THE ROLE OF THE INSULIN GROWTH FACTOR FAMILY IN DEVELOPMENT OF BARRETT’S ESOPHAGUS: A CASE-CONTROL STUDY

by

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List of abbreviations

95%CI: 95 Percent Confidence Interval
BE: Barrett’s esophagus
BMI: Body mass index
CV: Coefficient of Variation
CM: Centimeter
ECA: Esophageal adenocarcinoma
ERK: Extracellular signal regulated kinase
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
FFA: Free fatty acids
GERD: Gastroesophageal reflux disease
HOMA: Homeostasis Model Assessment
HOMA-IR: Homeostasis Model for Assessment of Insulin resistance
IGF-1/2: Insulin growth factor 1/2
IGFBP 1/3: Insulin growth factor binding protein 1/3
IGFR1: Insulin growth factor receptor 1
p-AKT: Phosphorylated Akt
p-IRS: Phosphorylated insulin receptor substrate
p-mTOR: Phosphorylated molecular target of rapamycin
IHC: Immunohistochemistry
IKKβ: I-kappa B kinase beta
IR: Insulin receptor
JNK: Jun N-terminal kinase
LSBE: Long segment Barrett’s Esophagus

OR: Odds ratio

PDGF: Platelet derived growth factor

PKC: Protein kinase C

PI3K: Phosphotidylinositol 3-kinase

RR: Relative risk

SIM: Specialized intestinal metaplasia

TNFα: Tissue necrosis factor alpha

VAT: Visceral adiposity

VBE: Visible Barrett’s epithelium

WHR: Waist-to- Hip ratio
Obesity is a well established risk factor for Barrett’s esophagus (BE). The mechanisms by which obesity leads to development of BE are not completely understood. High levels of insulin and IGF-1 were hypothesized to provide the link between obesity and BE. A case-control study comparing BE cases with controls with respect to measures of adiposity, levels of serum insulin as well as insulin growth factor proteins was conducted. The activation of the insulin signaling pathway was further assessed by immunohistochemistry of tissues obtained from BE cases. High serum insulin was associated with increased risk of BE, whereas high levels of IGFBP-3 were associated with decreased risk of BE. There was no demonstrable correlation between serum factors and tissue activation of phosphorylated insulin receptor substrate-1. Insulin signaling pathway is likely active in esophageal tumorigenesis. Further studies are needed to explore the complex interactions of this pathway with other molecular pathways activated by excess body weight.
INTRODUCTION

The epidemiology of esophageal cancer in the Western world has changed dramatically over the past three decades. Rates of esophageal squamous cell carcinoma have declined, whereas esophageal adenocarcinoma (EAC) has been on the rise.\textsuperscript{1-7} The absolute incidence of EAC in the United States increased over six-fold from 3.8 per million in 1975-1978 to 23.3 per million in 2001.\textsuperscript{8} It is believed that chronic irritation and inflammation initiate the sequence of genetic alterations in the damaged esophageal epithelium and progressively lead to tissue metaplasia, dysplasia, and finally cancer. Western diet and reflux of gastric contents are among the well studied environmental irritants that promote esophageal carcinogenesis. Diets rich in carbohydrates, unsaturated fats, red and processed meats but low in fruits and vegetables have been shown to increase risk of esophageal cancer in several large scale prospective studies.\textsuperscript{9-15} Gastroesophageal reflux disease (GERD) is strongly associated with esophageal adenocarcinoma and increases the risk of esophageal cancer eight to forty-five fold, depending on chronicity.\textsuperscript{16-20} It is quite puzzling, however, that an estimated forty percent of patients with EAC will give no clear-cut history of GERD.\textsuperscript{21} This suggests the possibility of an alternate, non-reflux mediated pathway.

