it may give rise to false-positive SS genotype frequencies and false-negative SL genotype frequencies. Unfortunately, this is unknown to clinicians who are not used to perform a PCR themselves. Unaware of the pitfalls of an inadequate PCR test they tend to uncritically accept the (written) conclusion of the laboratory investigator. An indication of the unawareness of clinicians of the importance of a good PCR test, is our finding that in 5 of the 6 studies essential information of the PCR has not been reported in the section materials and methods giving the impression as if the authors (and also the reviewers of their manuscripts) do not consider this information important for the reader. However, we would like to emphasise that for a good understanding and interpretation of the laboratory work future genetic studies of lifelong PE and all other studies should provide all the relevant data of the PCR procedure.

As 3 of the 6 studies were not-in-HWE based on inadequate PCR analysis of DNA fragments, it may be clear that a meta-analysis cannot be performed on the 6 studies as they differ on the most essential procedure of genetic research. The remaining 3 studies that were in-HWE (Janssen, Bakker et al. 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013) and did not show dramatic PCR insufficiencies, show no significant aberations of LL, SL or SS genotype frequencies compared to the normal population. In other words, these 3 studies (Janssen, Bakker et al. 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013) show that the genotype frequencies of men with lifelong PE is just normally distributed. However, and interestingly, one of these 3 studies, showed that men with lifelong PE and with a LL genotype have a significant shorter IELT than men with SS genotype (Janssen, Bakker et al. 2009).

It is of note that there are indications for a geographical spread of the S-allele occurrence of 5-HTTLPR around the world. In Western Europe the S-allele frequency is about 45%, where in Turkey and China it is 55% and 70%, respectively (Chiao and Blizinsky 2010). According to these general data, three studies of the six articles (Ozbek, Tasci et al. 2009, Safarinejad 2009, Luo, Wang et al. 2011) have been performed in countries with a natural higher S-allele frequency occurrence compared to Western European countries (Chiao and Blizinsky 2010). However, even when there is a natural higher S-allele frequency occurrence in non-Western European countries, this will not have any influence on our findings of the PCR test analysis. Our view and conclusion opposes that of Zhu (Zhu, Mi et al. 2013). These authors who performed a meta-analysis on the same 6 studies, argued that a meta-analysis is allowed in spite of the fact that they are aware that some of these studies are not-in-HWE. As SS genotype may be ethnically higher in Asian populations, Zhu (Zhu, Mi et al. 2013) separated the Asian population study of Luo (Luo, Wang et al. 2011) from the five other studies, which they labelled as Caucasian studies (Janssen, Bakker et al. 2009, Ozbek, Tasci et al. 2009, Safarinejad 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013).

In addition, Zhu (Zhu, Mi et al. 2013) calculated the pooled Odds ratio (OR) of these 5 single studies, as a measure of the strength of association between 5-HTTLPR gene polymorphism and lifelong PE.

Based on the L and S allele frequencies in patients and controls, Zhu (Zhu, Mi et al. 2013) reported a low OR value for both the Asian study (OR=0.64; CI 0.43-0.96) (Luo, Wang et al. 2011) and the five Caucasian studies (Janssen, Bakker et al. 2009, Ozbek, Tasci et al. 2009, Safarinejad 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013) together (OR=0.83; CI 0.80-0.98), indicating an all together weak association of 5-HTTLPR and lifelong PE. As also a lower OR was found in LL versus SS genotype frequencies in all Caucasian patients versus controls (OR=0.88; CI 0.80-0.98), and a lower OR was also found in LL+LS versus SS genotype frequencies in Caucasian patients versus controls (OR=0.83; CI 0.70-1.00), Zhu (Zhu, Mi et al. 2013) interpreted these results as that SS genotype and/or S-allele are risk factors of lifelong PE, and therefore they concluded that LL genotype and/or L-allele might be protecting factors for lifelong PE.

In much contrast to the study of Zhu (Zhu, Mi et al. 2013), we have not only demonstrated but also emphasised that a very high SS genotype frequency only occurs in the 3 studies not-in-HWE (Janssen, Bakker et al. 2009, Zuccarello, Ghezzi et al. 2012, Jern, Eriksson et al. 2013) and that this deviation most probably is caused by misinterpretation of the gel electroforesis of the PCR analysis or a PCR reaction disturbance. Out of curiosity, we calculated the ORs of the patients and controls in the 3 separate studies in-HWE (Janssen, Jern and Zucarello) (Figure 3) and in the 3 separate studies not-in-HWE (Safarinejad, Luo and Ozbeck) (Figure 4). In addition, we calculated the pooled Odds ratios of the three studies in-HWE (Figure 3) and the three studies not-in-HWE (Figure 4) regarding allele frequency.

