Sex steroid hormones in relation to Barrett's esophagus: An analysis of the FINBAR Study

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Abstract

Previously we observed strong positive associations between circulating concentrations of free testosterone and free dihydrotestosterone (DHT) in relation to Barrett's esophagus in a US male military population. To replicate these findings, we conducted a second study of sex steroid hormones and Barrett's esophagus in the Factors Influencing the Barrett/Adenocarcinoma Relationship (FINBAR) Study based in Northern Ireland and Ireland. We used mass spectrometry to quantitate EDTA plasma concentrations of nine sex steroid hormones and ELISA to quantitate sex hormone binding globulin in 177 male Barrett's esophagus cases and 185 male general population controls within the FINBAR Study. Free testosterone, free DHT, and free estradiol were estimated using standard formulas. Multivariable logistic regression estimated odds ratios (OR) and 95% confidence intervals (95%CI) of associations between exposures and Barrett's esophagus. While plasma hormone and sex hormone binding globulin concentrations were not associated with all cases of Barrett's esophagus, we did observe positive associations with estrogens in younger men (e.g., estrone + estradiol $OR_{continuous\ per\ ½\ IQR}$=2.92, 95%CI:1.08, 7.89), and free androgens in men with higher waist-to-hip ratios (e.g., free testosterone $OR_{continuous\ per\ ½\ IQR}$=2.71, 95%CI:1.06, 6.92). Stratification by body mass index, antireflux medications, and geographic location did not materially affect the results. This study found evidence for associations between circulating sex steroid hormones and Barrett's esophagus in younger men and men with higher waist-to-hip ratios. Further studies are necessary to elucidate whether sex steroid hormones are consistently associated with esophageal adenocarcinogenesis.

Introduction

During the natural history of esophageal adenocarcinoma from reflux, to Barrett's esophagus to cancer, the male-to-female sex ratio progressively increases, culminating in more than seven males for every female with cancer (Cook *et al.* 2005, Cook *et al.* 2009). This stark sex disparity has been repeatedly observed, yet underlying causes have not been identified (Cheng *et al.* 2000, Lindblad *et al.* 2005, Lofdahl *et al.* 2008, Cook *et al.* 2010, Rutegard *et al.* 2010, Bodelon *et al.* 2011, Freedman *et al.* 2011, Cook *et al.* 2012, Hoyo *et al.* 2012, Kubo *et al.* 2013, Cook *et al.* 2014). Sex steroid hormones have been hypothesized to be a possible cause (Lagergren *et al.* 1998) and the fact that sex steroid hormones are involved in inflammatory processes (Maggio *et al.* 2005, Schmidt *et al.* 2006, Kupelian *et al.* 2010, Liao *et al.* 2012) and that sex steroid hormone receptor proteins are expressed in esophageal tissues (Rashid *et al.* 2010, Yang *et al.* 2012) support such a proposition. This motivated us to conduct an analysis of sex steroid hormones in relation to Barrett's esophagus in a US male military population in which we observed strong positive associations with circulating concentrations of free testosterone and free dihydrotestosterone (DHT) (Cook *et al.* 2014). To replicate these findings, we have conducted a second study of sex steroid hormones and Barrett's esophagus in the Factors Influencing the Barrett/Adenocarcinoma Relationship (FINBAR) Study.

Materials and Methods

Study Population

The FINBAR Study has been described in detail elsewhere (Anderson *et al.* 2006). In brief, the study recruited incident and prevalent long segment (≥3 cm) Barrett's