Obesity increases the risk of esophageal adenocarcinoma two to eight fold.\textsuperscript{22-28} Obesity, more specifically central adiposity, has also been established as a risk factor for the precursor of esophageal adenocarcinoma, i.e. Barrett’s esophagus (BE). In a study by Edelstein et al. (2007),\textsuperscript{29} BMI above 30kg/m\textsuperscript{2} increased the odds of developing specialized intestinal metaplasia (SIM), visible BE (VBE) and long segment BE (LSBE) by approximately two-fold. The odds of BE were three fold higher when upper vs. lower tertiles of waist to hip ratio were compared in cases versus population controls. Further, when analyses of BMI and BE risk were adjusted for central adiposity (i.e. waist-to-hip ratio), associations between body mass index, short and long segment BE were
attenuated. Relationship between SIM and BMI remained statistically significant (OR= 2.0, 95%CI 1.1, 3.5) in analyses adjusted for central adiposity. Adjusting the analyses of waist to hip ratio and BE risk for BMI did not significantly alter the strength of observed associations. Risk of BE also increases with increasing visceral adiposity (VAT). In a study of US veterans, each 10cm² increase in VAT was associated with 9% increase in BE risk. These observations are likely due to the fact that increased waist to hip ratio correlates strongly with increasing visceral fat. Visceral fat is a metabolically active organ associated with increased levels of pro-inflammatory cytokines such as IL-6 and TNF-α, both of which have a role in development of hyperinsulinemia, insulin resistance, and perhaps tumorigenesis.

Although obesity contributes to GERD, it appears to be an independent risk factor for BE and esophageal adenocarcinoma. Hyperinsulinemia, which is often present in centrally obese patients with characteristics of metabolic syndrome, has been postulated to provide the key mechanistic link between obesity and esophageal carcinogenesis. Prolonged hyperinsulinemia leads to increased growth hormone release and increased production of insulin growth factor-1. Hyperinsulinemia also decreases hepatic production of insulin growth factor binding proteins. Decrease in the synthesis of insulin growth factor binding proteins during hyperinsulinemia increases the amount of ‘bioavailable’ insulin growth factor-1 (IGF-1). Given that IGF-1 is a growth factor with proliferative and antiapoptotic properties, its increased bioavailability has been associated with increased tissue proliferation and tumorigenesis (Figure 1).
Figure 1. Proposed mechanism linking insulin resistance and carcinogenesis.

Studies in breast, prostate, colorectal and endometrial cancer generally support the role of elevated insulin growth factor-1 in carcinogenesis.\textsuperscript{33-47} A recent meta-analysis of 21 case-control studies, which included 3609 cases and 7137 case controls concluded that high levels of IGF-1 were associated with an increased risk of prostate cancer and pre-menopausal breast cancer.\textsuperscript{48} To our knowledge, no published studies have examined the role of the insulin growth factor family in esophageal adenocarcinoma or its precursor, Barrett’s esophagus.

The aims of this observational study were following:

1) Determine whether hyperinsulinemia, insulin resistance and elevated levels of serum IGF-1 increase the risk of Barrett’s esophagus,

2) To examine the activation of the insulin axis in esophageal metaplasia by exploring the role of insulin receptor substrate activation in tissues derived from patients with Barrett’s esophagus, and lastly

3) To explore the relationship between serums levels of the components of the insulin growth factor family and tissue activation of the insulin receptor substrate and its downstream mediators.
We hypothesized that BE cases will have higher serum levels of IGF-1 and lower levels of IGFBP-1 and IGFBP-3 compared to GERD controls, consistent with the currently accepted “insulin-cancer” hypothesis. In addition, we hypothesized that increased serum levels of IGF-1 will result in increased tissue activation of insulin receptor substrate and related downstream proliferative mediators.
BACKGROUND

Barrett’s Esophagus and Esophageal Adenocarcinoma

This project addresses the role of hyperinsulinemia in development of BE. BE is a precursor condition to EAC where normal squamous epithelium is replaced with metastatic, specialized intestinal epithelium. The incidence of BE esophagus in the general population is 1-2%. Most cases of EAC arise in Barrett’s epithelium--metastatic Barrett’s epithelium can be demonstrated in 58-100% of EACs.49-52 Evidence supports the sequential paradigm of metaplasia-dysplasia-carcinoma in BE. Specialized intestinal epithelium progresses to low grade dysplasia, high grade dysplasia, intramucosal carcinoma and finally invasive carcinoma.53-59 The length of time for progression from high grade dysplasia to adenocarcinoma is unknown. In the setting of confirmed BE diagnosis, progression to esophageal adenocarcinoma is estimated at 1 per 250 patient-years, or 0.4% per year.60