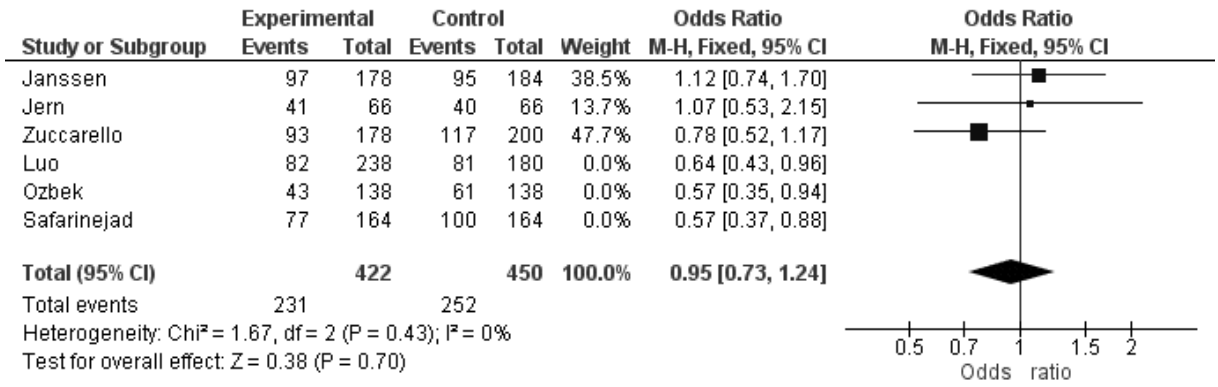

Figure 3: Odds Ratios of the Three Studies in-HWE

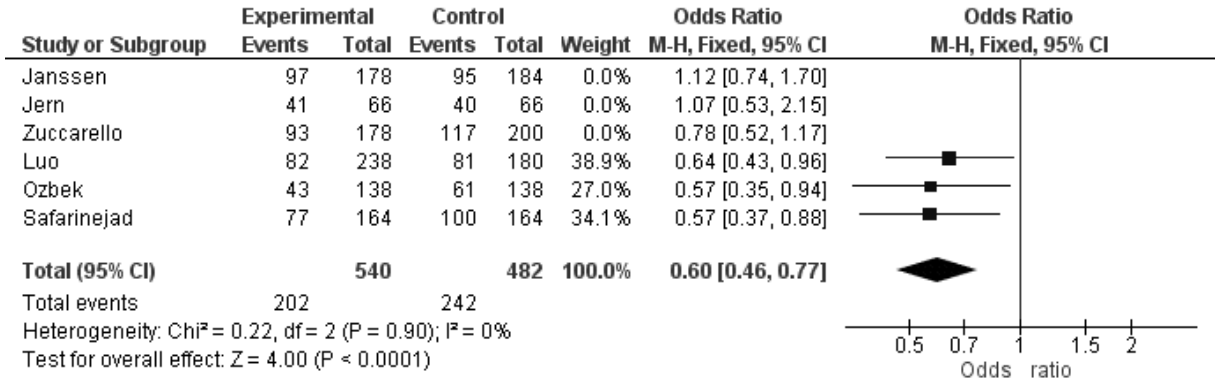

Figure 4:Odds Ratios of the Three Studies not-in-HWE

The ORs of the 3 studies in-HWE were for Janssen, Jern and Zucarello: OR 1.12 (CI 0.74-1.70), OR 1.07 (CI 0.53-2.15) and OR 0.78 (CI 0.52-1.17), respectively. The pooled OR of these 3 studies in-HWE was 0.95 (CI 0.73-1.14) (Figure 3). The ORs of the 3 studies not-in-HWE were for Luo, Safarinejad and Ozbeck: OR 0.64 (CI 0.43-0.96), OR 0.57 (CI 0.37-0.88) and OR 0.57 (CI 0.35-0.94), respectively. The pooled OR of these 3 studies not-in-HWE was 0.60 (CI 0.46-0.77) (Figure 4). In other words, according to the separate ORs of the 3 studies in-HWE, and according to the pooled OR of these 3 studies together, there is no association at all between 5-HTTLPR polymorphism and lifelong PE. In contrast, as the pooled OR of the 3 studies not-in-HWE was 0.60 (CI 0.46-0.77) and the separate ORs of these 3 studies were very low, it may erroneously be concluded that there is a strong association between 5-HTTLPR polymorphism and lifelong PE.

Unfortunately, in their meta-analysis, Zhu (Zhu, Mi et al. 2013) did not report the separate ORs of all 6 studies regarding the allele frequencies. Instead, as Zhu (Zhu, Mi et al. 2013) have pooled the ORs of 5 (Caucasian) studies, including the two studies not-in-HWE (Safarinejad and Ozbek), they erroneously calculated a low OR for all these 5 studies together.

Re-analysis of the Meta-analysis of Zhu et al.

Apart from our aforementioned critical analysis of the 6 articles, we re-analysed the data as reported by Zhu et al. (Zhu, Mi et al. 2013) for their OR calculations. As we were unable to replicate their outcome data, we used three statistical programs to calculate the ORs: Excel from Microsoft, Review Manager from Cochrane (version 5.2) and IBM SPSS version 19. By using the Review Manager we found a mistake made by Zhu (Zhu, Mi et al. 2013) in the use of their statistical program. After having recognized their probable mistake, we were able to exactly reproduce their tables and figures.

We found that Zhu (Zhu, Mi et al. 2013) did calculate the OR for the study of Luo (Luo, Wang et al. 2011), but instead of the OR they calculated the risk ratio (RR) for the 5 other studies, as represented in their table 2 of the allele frequencies , in spite of the fact that they claimed to have calculated the OR of these 5 studies. Moreover, instead of the OR they calculated the RR for all the 6 studies with regard to LL vs SS genotype (their figure 2) and with regard to LL+LS vs SS genotype (their figure 3). Apart from that miscalculation, we found that the legend of their figure 2 ought to refer to their figure 3, whereas the legend of their figure 3 ought to refer to their figure 2.

In Figure 5 we present all the data that belong to table 2 of the study of Zhu (Zhu, Mi et al. 2013) showing how they erroneously calculated the RR instead of the OR of the 5 Caucasian studies. In Figure 6 we present the separate ORs and pooled OR of all the six studies, as we have calculated using the Review Manager.

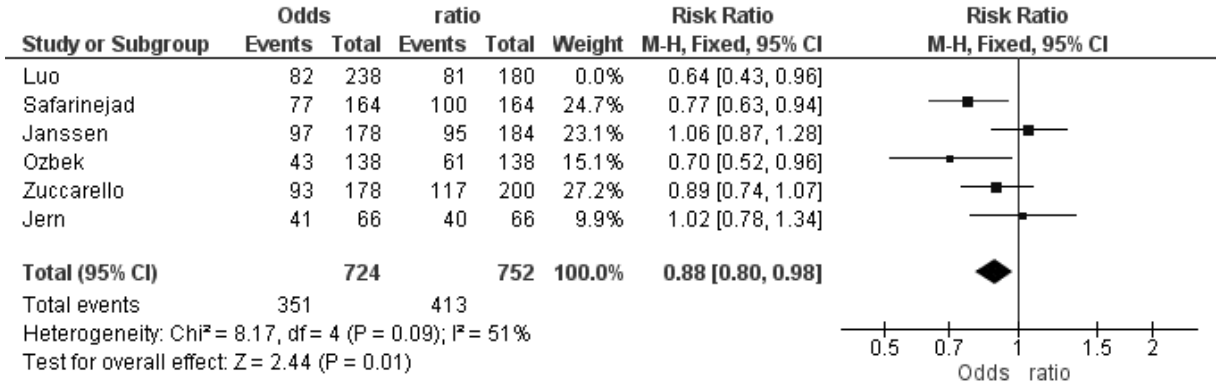

Figure 5: Risk Ratio of the 5 Studies and their pooled OR of Allelic Contrast, inadequately represented as Odds Ratio in Table 2 in the Meta-analysis of Zhu (Zhu, Mi et al. 2013)

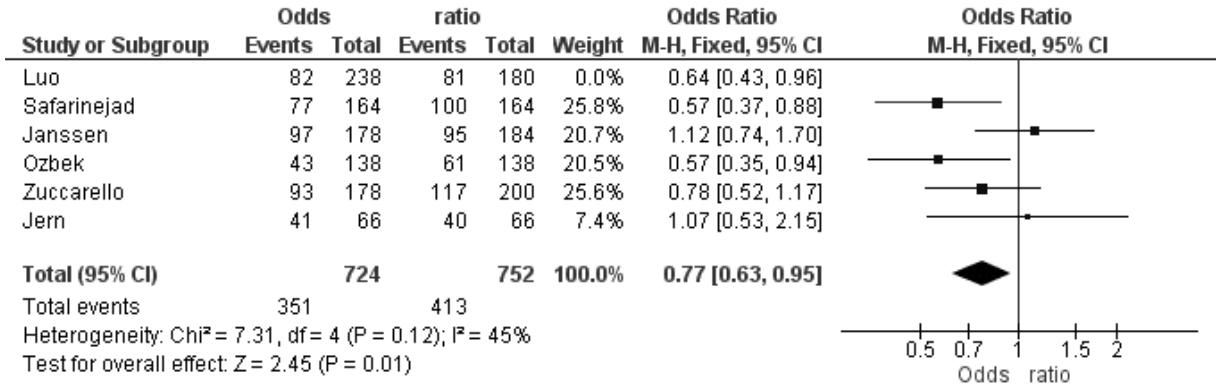

Figure 6: Separate ORs and pooled OR of the Six Studies regarding Allelic Contrast, as Calculated by the Current Authors using the Review Manager