esophagus cases, incident esophageal adenocarcinoma cases, and population controls during 2002–2004 in Northern Ireland and Ireland. All cases and controls in the FINAR Study were Caucasian. Barrett's esophagus cases were required to have histologic confirmation of specialized intestinal metaplasia from biopsy specimens. Patients with dysplasia were not included. In Northern Ireland, Barrett's esophagus cases were initially identified from pathology reports gathered from throughout Northern Ireland. Endoscopy note review was necessary in most patients to confirm long segment Barrett's esophagus. In the Republic of Ireland, clinicians in the Dublin and Cork areas sent details of Barrett's esophagus patients who met the inclusion criteria to the research personnel. Eligible control subjects for the FINBAR Study were adults without a prior diagnosis of Barrett's esophagus, esophageal cancer, or other gastrointestinal cancer. Eligible Northern Ireland controls were individuals listed in the General Practice Master Index (a province-wide database of all persons registered with a general practitioner). Eligible Republic of Ireland controls were individuals listed with one of four general practices (two urban and two rural) in the Dublin and Cork areas. For the primary FINBAR Study, population controls and Barrett's esophagus cases were each frequency-matched by region, sex, and 5-year age groups to the esophageal adenocarcinoma case group.

After giving written informed consent, each study participant underwent a structured computerized interview with a trained interviewer. Demographic, exposure, and medical information were ascertained. Frequent gastroesophageal reflux was defined as symptoms of heartburn and/or acid reflux occurring at least once weekly (>50 times per year) >5 years before interview. Subjects were classified as having gastroesophageal reflux if they responded positively to either of the following questions: (a) Have you ever

had frequent heartburn (i.e., a burning or ache behind the breastbone that is not due to heart problems) not including the last 5 years? or (b) Have you ever had frequent acid reflux (i.e., a bitter taste of stomach acid, which has come up to the back of your throat) not including the last 5 years? Current smoking status was defined as smokers who had smoked at least one cigarette daily for ≥6 months 5 years before interview date. Previous smokers were those smokers who had quit smoking >5 years before interview date. People who had never smoked, who had smoked <100 cigarettes in their lifetime, or who had smoked <1 cigarette daily for <6 months were defined as never smokers. Alcohol consumption was measured using the European Prospective Investigation of Cancer food frequency questionnaire developed for the Norfolk area of England (Day *et al.* 1999). Alcohol consumption 5 years before interview date was defined as the number of grams of alcohol consumed daily. At the interview visit, height, weight and waist-to-hip ratio were measured by the interviewer. In addition, a 30 ml sample of peripheral venous blood (non-fasting) was taken, transported on ice, and then—for 97% of subjects—centrifuged within 5 hours (the maximum time between blood draw and centrifugation was 12 hours). Serum, plasma, and buffy coat were stored at −80°C. EDTA plasma samples were used for quantitation of circulating sex steroid hormones and SHBG. For this analysis of circulating hormones, comparing Barrett's esophagus cases to population controls, the study population was restricted to males because there were too few females to provide adequate statistical power for a female-only analysis.

Laboratory analysis

In collaboration with the Pharmacogenomics Laboratory of Laval University, Quebec, Canada, we quantitatively assessed: dehydroepiandrosterone (DHEA), androstenediol, androstenedione, progesterone, testosterone, dihydrotestosterone (DHT), androsterone (ADT), estrone and estradiol using gas chromatography–mass spectrometry (GC-MS); and sex hormone binding globulin (SHBG) using ELISA (Diagnostics Biochem Canada, Inc.). These selected sex steroid hormones cover a wide array and key positions of the sex steroid biosynthesis pathway (Figure). Three low hormone concentration quality control replicates and three high hormone concentration quality control replicates were included in each of the nine batches. All metabolite coefficients of variation (CV) were less than 10% (range: 3.5–8.8%; Supplementary Table 1). Further information on the methods can be found in our recent publication.(Caron *et al.* 2015)

Statistical Analysis

Participant characteristics of Barrett's esophagus cases were compared with controls using the t-test for continuous variables and the chi-squared test for categorical variables. Partial correlation coefficients adjusted for age at interview (continuous) and body mass index (BMI, kg/m^2) at interview (continuous) were calculated using population controls. Univariate linear regressions and univariate logistic regressions were conducted to assess whether study participant variables were associated with log hormone concentrations and with case-control status. Study participant variables assessed included age at interview (quartiles), education (<10, 10–12, 13–20 years), occupation (non-manual/manual), BMI at interview (<25, 25–<30, ≥30 kg/m^2), waist-to-hip ratio (<0.97, ≥0.97) gastroesophageal reflux symptoms (yes/no), ever-cigarette smoking status (ever/never),