Obesity and Cancer: Insulin growth factor pathway and carcinogenesis

Obesity potentially increases the risk of various cancers through a number of mediators including diet,61 physical activity,61 or genetic susceptibility. Oxidative stress/DNA damage, non-inflammatory changes in immune function, inflammatory mediators and hormones also promote obesity associated carcinogenesis.62,63 Increasing evidence points toward involvement of the insulin like growth factor family.64 Insulin like growth factor (IGF)-1 is a hormone related to insulin and it plays an important role in normal cellular proliferation. IGF-2 is another growth factor that is important in normal fetal development but has also been associated with increased risk of cancer.65 Gene disruption experiments show that combined absence of IGF-1 and IGF-2 results in severe organ hypoplasia and death shortly after birth due to respiratory failure. Both IGF-1 and 2 can bind to the insulin growth factor receptor with equal affinity. In the circulation, both IGF-1 and
IGF-2 are bound to IGFBPs. Seven IGFBP species have been cloned to date.\textsuperscript{66} Seventy-five percent of circulating IGF-1 is bound to IGFBP3. IGFBPs have been shown to augment or inhibit actions of IGF-1 by altering receptor/ligand interactions.

IGF-1 and -2 mediate intracellular activity through insulin growth factor type 1 receptor (IGF1R), which is homologous in structure to the insulin receptor. IGF1R is a glycosylated heterotetramer complex composed of two extracellular α subunits and two β transmembrane subunits that have tyrosine kinase activity. IGF-1/insulin binding to the extracellular α subunit causes conformational changes of the IGF1R that activate the tyrosine kinase domain of the intracellular portion of the β subunit. Once the tyrosine kinase domain of insulin receptors is activated, it promotes auto-phosphorylation of the β unit itself. Phosphorylation of three tyrosine residues (Tyr-1158, Tyr-1162, Tyr-1163) is required for amplification of the tyrosine kinase activity. Activation of the tyrosine kinases of the insulin receptor leads to rapid phosphorylation of insulin receptor substrate (IRS) proteins 1, 2, 3, and 4 and several Shc proteins. These subsequently attract multiple intracellular signaling molecules.\textsuperscript{67}

The IRS and Shc play an important regulatory role in the insulin signaling cascade because they are the first step in dispersing of insulin signaling intracellularly. Metabolic and anti-apoptotic effects of insulin signaling are mediated through IRS proteins, activation of phosphoinositol (PI) 3-kinase, protein kinase B (Akt), molecular target of rapamycin (mTOR), and p70 S6 kinase. Activation of PI 3-kinase, Akt, and atypical protein kinase C through phophoinositide dependent protein kinase is critical in the effects of insulin on glucose transport. Hyperactivation of m-tor by amino acids, Akt, or hyperinsulinemia results in serine phosphorylation of IRS-1 by p70S6 kinase, with a subsequent decrease in strength of the IRS-1/PI 3-kinase signaling.\textsuperscript{68} IRS-1 phosphorylation can be also promoted by Jun-N-terminal kinase(JNK), protein kinase C (PKC), I-kappa B kinase beta (IKKβ), and tumor nectorsis factor alfa (TNFα). \textit{Insulin resistance} is mediated through the increased
expression of the p85α monomer, which tends to compete and displace p85-p110 heterodimer from IRS-1 binding sites. The resultant decrease in p110 binding with IRS-1 diminishes the PI 3-kinase activity and its downstream effects. Steroids, growth hormone, short term overfeeding, obesity and type 2 diabetes mellitus have all been shown to increase p85 activity \(^{69}\) (see Figure 2).

**Figure 2.** Intracellular activation of insulin receptor substrate.

A. Aminoacids, hyperinsulinemia or Akt hyperactivate mTOR and result in serine phosphorylation of IRS-1 by p70S6 kinase, with subsequent decrease in IRS/PI3kinase signaling. Serine phosphorylation can also be promoted by JNK, PKC, IKKβ and TNFα.

B. Increased expression of p85 monomer displaces the p85-p110 heterodimer from IRS binding sites. Decrease in the association of p110 with IRS diminishes PI 3-kinase signaling. Obesity is known to increase p85 monomer expression. Adapted from Draznin B et al. *Diabetes* 2006; 55:2393.