Figure 5 shows figure 3 of Zhu (Zhu, Mi et al. 2013), that actually represents the lifelong PE risk associated with the 5-HTTLPR gene polymorphism (LL vs SS) instead of the (LL+LS vs SS) as is erroneously represented in their article. Figure 5 shows the RR as calculated by Zhu (Zhu, Mi et al. 2013), whereas figure 6 shows the ORs of all the six studies with regard to the lifelong PE risk associated with the 5-HTTLPR gene polymorphism (LL vs SS). Notably, figure 2 of the study of Zhu (Zhu, Mi et al. 2013) contains the same miscalculations as their figure 3 (not represented here).

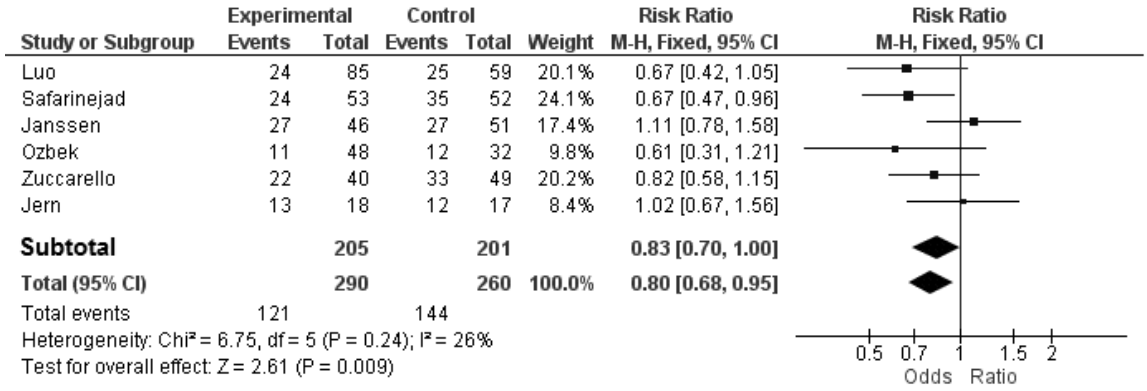

Figure 7: Risk Ratio LL vs SS, as misrepresented as Odds Ratio in figure 3 of the meta-analysis of Zhu (Zhu, Mi et al. 2013)

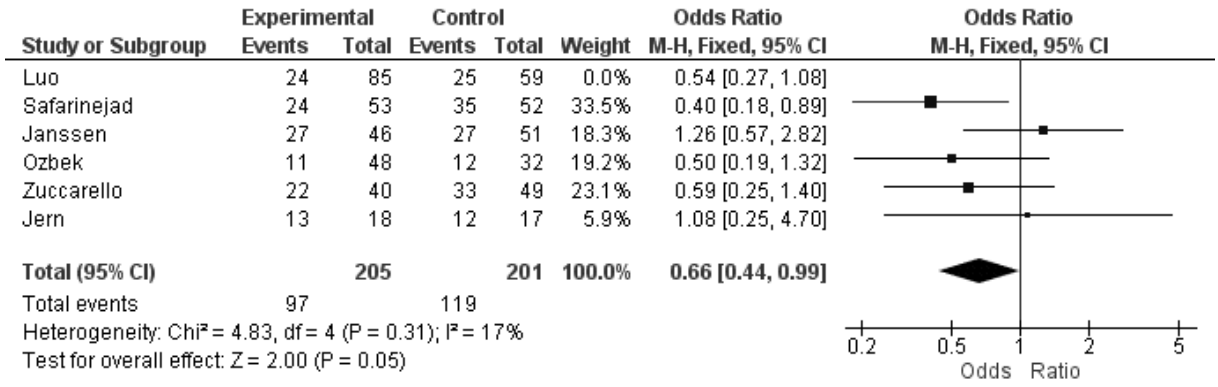

Figure 8: Odds Ratio LL vs SS, as calculated by us using the Review Manager

According to our OR calculations, the OR values of the 3 studies not-in-HWE are even lower than the RR values presented as ORs by Zhu (Zhu, Mi et al. 2013). Nevertheless, our finding of 3 seriously disturbed PCR tests which are at the basis of a deviated HWE and a low OR, show that these 3 studies are completely inadequate to be used in a meta-analysis that includes 3 other studies that have a normal PCR, are in-HWE and have a normal OR.

Conclusion

In our analysis of 6 studies that were previously used by Zhu (Zhu, Mi et al. 2013) for a meta-analysis of 5-HTTLPR polymorphism and lifelong PE, it was found that 3 of these studies were not-in-HWE. In these 3 studies, SL genotype frequency was very low whereas the SS genotype frequency was very high compared with the 3 other studies that were in-HWE. As we assume that this very low SL/very high SS genotype combination is caused by an inadequate visual interpretation of the PCR test or a disturbed PCR test, we investigated the PCRs of the 6 studies. It was found that 5 of the 6 studies did not provide all the required information of the PCR procedure. Moreover , there were important differences in the PCR reaction mixture, the PCR program and the gel-electrophoresis, particularly in the studies that were not in HWE. Therefore, we suggest that in the 3 studies that were not-in-HWE, the PCR test had a preference for the short allele to become visible for the laboratory investigator. Consequently, part of the heterozygotes (SL) have erratically been interpreted as homozygote mutant (SS), leading to a false high percentage of SS genotypes. Indeed, in the 3 studies not-in-HWE there is a very high frequency of SS and a very low frequency of SL genotype, compared to the studies who are in-HWE , wheras the percentage of the homozygote LL genotype does not appear to be affected.

Our finding of very high SS and very low SL genotype distribution in the 3 studies not-in-HWE in relation to disturbances of their PCR test and/or misinterpretation of their gel electrophoresis, supports our view that understanding of the PCR procedure is pivotal for clinicians in general, and obviously for those who are involved in genetic research of 5-HTTLPR polymorphism and ejaculation. Moreover, as the outcome of a genetic research study in men with lifelong PE is heavily dependent on an adequate PCR procedure, we argue that an inadequate PCR test may behave as a confounding factor in genetic studies, particularly when the details of the PCR test are unknown to the clinician.

Notably, as the PCRs of 3 studies not-in-HWE produced false SL and SS genotype frequencies, their inclusion together with the 3 studies in-HWE for a meta-analysis is inadequate. Our calculation of the ORs regarding allele frequencies (S and L) of patients and controls, yielded normal ORs in the 3 studies in-HWE and a low OR in the 3 studies not-in-HWE. In other words, the normal ORs of the three studies in-HWE demonstrate that there is not any association between 5-HTTLPR polymorphism and lifelong PE.