alcohol consumption (<6.3, 6.3–23.1, 23.2–44, ≥44.1 g/day) physical activity (not at all active/not very active/fairly active), and serologic *Helicobacter pylori* (*H. pylori*) status (positive/negative) (Anderson *et al.* 2008). Hormone concentrations were assessed as quartiles using cutpoints based on population control distributions, as well as continuous variables with standardization to half the value of the interquartile range (which approximates a single quartile increase in exposure). In addition to assessing individual hormone exposures, we also assessed *a priori* specified combinations and ratios of hormones that are in close proximity within the metabolic pathway: parent estrogens (the sum of estrone and estradiol), testosterone:parent estrogens ratio, testosterone:estradiol ratio, and androstenedione:estrone ratio. We calculated free estradiol (Sodergard *et al.* 1982), free testosterone (Vermeulen *et al.* 1999), and free DHT (Starka *et al.* 2009) using formulas that include the individual hormone, SHBG, and a constant for albumin.

Variables that were associated with Barrett's esophagus cases and with at least one exposure were considered as potential confounding variables and were entered into an age-adjusted multivariable stepwise logistic regression with entrance p value set at 0.05 and removal p value set at 0.10 in order to estimate ORs and 95% confidence intervals (95%CI). Adjusted logistic regression models, using stepwise selection, resulted in adjustment for age (quartiles), gastroesophageal reflux symptoms (yes/no) and *H. pylori* seropositivity (yes/no). We also conducted a "fully adjusted" model which included age (categorical), ever-cigarette smoking status, alcohol consumption (categorical), BMI at diagnosis (categorical), and gastroesophageal reflux disease symptoms in consideration of previous evidence that these exposures are associated with Barrett's esophagus. We also assessed whether relationships between exposures and

Barrett's esophagus were modified by age, BMI, waist-to-hip ratio, waist circumference, proton pump inhibitor use, H2 receptor antagonist use, and geographic location (Northern Ireland/Ireland) by conducting stratified analyses. Exposures were modelled as both categorical and continuous variables in this assessment of effect modification. The likelihood ratio test was used to assess whether strata were statistically significantly different to one another. All tests were two-sided and p-values <0.05 were considered to be statistically significant. Analyses were conducted using STATA version 13.1 (Stata-Corp LP, College Station, TX).

Results

Descriptive characteristics of male Barrett's esophagus cases and male population controls were largely similar (Table 1). Notable differences existed for gastroesophageal reflux disease symptoms (72% Barrett's vs 20% controls; p<0.001), *H. pylori* positivity (44% vs 63%; p<0.001), and manual occupation (61% vs 51%; p=0.046).

Partial correlation coefficients adjusted for age and BMI at interview using the control population were strongest for estradiol and free estradiol (0.86), testosterone and DHT (0.72), DHT and free DHT (0.68), free testosterone and free DHT (0.64), and estradiol and estrone (0.63) (Supplementary Table 2).

The primary statistical models, adjusted for age at interview, gastroesophageal reflux symptoms and *H. pylori* seropositivity, are shown in Table 2. None of the hormones or SHBG demonstrated a consistent association with Barrett's esophagus. Additional adjustment for ever-cigarette smoking status and alcohol consumption had no material effect on the results obtained (Supplementary Table 3).