Non-metabolic and proliferative actions of insulin are mediated through activation of Ras, Raf, and mitogen activated protein kinases Erk-1 and Erk-2.\(^{70}\) This paper will explore the elements of the above described mechanisms of insulin resistance in BE patients by looking at how hyperinsulinemia alters the serum levels of IGF-1 and its binding proteins, activation of the IGF-R and what is its role in post-receptor activation of mTOR, Akt, and ki-67.
METHODS

Study Population, anthropometric measurements and biological samples.

Study patients were chosen from adult Northeast Ohio residents undergoing endoscopy for evaluation of refractory GERD symptoms at two referral institutions between January 2005 and May 2009. Potential participants were recruited at the time of their endoscopy visit. Study subjects had four quadrant biopsies taken at 2cm intervals along the tubular esophagus above the squamocolumnar junction. Patients with biopsy results that were consistent with intestinal metaplasia, i.e. Barrett’s esophagus, and abnormal metaplastic tissue specimens at least 1 cm in length, were defined as cases. Those without the presence of intestinal metaplasia were used as controls. Recruitment continued until projected sample size was reached. We excluded subjects with prior history of cancer other than that of skin, history of diabetes mellitus, those who underwent obesity surgery or were enrolled in chemoprevention trials. All study patients underwent an initial structured interview. At this visit, anthropometric measurements were obtained. We collected data on patient weight (kg), height (cm), and waist and hip circumference (cms). Subject weight was obtained on a leveled platform scale. Subjects were instructed to stand over the center of the platform with body weight evenly distributed. Height was measured in fully erect position with subjects inhaling. The measurements were recorded to the nearest 0.1cm. Waist circumference was measured in a horizontal plane at the narrowest part of the torso. In obese subjects where it was difficult to observe waist narrowing, we accepted the smallest horizontal circumference between the ribs and the iliac crest as the correct measurement. Hip circumference was measured at the level of the greatest lateral extension of the hips, using the greater trochanter as an anatomic landmark. Patients who had waist to hip ratios (WHR) greater than 0.90 were considered to have central adiposity. All subjects provided fasting venous blood
samples at the time of initial interview or subsequent endoscopy. The serum was divided into multiple aliquots and stored within 4 hours of blood draw at minus 70 degrees Celsius. Hemolyzed, icteric or grossly contaminated samples were discarded. Serum glucose concentrations were determined by the glucose oxidase method on the YSI 2300 Stat Plus Glucose and Lactate Analyzer (YSI, Yellow Spring, OH). We opted to use a single blood draw for determination of serum insulin level. This decision was based on data regarding the reliability of Homeostatic Model Assessment (HOMA) calculated from three insulin samples compared to a single sample; the values show nearly perfect correlation (r=0.99, p<0.0001).72 Serum insulin concentration was measured by radioimmunoassay method using Siemens analyzer (Siemens Medical Solutions Diagnostics, Los Angeles, CA). This assay does have 32% cross-reactivity with pro-insulin. The assay has no cross-reactivity with C-peptide or glucagon. Hyperinsulinemia was defined as a value in the highest quartile of serum insulin measured in non-diabetic study individuals. Serum concentrations of IGF-1, IGF-2, IGFBP-1 and IGFBP-3 were measured by enzymatically amplified two step immunoassay available from Diagnostic Systems Laboratories (Diagnostic Systems Laboratories, Inc. Webster, TX). IGF-1 measurement included an extraction step in which IGF-1 was separated from its serum binding proteins. In addition to kit controls, aliquots of in house serum controls were used to determine assay performance for all serum markers. Coefficients of variation (CV) were calculated by summing up intra-assay and inter-assay variability. CV for glucose, insulin, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 were 3.53%, 14%, 12%, 9.4%, 9.7%, and 7.8%, respectively.
Assessment of insulin resistance

This study relied on the Homeostatic Model Assessment (HOMA) method for estimation of basal insulin resistance. HOMA was calculated from fasting serum insulin and glucose concentrations obtained at the time of EGD using the following simplified formula:

\[ \text{HOMA-IR} = \frac{\text{FPI} \times \text{FPG}}{22.5}, \]

where FPI is fasting plasma insulin concentration in (mU/L) and FPG is fasting plasma glucose (mmol/L)\(^7\). Estimates from the HOMA model correlate well with measurements obtained from the euglycemic clamp \((R_s = 0.88, P < 0.0001)\).\(^7\)