In conclusion, our analysis demonstrate that 3 of the 6 studies who are not-in- HWE have a disturbed PCR test and a low OR and therefore are inadequate to be compared in a meta-analysis with 3 other studies who are in-HWE, have a normal PCR test and a normal OR.

From our analysis we also conclude that a PCR test may form a confounding factor to clinicians who do not understand the details of a PCR test, and that there is not any indication that 5-HTTLPR is associated with lifelong PE. In other words, according to our conclusion there is not any indication that L alleles might protect against lifelong PE as Zhu et al have erroneously concluced in their meta-analysis.

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Chapter 8:
Discussion an conclusions

Lifelong Premature Ejaculation, Serotonin and Genetics

Lifelong premature ejaculation (PE) is a male sexual disorder characterized by a consistent very short ejaculation time of less than about 1 minute, in more than 90% of intercourses, with nearly every female partner, since the first sexual activities of a man in puberty or adolescence (Waldinger 2007). In about 30% of these men, ejaculation becomes even more faster at the age of 30-35 years (Waldinger 2007). In the 1990s, it has been hypothesised that the short ejaculation time, e.g., the intravaginal ejaculation latency time (IELT) which is the time between intravaginal penetration and the moment of intravaginal ejaculation, is associated with a diminished central serotonin (5-hydroxytryptamine; 5-HT) neurotransmission, a hyperfunction of 5-HT_{1A} receptors and a hypofunction of 5-HT_{2C} receptors (Waldinger, Berendsen et al. 1998).

The aim of this thesis was to study the role of serotonergic gene polymorphisms in men with lifelong premature ejaculation (PE) with regard to the duration of their IELT. In order to do this, we investigated the duration of their IELT by using a stopwatch that had to be handled by the female partner of the male. After an explanation was given on how to use the stopwatch during intercourse, the IELT was measured at home in a one month baseline period and during a period of daily paroxetine treatment. In addition, we investigated whether the duration of daily paroxetine treatment-induced ejaculation delay was associated with polymorphism of the 5-HT transporter gene (**Chapter 2**), polymorphisms of the 5-HT_{1A} receptor gene (**Chapter 3**) and the 5-HT_{2C} receptor gene (**Chapter 4**). In **Chapter 5** it was investigated whether paroxetine-induced ejaculation delay is associated with polymorphism of the 5-HT transporter gene. Furthermore, it was investigated whether in these men response and non-response to paroxetine treatment was associated with paroxetine serum concentration, CYP2D6 genotype, and a number of other factors, such as an intact hypothalamic-pituitary gonadal axis, thyroid function and serum leptin levels (**Chapter 6**). In **Chapter 7** we analyzed six studies that were published in recent years on 5-HTTLPR polymorphism and premature ejaculation.

Lifelong Premature Ejaculation and Genetic Polymorphisms of the Central 5-HT System

As described in **Chapter 1**, the history of lifelong premature ejaculation is characterized by a development of mere psychological into neurobiological and genetic thinking.

Although in 1943 familial occurrence of lifelong PE has been noted by Bernhard Schapiro (Schapiro 1943), attention to the influence of genetic factors on premature ejaculation has been ignored for more than 50 years. However, in 1998, Waldinger (Waldinger, Berendsen et al. 1998) suggested that the short IELT of less than about 1 minute might be associated with genetic factors and central serotonergic mechanisms, e.g., a diminished central 5-HT neurotransmission, a hyperfunction of 5-HT_{1A} receptors and hypofunction of 5-HT_{2C} receptors (Waldinger, Berendsen et al. 1998). Moreover, in a cohort of 110 Dutch men with lifelong PE, it was found that the majority of men was not willing to discuss the occurrence of PE with their family members due to embarrassment related to the taboo on PE and the unwillingness to talk about private sexual issues with parents and other family members (Waldinger, Rietschel et al. 1998). Despite the unwillingness to talk about PE, this cohort study showed some indications that there is a tendency of familial occurrence of lifelong PE in first degree male relatives, confirming the observation of Schapiro in 1943 of familial occurrence of lifelong PE. The observation of Schapiro and the hypothesis of Waldinger have been the basis for the genetic studies, as described in this thesis.

However, for a better understanding of the studies mentioned in this thesis, it is important to bear in mind that prior to our studies nothing was known or published about DNA research in lifelong PE. Therefore, and with no financial support, we started our genetic research from scratch. It has only become possible to conduct these studies as a result of the willingness of the heads of the four participating laboratoria to give permission to me to perform the laboratory genetic work myself without additional costs.

On the basis of data derived from sexual animal research (Ahlenius, Larsson et al. 1981, Berendsen and Broekkamp 1987, Foreman, Love et al. 1988) we hypothesized that the odds to find an association between 5-HT and the IELT duration was higher when investigating polymorphism of the 5-HT-transporter (5-HTT) gene. Therefore, we started our first study (**Chapter 2**) investigating 89 men with lifelong PE. The majority of them ejaculated within 1 minute, whereas 6 men ejaculated between 1 and 2 minutes. In this group of men, those with LL genotype ejaculated within 13.2 seconds, expressed in geometric mean IELT, whereas men with SL and SS genotype ejaculated within 25.3 and 26.0 seconds, respectively ($p<0.05$). In other words, men with LL genotype ejaculated 100% faster than men with SS genotype. Importantly, the genotype distribution of these men with lifelong PE did not differ from the genotype distribution of the general male population in the Netherlands.

In the second study (**Chapter 3**) in the same cohort of men with lifelong PE, we investigated 54 men with respect to the role of the C(1019)G polymorphism of the 5-HT_{1A} receptor gene on the duration of the IELT. It was shown that men with CC genotype ejaculated within 14.5 seconds, whereas men with CG and GG genotype ejaculated within 27.7 seconds , and 36.0

sec, respectively. Therefore, it was concluded that men with CC genotype ejaculated 250% earlier than men with GG genotype.

Similarly, in the third study (**Chapter 4**) in 64 men with lifelong PE, we investigated the role of the Cys23Ser polymorphism of the 5-HT_{2C} receptor on the duration of the IELT. As this polymorphism is only present at the X chromosome, only homozygote wildtypes and mutants were measured in these men. It was shown that the wildtypes (CysCys) had an IELT of 22.6 seconds, whereas the mutants (Ser/Ser) had an IELT of 40.4 seconds. Thus, the men with CysCys genotype ejaculated 79% faster than the monozygote mutant (SerSer) men.