Upon stratified analysis, age did appear to be an effect modifier of hormone-Barrett's esophagus associations, with estrone ($OR_{continuous}$=2.53, 95%CI: 1.07, 5.97), estradiol ($OR_{continuous}$=2.11, 95%CI: 0.80, 5.56), the sum of these estrogens (parent estrogens: $OR_{continuous}$=2.92, 95%CI:1.08, 7.89), and free estradiol ($OR_{continuous}$=2.66, 95%CI:0.96, 7.38) providing evidence of associations with Barrett's esophagus in younger aged (<65.9 years) men, but not in older aged men (Table 3 and Supplementary Table 4). However, older aged men did appear to exhibit an association between androstenedione:estrone ratio ($OR_{continuous}$=2.82, 95%CI: 1.15, 6.93) which was absent in younger men. BMI was not an effect modifier of hormone-Barrett's esophagus associations (data not shown) but waist-to-hip ratio was (Table 4 and Supplementary Table 5). In men with a waist-to-hip ratio greater than or equal to the median (0.97), free testosterone ($OR_{continuous}$=2.71, 95%CI:1.06, 6.92) and free DHT ($OR_{continuous}$=2.66, 95%CI:1.29, 5.48) were each positively associated with Barrett's esophagus. Stratified analyses by waist circumference (Supplementary Table 6), proton pump inhibitor use, H2 receptor antagonist use, and geographic location (Northern Ireland/Ireland) did not affect results obtained (unless specified, results not shown). Adjustment for age continuous instead of age categorical had negligible effects on the analyses conducted.

Discussion with Conclusions

In this study of participants from the island of Ireland, we find no overall association between circulating sex steroid hormone concentrations and Barrett's esophagus in males. Barrett's esophagus was, however, positively associated with estrogens in younger men and free androgens in men with higher waist-to-hip ratios. The latter associations—

in which free testosterone and free DHT each demonstrated strong, dose-response associations with Barrett's esophagus—concur with the results of the overall analysis of our previous US-based study (Cook *et al.* 2014).

The positive results of our prior US-study were due to a combination of higher concentrations of testosterone and DHT and lower concentrations of SHBG in cases relative to controls. In this analysis based within the FINBAR Study, free testosterone and DHT were only associated with BE for men with higher waist-to-hip ratios. The reason for this is unclear and waist-to-hip ratio was not available in our previous US study. It is possible that the associations within the prior US study would have been stronger—or restricted to—men with a higher waist-to-hip ratio, and that the overall analysis was also positive because of higher waist-to-hip ratios. This idea has support from the fact that the mean BMI values were slightly higher for cases (28.4) and for controls (28.4) in the prior US study relative to the study presented here, and this was despite the younger mean ages of the prior study. Although no associations were observed in the present study when stratified by BMI or waist circumference, waist-to-hip ratio is the highest anthropometric correlate of visceral adipose tissue/subcutaneous adipose tissue ratio in men (Bazzocchi *et al.* 2014) and both of these fat depots have been implicated in risk of Barrett's esophagus, with waist circumference positively associated (Kubo *et al.* 2013) and hip circumference inversely associated (Kendall *et al.* 2016). Similar effects have been noted for esophageal adenocarcinoma (Steffen *et al.* 2015). Visceral adipose tissue is known to have a high metabolic rate with detrimental consequences on health (Walker *et al.* 2014). Conversely, gluteofemoral adipose tissue has been postulated to act as metabolic sink, reducing concentrations of adipocytokines,

insulin and carbohydrates (Manolopoulos *et al.* 2010). Thus it is conceivable that waist-to-hip ratio—as a proxy of these underlying exposures—modifies associations between sex steroid hormones and Barrett's esophagus. The slight age differences between the studies is unlikely to explain the differences in free androgen-Barrett's esophagus associations, given that age was not an effect modifier of these associations in either population. In addition, the findings are unlikely to differ due to a technicality given that the same laboratory, personnel, machines and assays were used for both studies.

Another possible reason for the slight differences in results of our two studies is the use of different control groups—population controls in this study and endoscopy-negative controls in the prior US-study. Our original hypothesis—that the inverse associations between androgens and wound healing (Ashcroft *et al.* 2002, Engeland *et al.* 2009, Gilliver *et al.* 2009) may expand the interval for opportunistic metaplastic re-population—may be better tested using endoscopy-negative (or even reflux-positive) controls, as opposed to the population controls used in this study. Further studies comparing Barrett's esophagus cases with both types of control groups may help further elucidate any consistent relationship between free androgens and Barrett's esophagus.