Immunohistochemistry staining

Immunohistochemical staining was performed for all cases with a tissue diagnosis of Barrett’s esophagus. Phosphorylated mouse monoclonal antibodies against ki-67 were obtained from Dakocytomation (Glostrup, Denmark). Rabbit monoclonal antibodies against p-mTOR and p-Akt, as well as rabbit polyclonal antibody against p-IRS1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Tissue sections were freshly cut, processed, and immediately refrigerated to enhance immunoreactivity. At the time of tissue staining, sections were deparaffinized in xylene, rehydrated through a series of graded ethanol washes, and then rinsed in dehydrogenated water. Slides were further prepared for antigen retrieval by incubation in citrate buffer at 98 degrees Celsius for 20 minutes. Endogenous peroxidase activity was blocked in sections that would be treated with Ki-67 antibody. Serum-free protein block was further used to block nonspecific background staining. Above mentioned primary antibodies were then applied and slides were allowed to incubate for an additional 60 minutes. After incubation, slides were rinsed in TBS buffer. Bound antibody was detected using the BioCare Medical polymer detection
system (Biocare Medical, Concord, CA). Mach-3 rabbit probe was applied to slides treated with p-Akt, p-mTOR and p-IRS1 antibody. Slides treated with ki-67 antibody were treated with Mach 4 mouse probe. Slides were incubated for 20 minutes with a peroxidase based polymer conjugated to mouse or rabbit antibodies. Immunogenic complexes with p-Akt, p-mTOR, p-IRS1 were visualized with Vulcan Fast Red kit, whereas antigen complexes with ki-67 antibody were visualized with Cardassian DAB kit. Both chromogenins were available through Biocare Medical (Concord, CA). Slides were counterstained with hematoxylin, mounted and submitted to an expert gastrointestinal pathologist (J.E.W) for interpretation. The pathologist was blinded to the patient’s BMI and results of serologic studies. Staining was compared between specialized intestinal epithelium and adjacent squamous or fundic epithelium in biopsies. It was classified as intense or weak (>50% vs. <50% of visualized field with positive immunohistochemical reaction).

Statistical considerations

Simple descriptive statistics were performed to help define the study population and to describe the frequencies of risk factors among cases and controls. Assumptions of normal distribution were tested for each variable of interest. We explored logarithmic transformation of variables with skewed distributions and included them in further analysis as appropriate. Differences in the distribution of baseline characteristics between the case subjects and controls were compared using the Wilcoxon rank sum test (for continuous data) or the Pearson $X^2$ test (for categorical data). Continuous variables were explored as such and also grouped into quartiles. Division of variables into quartiles was performed based on data distribution. Relationships between BMI, central adiposity and case status were explored through contingency tables. We also explored the correlation of anthropometric measures with insulin and the insulin growth peptide family through the use of Spearman correlation coefficients.
Multivariate logistic regression analysis was performed to help describe the complex relationships between the covariates and case status while adjusting for relevant risk factors. For variables that were split into quartiles, odds ratio and the associated 95% confidence intervals were calculated by comparing highest quartiles to the lowest. Odds ratio estimates for the insulin growth factor family were adjusted for age; gender (male vs. female); race (white vs. non-white); BMI (BMI<25[referent], 25 to <30, or >=30kg/m²) and central adiposity (waist to hip ratio >0.9 vs. <0.9). Multivariate regression models utilized reciprocal adjustments of IGF-1 for IGFBP-3 and vice versa. The molar ratios of IGF-1 to IGFBP-3 were also assessed as an indicator of IGF-1 bioavailability. For conversion, 1μg/L (or 1ng/μL) was equal to 0.130nmol/L for IGF-1 and 0.036nmol/L for IGFBP-3.75 Cochran-Armitage test was applied in the analysis of trends. As there is evidence that single measurement of IGF-1 and IGFBP-3 is reflective of long term levels, odds ratios obtained from our models were also taken as estimates of the relative risk (RR).76

Imputation of mode or median was used when data were missing for variables with categorical or continuous distribution, respectively. Indicator variables for missing data points were included in the multivariate logistic regression models when appropriate.