The fourth study of this thesis (**Chapter 5**) describes the investigation of whether there is an association between paroxetine-induced ejaculation delay and polymorphism of the 5-HT transporter gene. In this study in 54 men with lifelong PE, it was found that there is no association between paroxetine-induced ejaculation delay, expressed in the fold-increase of the geometric mean IELT compared to baseline values in men with lifelong PE, and 5-HTTLPR polymorphism. Interestingly, about 80% of men measured and reported a clinically relevant ejaculation delay induced by daily paroxetine treatment, whereas in 20% of men, paroxetine treatment did not result in a clinically relevant ejaculation delay.

In the fifth study (**Chapter 6**) a sofar in the literature not reported phenomenon was investigated, e.g., complete ejaculation delay nonresponse to paroxetine and another serotonergic antidepressant treatment. For the purpose of this study, five previously with paroxetine unsuccessfully treated men with lifelong PE were recruited and compared with eight newly recruited men. The study showed that one of the eight newly recruited men did not respond to paroxetine treatment. Therefore, this male patient was classified into the non responder group. It was found that in the responder group (n=7), paroxetine treatment resulted in a statistically significant increased serum prolactine level compared to the serum prolactine levels of the non-responders (n=6) after paroxetine treatment (p=0.04). However, the serum prolactine level in the responder group after paroxetine treatment (X ±SD: 147,0 mU/L ± 30,8) were not significantly different compared to baseline prolactine levels (X±SD: 109,4 mU/L ± 46,2) (p=0,09). In this respect, it is important to note that in the non-responder group a tendency was noted of prolactine levels to become reduced after paroxetine treatment. Intriguingly, it was also found that with regard to 5-HT_{1A} receptor gene polymorphism, all non-responder were heterozygote (CG) whereas all responder appeared to be homozygote wildtype(CC). However, the number of participating men in this study has been extremely low. Therefore no conclusions can be drawn from this finding. Notably, we are currently investigating whether paroxetine-induced ejaculation delay is associated with 5-HT_{1A} receptor gene polymorphism in men with lifelong PE. Importantly, there are indications that expression of the 5-HT_{1A} receptor gene is also related to C(1019)G polymorphism of this 5-HT receptor gene (Albert, Le Francois et al. 2011).

There are indications that in homozygote wildtypes (CC genotype) more 5-HT_{1A} receptors become expressed, whereas in case of a mutation (CG en GG) less 5-HT_{1A} receptors become to expression (Le Francois, Czesak et al. 2008, Albert, Le Francois et al. 2011).
In the final and sixth study of this thesis (**Chapter 7**) we investigated six studies on 5-HTTLPR polymorphism and PE, which have been used by Zhu (Zhu, Mi et al. 2013) in a meta-analysis of these six studies on the influence of this polymorphism on lifelong PE. We have shown that three of the six studies were not in Hardy-Weinberg equilibrium (HWE), and that the polymerase chain reaction (PCR) reaction in the latter 3 studies was aberant compared to the PCR reaction in the studies who were in HWE. We provided arguments to show that the deviation from HWE was caused by a lower SL genotype frequency and a higher SS genotype frequency. This deviation was present in all the three studies that were not in HWE, whereas the deviation did not occur in the other three studies which were in HWE. It is most likely that the uniform shift from HWE was caused by a disturbance of PCR test favouring the visibility of the short arm, erroneously leading to a misreading of SL genotypes. Based on this phenomenon we postulated that insufficient knowledge of proper PCR analysis among clinicians may be a confounding factor in the interpretation of PCR analysis outcome data. As the three studies in HWE did not show an association between lifelong PE and 5-HTTLPR polymorphism, we concluded that, in contrast to the conclusion of Zhu et al (Zhu, Mi et al. 2013), there are no indications that 5-HTLPR polymorphism is associated with lifelong PE.

Future perspectives and limitations

In the current thesis three separate studies of single 5-HT gene polymorphisms have been conducted in a single cohort of Dutch men with lifelong PE. It was found that three 5-HT gene polymorphisms are associated with the duration of the IELT. Despite the association of 5-HTTLPR polymorphism and IELT duration (**Chapter 2**), we have not found any difference in genotype distribution of this polymorphism with a Dutch control group, that was analyzed by another research group with regard to 5-HTT gene polymorphism. In the studies investigating 5-HT_{1A} en 5-HT_{2C} gene polymorphism (**Chapter 3 and 4**), a control group was not part of the study as these polymorphism genotype data were not available to us at the time. However, there are no indications that the 5-HT_{1A} en 5-HT_{2C} gene polymorphism genotype distributions from our study differ from the general Dutch male population, as our genotype distribitions were in Hardy Weinberg equilibrium. Nevertheless, as we have only investigated the genotype distributions of Dutch males, it would have been better if we would have compared our genotype distributions with the genotype distribution of Dutch males controls. However, these data have not been available to us.

In our studies we have focused on the investigation of gene polymorphisms of men who ejaculated within 1 minute, as measured with a stopwatch. In the control group of the 5-HTT gene polymorphism study, the genotype distribution has been available, but the IELT data of the control group have not been measured. Nevertheless, for future reseach it is of relevance that also in the control group, IELT data should be measured.

It is important to note that within the group of males who ejaculate within a minute, a distinction can be made in rapid (20-40 sec) to very rapid (1-20 sec) males, which distinction is associated with their genotype distribution. Therefore, it is intriguing, that within the general male population approximately 2-3% ejaculates within 1 minute, whereas, as far is known on the basis of our first study, their 5-HTTLPR genotype distribution does not differ from the genotype distribution of males in the general population.

With regard to the association that we have found between the C(-1019) G polymorphism of the 5-HT_{1A} receptor gene and the IELT **(Chapter 3**), we would like to emphasise that this finding occurred in a small sample of participants. Moreover, it remains unclear whether this association involves presynaptic 5-HT_{1A} receptor and/or post-synaptic 5-HT_{1A} receptor activity.

The negative finding of our study regarding a potential association between the plasma paroxetine level and the fold-increase of the IELT (**Chapter 6**), does not exclude the possibility of a potential positive association between the fold-increase of the IELT and one of the four metabolites (e.g., glucuronides) of paroxetine (Bourin, Chue et al. 2001).

Despite the fact that our studies in men with lifelong PE did not provide any indication that the genotype distributions of the investigated gene polymorphisms differ from the genotype distribution of the general male population, we did find a statistically significant difference within the IELT values were compared with the IELT data. This result has only become manifest by the exact method, e.g., the use of a stopwatch, that we used to measure the duration of the IELT. Therefore, for genetic research of the IELT the stopwatch method is of utmost importance.

In the near future we would like to investigate a large group of men with lifelong PE in order to investigate whether combinations of these genetic variations are associated with the IELT duration. From a retrospective view, it appears that the current group of men is too small to stratify on more than one genetic variation. In addition, for a better understanding , a very large group of men with lifelong PE and a control group are required to investigate the various combinations of the genetic variations. The more genetic variations associated with the IELT will be found, the larger both groups have to be to find an association. Although this is argued from a retrospective view, it should be noted that even when we would have argued this prior to our studies, the lack of financial support would have hampered us to perform such research.