Other factors that differ between the studies that could underlie the contrasting results of the main analyses include different countries (Northern Ireland & Ireland vs United States), different underlying population types (general population vs military), and different specimen types (EDTA plasma vs serum). Although circulating androgen and SHBG concentrations have been quantitated in healthy US men, no study has published on such concentrations using healthy men from Northern Ireland or Ireland. In general, age-specific concentrations of androgens were lower and SHBG was higher in this

population relative to the US population of the prior study. Regardless, any differences in hormone levels due to the underlying population would be unlikely to explain the relative case-control differences between studies. This is also true for the different specimen types used, despite few studies of the comparability of blood hormone levels from plasma and serum samples.

Strengths of this study include the use of a robust quantitative technology for quantitation of sex steroid hormones and a sample size more than adequate to detect the magnitude of associations we reported in our prior study. A limitation is the fact that we did not have an endoscopy-negative or gastroesophageal reflux disease control population, which would have provided for a more direct comparison with our prior US-study. In addition, statistical power was less for stratified analyses in which associations have been detected and future studies should be designed with these findings in mind.

In summary, this study found evidence that Barrett's esophagus was positively associated with estrogens in younger men and positively associated with free androgens in men with higher waist-to-hip ratios. Additional studies of esophageal adenocarcinogenesis are warranted to further elucidate the hypothesis that sex steroid hormones may underlie the stark sex differences of this disease.

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Author contributions

Conception: MBC, LAA, LJM; Design: All authors; Statistical Analysis: SN, MBC, LAA, RTF; Laboratory Analysis: CG, PC; Interpretation: All authors; Drafting of the manuscript: All authors; MBC is responsible for the overall content and is guarantor of the article.

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Figure. Schematic of Sex Steroid Hormone Metabolism.

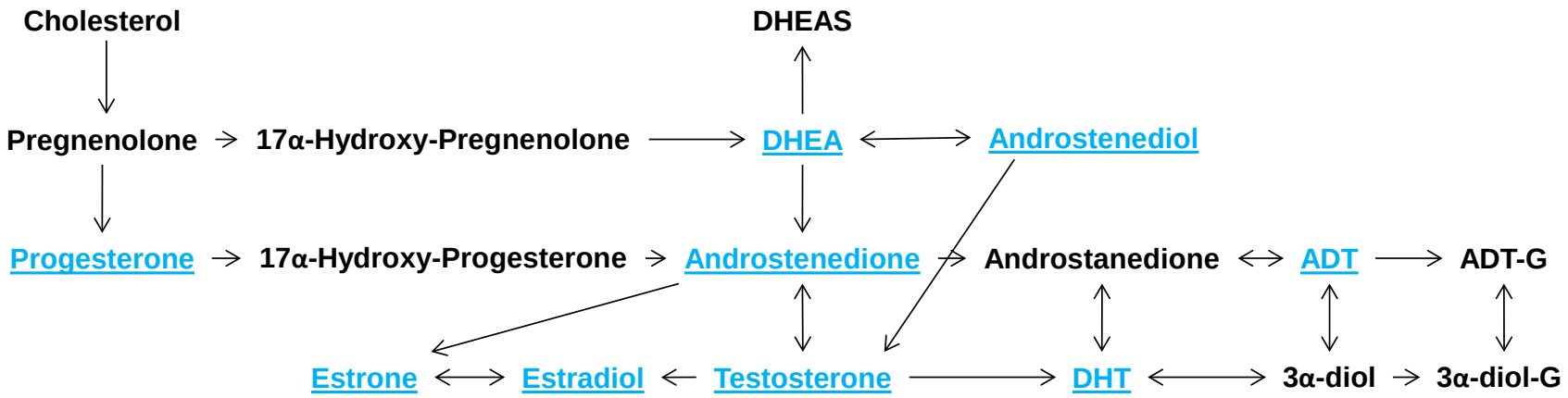

Figure Legend: Sex steroid hormones that were quantitated are shown in blue font. Note that Sex Hormone Binding Globulin (SHBG) is not shown as it is not a part of the sex steroid biosynthesis pathway. Abbreviations: 3α-diol, 3-androstanediol; 3α-diol-G, 3-androstanediol glucuronide; ADT, androsterone; ADT-G, androsterone glucuronide; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone-sulphate; DHT, dihydrotestosterone.