Strength of association between p-Akt, p-IRS1, p-mTOR and ki-67 was tested by Spearman correlation statistic. All tests of statistical significance were two-sided and p values less than 0.05 were considered significant.

All statistical analyses were performed in Statistical Analysis Systems software package 9.1 (Cary, NC). Graphical output was obtained through the R statistical software package.

Sample size consideration

We expected that 20 to 40% of cases will have increased insulin resistance, BMI>30, elevated IGF-1, and other “high risk” exposures. Further, we were interested in identifying effects that would
increase relative risk of Barrett’s esophagus 2 to 4-fold. We estimated the magnitude of this effect based on previous data regarding the relationship of obesity and esophageal adenocarcinoma. Assuming frequency of 0.3 for high risk exposures, alpha of 0.05 and 80% power, we aimed to recruit 120 cases with Barrett’s esophagus and 120 controls.

RESULTS

We recruited 97 patients with Barrett’s esophagus and 119 GERD controls. Twenty two patients were excluded from the study population due to a history of diabetes mellitus. There were no significant differences in the proportion of patients with diabetes among cases and controls (13/97 vs. 9/119, p=0.17). The majority of study subjects with diabetes were on insulin sensitizing agents as a sole medication. Insulin was used for diabetes mellitus management in 1/22 cases. Baseline characteristics of the final study population (n=194) are shown in Table 1. Case subjects compared to controls were older, predominantly male and of white race. There were significant differences in mean BMI among the two study groups. There were no significant differences in mean WHR, however, statistically significant differences were found in unadjusted analyses of WHR when this variable was analyzed as binary (WHR>0.9 vs. WHR<0.9). High versus low WHR showed a strong positive association with case status (OR=2.88, 95% CI 1.17, 7.13) and this risk was further increased by adjustment for BMI (OR=3.10, 95% CI 1.23, 7.82). Adjusting for age, gender, and BMI attenuated the relationship between case status and WHR (OR= 1.82 95%CI 0.65, 5.34). Association of case status with BMI was weaker than central adiposity (OR=1.05, 95% CI 1.01, 1.11).
Table 1. Baseline characteristics of study patients.

<table>
<thead>
<tr>
<th></th>
<th>Subjects</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n=86)</td>
<td>Controls (n=108)</td>
</tr>
<tr>
<td>Age (SD)</td>
<td>63.3(10.6)</td>
<td>55.9 (11.5)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>65/86 (75.6%)</td>
<td>64/108 (59.3%)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>21/86 (24.4%)</td>
<td>44/108 (40.7%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>77/86 (89.5%)</td>
<td>91/108 (84.3%)</td>
</tr>
<tr>
<td>Non-white (%)</td>
<td>9/86 (10.5%)</td>
<td>17/108 (15.7%)</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, kg/m² (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25 kg/m² (%)</td>
<td>31.05(5.9)</td>
<td>29.2(5.5)</td>
</tr>
<tr>
<td>25-30 kg/m² (%)</td>
<td>18/86 (20.9%)</td>
<td>30/108 (27.8%)</td>
</tr>
<tr>
<td>&gt;30 kg/m² (%)</td>
<td>21/86 (24.4%)</td>
<td>37/108 (34.3%)</td>
</tr>
<tr>
<td>Central adiposity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean WHR (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0.98(0.06)</td>
<td>0.96(0.07)</td>
</tr>
<tr>
<td>Present†</td>
<td>7/86 (8.1%)</td>
<td>22/108 (20.4%)</td>
</tr>
<tr>
<td></td>
<td>79/86 (91.9%)</td>
<td>86/108 (79.6%)</td>
</tr>
</tbody>
</table>

† Central adiposity was defined as waist to hip ration greater than 0.9.