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Samenvatting in het Nederlands

Lifelong Premature Ejaculatie, Serotonine en Genetica

Lifelong premature ejaculatie (levenslange vroegtijdige zaadlozing; lifelong PE) is een mannelijke seksuele stoornis die gekenmerkt wordt door een ejaculatie tijd van minder dan 1 minuut die optreedt in meer dan 90% van de gevallen van seksuele gemeenschap, met vrijwel elke vrouwelijke partner, vanaf de eerste seksuele activiteiten van de man in zijn puberteit of adolescentie (Waldinger 2007). Bij ongeveer 30% van deze mannen, wordt de duur van de ejaculatie nog korter rondom de leeftijd van 30-35 jaar (Waldinger 2007). In 1998 werd gepostuleerd dat de korte ejaculatie tijd, i.e., de intravaginale ejaculatie latentie tijd (IELT), hetgeen de tijd is tussen intravaginale penetratie en het moment van intravaginale ejaculatie, geassocieerd is met een verminderde centrale serotonine (5-hydroxytryptamine; 5-HT) neurotransmissie, een hyperfunctie van 5-HT_{1A} receptors en een hypofunctie van 5-HT_{2C} receptors (Waldinger, Berendsen et al. 1998).

De studies in dit proefschrift richten zich op de vraag of polymorfismen van genen die betrokken zijn bij het centraal serotonerge systeem bij mannen met lifelong premature ejaculatie invloed hebben op de duur van hun IELT. Teneinde dit objectief te kunnen bestuderen, hebben wij studies uitgevoerd waarbij de duur van de IELT met behulp van een stopwatch werd gemeten. De stopwatch moest gehanteerd worden door de vrouwelijke partner. Na uitleg te hebben gegeven over hoe de stopwatch gebruikt moet worden bij seksuele gemeenschap, werd de IELT thuis gemeten bij elke coitus gedurende 1 maand (baseline periode) waarin de man geen medicatie gebruikte, en gedurende een periode waarin de man dagelijks paroxetine in verschillende doseringen innam. Wij onderzochten tevens of de door paroxetine veroorzaakte vertraagde duur van de IELT geassocieerd was met het polymorfisme van het 5-HT transporter gen (**Hoofdstuk 2**), het polymorfisme van het 5-HT_{1A} receptor gen (**Hoofdstuk 3**) en het polymorfisme van het 5-HT_{2C} receptor gen (**Hoofdstuk 4**). In **Hoofdstuk 5** werd onderzocht of de door paroxetine veroorzaakte ejaculatie vertraging geassocieerd is met het polymorfisme van het 5-HT transporter gen. Verder werden bij deze mannen twee groepen onderscheiden: enerzijds mannen die bij paroxetine behandeling een vertraging van de zaadlozing kregen (paroxetine respons) en anderzijds mannen die bij paroxetine behandeling geen vertraging van de zaadlozing kregen (paroxetine non-respons). Wij onderzochten of de paroxetine respons en de paroxetine non-respons geassocieerd was met de paroxetine serum concentratie, het CYP2D6 genotype, en een aantal andere factoren, zoals een intacte hypothalamus-hypofyse gonadale as, schildklier functie en serum leptine concentraties (**Hoofdstuk 6**).

In **Hoofdstuk 7** analyseerden wij zes studies over de relatie tussen premature ejaculatie en het 5-HTTLPR polymorfisme, die door Zhu et al. (Zhu, Mi et al. 2013) gebruikt waren in een door hen uitgevoerde meta-analyse.

Lifelong Premature Ejaculatie en Genetisch Polymorfisme van het Centrale 5-HT Systeem

In **Hoofdstuk 1** wordt de historische ontwikkeling van lifelong premature ejaculatie over de afgelopen eeuw beschreven. Aanvankelijk werd lifelong premature ejaculatie alleen verklaard vanuit psychologische factoren. Pas in de afgelopen twee decennia wordt steeds meer gedacht dat lifelong premature ejaculatie een neurobiologisch substraat heeft waarbij genetische factoren een rol spelen. Hoewel Bernhard Schapiro (Schapiro 1943) in 1943 al schreef dat het hem was opgevallen dat lifelong premature ejaculatie ook bij familieleden van zijn patienten voorkwam, is er meer dan 50 jaar geen enkele aandacht besteed aan zijn opmerkelijke uitspraak. Pas in 1998 postuleerde Waldinger (Waldinger, Berendsen et al. 1998) dat IELTs van minder dan een minuut waarschijnlijk geassocierd zijn met genetische factoren en centraal serotonerge mechanismen, zoals een verminderde centrale 5-HT neurotransmissie, een hyperfunctie van 5-HT_{1A} receptoren en een hypofunctie van 5-HT_{2C} receptoren (Waldinger, Berendsen et al. 1998). Tevens vonden Waldinger et al (1998) dat in een cohort van 110 Nederlandse mannen met lifelong premature ejaculatie de meerderheid van deze mannen niet bereid was familieleden te vragen of zij ook last hadden van premature ejaculatie. Dit berustte op schaamte te moeten erkennen aan vroegtijdige zaadlozingen te lijden en het taboe dat hierover (nog steeds) bestaat, hetgeen des te sterker was bij de gedachte dit te moeten bespreken met ouders en andere familieleden (Waldinger, Rietschel et al. 1998). De studie gaf desondanks toch enige aanwijzingen voor het bestaan van een familiair voorkomen van lifelong premature ejaculatie in eerste graads manlijke familieleden, hetgeen overeenkwam met de klinische observatie van Schapiro in 1943 over een familiair optreden van lifelong premature ejaculatie. De observatie van Schapiro en de hypothese van Waldinger et al. hebben de basis gevormd voor de genetische studies die in dit proefschrift worden beschreven.

Voordat wij waren begonnen met de eerste studie van dit proefschrift, was er helemaal niets bekend of gepubliceerd over DNA onderzoek bij lifelong premature ejaculatie. Derhalve moesten wij eerst zelf uitzoeken hoe dit type onderzoek opgezet kon worden. De studies die wij gedaan hebben, konden wij alleen maar uitvoeren omdat ik toestemming had gekregen van de hoofd leidinggevenden van de vier deelnemende laboratoria om zelf het genetisch onderzoek te doen in hun laboratoria zonder dat daarvoor bijkomende kosten werden berekend.

Op basis van dierexperimentele bevindingen van andere onderzoeks groepen (Ahlenius, Larsson et al. 1981, Berendsen and Broekkamp 1987, Foreman, Love et al. 1988) hebben wij gemeend dat de kans een associatie te vinden tussen 5-HT en de duur van de IELT het grootst was bij onderzoek van het polymorfisme van het 5-HT-transporter (5-HTT) gen. Hierop werd dan ook de onderzoeksvraag van onze eerste studie geformuleerd (**Hoofdstuk 2**) waarbij wij 89 mannen met lifelong premature ejaculatie hebben onderzocht. Het merendeel van deze mannen ejaculeerde binnen 1 minuut: slechts 6 mannen ejaculeerden tussen de 1 en 2 minuten. De studie toonde aan dat in dit cohort, mannen met een LL genotype binnen 13.2 seconden ejaculeerden (uitgedrukt in de geometrisch gemiddelde IELT), terwijl mannen met SL en SS genotype, binnen 25.3 en respectievelijk 26.0 seconden ejaculeerden ($p<0.05$). Anders gezegd, mannen met het LL genotype ejaculeerden 100% sneller dan mannen met het SL en SS genotype. Het is van belang hierbij op te merken dat deze genotype verdeling niet verschilde van de genotype verdeling van mannen in de algemene bevolking in Nederland.
In de tweede studie (**Hoofdstuk 3**) uitgevoerd in hetzelfde cohort mannen met lifelong premature ejaculatie, onderzochten wij 54 mannen met de vraag of het C(1019)G polymorfisme van het 5-HT_{1A} receptor gen een rol speelde bij de duur van de IELT. De studie toonde aan dat mannen met het CC genotype binnen 14.5 seconds ejaculeerden, terwijl mannen met het CG en GG genotype binnen respectievelijk 27.7 seconden en 36.0 sec ejaculeerden. Derhalve was de conclusie van deze studie dat mannen met het CC genotype 250% sneller ejaculeerden dan mannen met het GG genotype.
In de derde studie (**Hoofdstuk 4**) uitgevoerd bij 64 mannen met lifelong premature ejaculatie, onderzochten wij de rol van het Cys23Ser polymorfisme van de 5-HT_{2C} receptor ten aanzien van de duur van de IELT. Aangezien dit polymorfisme alleen aanwezig is op het X chromosoom, werden alleen homozygote wildtypes en mutanten bij deze mannen gemeten. Aangetoond werd dat de wildtypes (CysCys) een IELT van 22.6 seconden hadden, terwijl de mutanten (Ser/Ser) een IELT van 40.4 seconden hadden. Met andere woorden, de mannen met het CysCys genotype ejaculeerden 79% sneller dan de monozygote mutant (SerSer) mannen.
De vierde studie van dit proefschrift (**Hoofdstuk 5**) beschrijft een studie waarbij de onderzoeks vraag was of er een associatie is tussen de door paroxetine behandeling veroorzaakte ejaculatie vertraging en het polymorfisme van het 5-HT transporter gen. In deze studie van 54 mannen met lifelong premature ejaculatie, werd gevonden dat er geen associatie is tussen de door paroxetine veroorzaakte ejaculatie vertraging, uitgedrukt in de fold-increase van de geometrisch gemiddelde IELT vergeleken met de uitgangswaarden bij mannen met lifelong premature ejaculatie, en 5-HTTLPR polymorfisme.

Interessant bij deze studie was verder dat ongeveer 80% van de mannen een door dagelijkse paroxetine behandeling veroorzaakte klinisch relevante ejaculatie vertraging hadden gemeten, terwijl anderzijds bij 20% van de mannen, paroxetine behandeling niet resulteerde in een klinisch belangrijke ejaculatie vertraging. Met andere woorden bij 20% van de mannen heeft dagelijkse paroxetine behandeling vrijwel geen tot geen ejaculatie vertragend effect.
In de vijfde studie (**Hoofdstuk 6**) is een vooralsnog niet in de literatuur genoemd fenomeen onderzocht, i.e., complete ejaculatie-vertraging non-response voor dagelijkse paroxetine behandeling en voor een behandeling met een ander serotonerg antidepressivum. Voor het doel van deze studie werden vijf, voorheen dagelijks met paroxetine behandelde mannen met lifelong premature ejaculatie, gevraagd deel te nemen. Deze mannen hadden bij die eerdere behandeling tot hun teleurstelling geen vertraagde zaadlozing opgemerkt. In de huidige studie werden zij vergeleken met acht mannen met lifelong premature ejaculatie die voor vroegtijdige zaadlozing behandeld wilden worden. De studie toonde aan dat 1 van de acht nieuw gerecruiteerde mannen geen ejaculatie vertragend effect van paroxetine had gekregen. Derhalve werd deze patient achteraf alsnog geclassificeerd in de non responder groep. De studie toonde aan dat in de responder groep (n=7), dagelijkse behandeling met 20 mg paroxetine een statistisch significante toename gaf van het serum prolactine gehalte ten opzichte van het serum prolactine gehalte van de non-responders (n=6) na paroxetine behandeling (p=0.04). Het serum prolactine gehalte van de responder groep na paroxetine behandeling (X ±SD: 147,0 mU/L ± 30,8) was echter niet significant verschillend ten opzichte van het uitgangswaarde gemeten prolactine gehalte (X±SD: 109,4 mU/L ± 46,2) (p=0,09). In de non-responder groep bestond een tendens dat het prolactine gehalte verlaagd werd na paroxetine behandeling. Deze studie gaf een opmerkelijke uitslag van het 5-HT_{1A} receptor gen polymorfisme. Alle non-responders ware namelijk heterozygoot (CG) terwijl alle responders homozygoot wildtype (CC) bleken te zijn. Maar omdat het aantal mannen in deze studie echter buitengewoon klein was, kan uit deze bevinding geen enkele conclusie worden getrokken. Niettemin, voeren wij thans een studie uit waarbij onderzocht wordt of de door paroxetine veroorzaakte ejaculatie vertraging geassocieerd is met het 5-HT_{1A} receptor gen polymorfisme bij mannen met lifelong premature ejaculatie. Er zijn overigens aanwijzingen uit de literatuur dat expressie van het 5-HT_{1A} receptor gen ook gerelateerd is aan het C(1019)G polymorfisme van dit 5-HT receptor gen (Zhu, Mi et al. 2013). Er zijn aanwijzingen dat in homozygote wildtypes (CC genotype) meer 5-HT_{1A} receptoren tot expressie komen, terwijl in het geval van een mutatie (CG en GG) minder 5-HT_{1A} receptoren tot expressie komen (Albert, Le Francois et al. 2011).
In de zesde studie van dit proefschrift (**Hoofdstuk 7**) hebben wij zes studies over 5-HTTLPR polymorfisme en premature ejaculatie onderzocht.

Deze zes studies zijn door Zhu et al (Zhu, Mi et al. 2013)gebruikt in een door hen uitgevoerde meta-analyse over de invloed van deze polymorfismen op lifelong premature ejaculatie. Onze studie toonde echter aan dat drie van deze zes studies niet in Hardy-Weinberg evenwicht (HWE) waren, en dat de polymerase chain reactie (PCR) testen in de studies die niet in HWE waren afwijkend bleken te zijn vergeleken met de PCR reactie testen in de studies die wel in HWE waren. Wij hebben hierbij met verschillende argumenten beredeneerd dat de afwijking van HWE werd veroorzaakt door een lager SL genotype frequentie en een hoger SS genotype frequentie. Deze afwijkende genotype frequenties waren aanwezig in alle drie studies die niet in HWE waren, terwijl deze afwijkingen niet optraden in de andere drie studies die wel in HWE waren. Het is daarom zeer waarschijnlijk dat de uniforme verschuiving van het HWE is veroorzaakt door een gestoorde PCR test waarbij de zichtbaarheid van de korte arm eenzijdig relatief versterkt werd, hetgeen dus bij vergissing leidde tot het verkeerd aflezen van de SL genotypes. Op basis van dit fenomeen, hebben wij gepostuleerd dat onvoldoende kennis over het op een goede manier uitvoeren van een PCR analyse bij klinici als een "confounding" factor kan werken bij de door hen te geven interpretatie van gegevens die uit een PCR analyse zijn gekomen. Omdat de drie studies in HWE geen enkele associatie lieten zien tussen lifelong premature ejaculatie en het 5-HTTLPR polymorfisme, hebben wij geconcludeerd dat er, in tegenstelling tot de conclusie van Zhu et al (Zhu, Mi et al. 2013), geen aanwijzingen zijn de het 5-HTLPR polymorfisme geassocieerd is met premature ejaculatie.

Toekomst en kritische kanttekeningen

In dit proefschrift zijn vijf afzonderlijke studies van een 5-HT gen polymorfisme uitgevoerd in een cohort Nederlandse mannnen met lifelong premature ejaculatie. Deze studies toonden aan dat op basis van de door ons gehanteerde methode er drie 5-HT gen polymorfismen geassocieerd zijn met de duur van de IELT bij deze mannen. Ondanks de gevonden associatie tussen het 5-HTTLPR polymorfisme en de duur van de IELT (**Hoofdstuk 2**), zijn er geen verschillen gevonden tussen de genotype verdeling van dit polymorfisme bij de Nederlandse mannen met lifelong premature ejaculatie en een Nederlandse controle groep, die door een andere onderzoeksgroep was onderzocht op dit 5-HTT gen polymorfisme. In de studies waarbij de 5-HT_{1A} en 5-HT_{2C} gen polymorfismen zijn onderzocht (**Hoofdstuk 3 en 4**), was een controle groep niet aanwezig omdat de gegevens van deze genotype polymorfismen destijds niet voor ons beschikbaar waren. Er zijn echter geen aanwijzingen dat de 5-HT_{1A} en 5-HT_{2C} gen polymorfisme genotype verdelingen uit onze studies afweken van die in de algemeen manlijke bevolking in Nederland, omdat onze genotype verdelingen in Hardy Weinberg evenwicht waren.

Niettemin, aangezien wij de genotype verdelingen van Nederlandse mannen hebben onderzocht, zou het beter zijn geweest indien wij de door ons gevonden genotype verdelingen vergeleken hadden met de genotype verdelingen van een Nederlandse controle groep mannen. In onze studies hebben wij ons beperkt tot het onderzoek van gen polymorfismen bij mannen die bij een coitus binnen 1 minuut tot een zaadlozing komen, zoals dit met een stopwatch is gemeten. In de controle groep van de 5-HTT gen polymorfisme studie (**Hoofdstuk 2**) was de genotype verdeling beschikbaar, maar de IELT gegevens van de controle groep mannen waren niet beschikbaar omdat deze niet waren gemeten. Niettemin, is het voor toekomstig onderzoek van belang dat de IELT ook gemeten wordt in een controle groep.

In onze studies hebben wij aangetoond dat binnen de groep mannen die binnen 1 minuut tot een zaadlozing komen, een onderscheid gemaakt kan worden in snelle (20-40 sec) en zeer snelle (1-20 sec) mannen. Onze studies toonden aan dat dit onderscheid in de IELT duur is geassocieerd met de genotype verdeling. Het is derhalve intrigerend dat voor zover bekend binnen de algemeen manlijke bevolking circa 2-3% van de mannen binnen 1 minuut tot een zaadlozing komt, terwijl, voorzover bekend op basis van onze eerste studie, hun 5-HTTLPR genotype verdeling niet verschilt van de genotype verdeling van de mannen in de algemeen manlijke bevolking in Nederland.

Wat betreft de associatie die we hebben gevonden tussen het C(-1019) G polymorfisme van het 5-HT_{1A} receptor gen en de IELT **(Hoofdstuk 3**), moet benadrukt worden dat wij dit gevonden hebben in een zeer kleine groep mannen. Bovendien blijft het onduidelijk of deze associatie de presynaptische 5-HT_{1A} receptor en/of de post-synaptische 5-HT_{1A} receptor activiteit betreft.

De negatieve bevinding van onze studie ten aanzien van de mogelijke associatie tussen de plasma paroxetine concentratie en de fold-increase van de IELT (**Hoofdstuk 6**) sluit de mogelijkheid niet uit van een potentieel positieve associatie tussen de fold-increase van de IELT en een van de vier metabolieten (i.e., glucuroniden) van paroxetine (Bourin, Chue et al. 2001).

Ondanks het feit dat onze studies in mannen met lifelong premature ejaculatie geen enkele aanwijzing gaven dat de genotype verdelingen van de onderzochte gen polymorfismen verschilden van de genotype verdelingen in de algemeen manlijke populatie, vonden we wel een statistisch significant verschil wanneer de IELT waarden binnen deze groep onderling werden vergeleken. Deze bevinding is alleen mogelijk geweest door de exacte methode die wij gehanteerd hebben, i.e., het gebruik van een stopwatch, om de duur van de IELT te meten. Derhalve is voor (toekomstig) genetisch onderzoek van de IELT de stopwatch methode van eminent belang.

In de nabije toekomst, willen wij een grote groep mannen met lifelong premature ejaculatie onderzoeken ten einde de vraag te beantwoorden of combinaties van de door ons gevonden genetische variaties geassocieerd zijn met de duur van de IELT. Achteraf beschouwd, blijkt dat de huidige groep mannen te klein is om meer dan 1 genetische variatie te stratificeren. Om de genetica van de IELT beter te begrijpen, zijn een zeer grote groep mannen met lifelong premature ejaculatie en een zeer grote controle groep noodzakelijk om deze verschillende combinaties van genetische variaties te onderzoeken. Hoe meer genetische variaties ten aanzien van de IELT worden gevonden, des te groter beide groepen moeten zijn om een associatie te vinden. Hoewel dit achteraf is beredeneerd, is het belangrijk in het oog te houden dat zelfs indien wij dit van te voren hadden beredeneerd, het gebrek aan financiele ondersteuning een dergelijk onderzoek onmogelijk had gemaakt.

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Curricilum Vitae

Paddy Koen Camiel Janssen werd geboren 6 juli 1973 in Roermond. In 1994 start hij met de studie farmacie aan de Universiteit Utrecht. Tijdens zijn bijvak deed Paddy farmacodynamisch onderzoek bij Janssen Cilag in Beerse (België). In mei 2001 behaalt hij zijn apothekersdiploma.
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