Associations for WHR and BE case status could not be estimated in males due to complete quasi-separation of data points and low cell frequencies. All male subjects with Barrett’s esophagus had WHR>0.9 and 62/64 (97%) of male GERD controls were also centrally adipose. Associations were strongest for those who were overweight and obese, yet they did not reach statistical significance (Table 2).
Table 2. Odds ratios for High Waist to Hip Ratio Among Subgroups.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Controls No. high/low</th>
<th>Cases No. high/low</th>
<th>OR (95% CI)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males Femaless</td>
<td>62/2 24/20</td>
<td>65/0 14/7</td>
<td>1.54 (0.51, 4.65)</td>
</tr>
<tr>
<td>Less than 55 years old</td>
<td>47/16 39/6</td>
<td>20/3 59/4</td>
<td>1.73 (0.34, 8.90) 2.00 (0.51, 7.88)</td>
</tr>
<tr>
<td>More than 55 years old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI&lt;25</td>
<td>18/4 68/18</td>
<td>10/2 69/5</td>
<td>0.41 (0.02, 6.39) 2.45 (0.74, 8.17)</td>
</tr>
<tr>
<td>BMI&gt;25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† All odds ratio estimates were also adjusted for age (<55/55+) and sex.

Figure 3. Skewed distribution of serum Insulin growth factor binding protein-1. Figure on the right illustrates how logarithmic transformation of IGFBP-1 normalized the distribution of this variable. IGFBP-1 was measured in ng/mL.

Levels of IGF-1, IGF-2 and IGFBP-3 were normally distributed. A right skewed distribution was observed for serum levels of IGFBP-1; log of IGFBP-1 was therefore used in all statistical analyses (Figure 3).

Further, we correlated serum concentrations of insulin growth factors and their binding proteins with anthropometric measurements. Based on our belief that serum levels of insulin growth factors would be altered in esophageal metaplasia, we included only GERD controls in the
analysis of correlations. Serum IGF-1 correlated with IGFBP-3, the major binding protein in blood (r=0.44, p<0.0001). Of interest, IGF-2 showed even stronger correlation with IGFBP-3 (r=0.88, p<0.0001). BMI correlated strongly with insulin and HOMA-IR (r>0.5, p<0.001). There was a strong negative correlation between BMI and IGFBP-1 (r=-0.52, p<0.0001). IGF-1 also showed moderately strong correlations with body height (r=0.37, p=0.001). Central adiposity did not correlate with serum levels of insulin growth factors or their binding proteins (Table 3). Insulin growth factors and IGFBP-3 negatively correlated with age (IGF1/age r=-0.21, p=0.02; IGF2/age r=-0.14, p=0.12; IGFBP3/age r=-0.20, p=0.03). Logarithm of IGFBP-1 had a non-significant positive correlation with age (r=0.16, p=0.09).

Insulin growth factors did not show linear association with indices of body fat such as BMI (Figure 4). Associations of linearity were examined among control subjects only. There were no significant differences in these patterns when the two study groups were combined. When serum levels of IGF-1 were plotted against BMI, peak serum levels of IGF-1 were observed in patients with BMI between 22-24 kg/m² (table 4). IGF-1 concentrations displayed a decreasing trend toward BMI<20 and BMI>30. IGFBP-3 levels also peaked in the BMI range of 22-24 kg/m², and dropped thereafter. The pattern of variation in mean IGFBP-3 concentrations across BMI categories paralleled that of IGF-1. Due to small numbers of patients in the above listed BMI categories, we could not meaningfully explore differences in these patterns based on gender.
**Table 3.** Correlations of serum factors with anthropometric measures.

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<th></th>
<th>Height</th>
<th>BMI</th>
<th>WHR</th>
<th>Insulin</th>
<th>HOMA-IR</th>
<th>IGF1</th>
<th>IGF2</th>
<th>IGFBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI</strong></td>
<td>0.04</td>
<td>(0.67)</td>
<td>-----</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WHR</strong></td>
<td>0.42</td>
<td>(&lt;0.01)</td>
<td>0.20</td>
<td>(0.04)</td>
<td>-----</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td>0.11</td>
<td>(0.23)</td>
<td>0.51</td>
<td>(&lt;0.01)</td>
<td>0.13</td>
<td>(0.18)</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>0.15</td>
<td>(0.12)</td>
<td>0.53</td>
<td>(&lt;0.01)</td>
<td>0.19</td>
<td>(0.05)</td>
<td>0.93</td>
<td>(&lt;0.01)</td>
</tr>
<tr>
<td><strong>IGF-1</strong></td>
<td>0.37</td>
<td>(&lt;0.01)</td>
<td>-0.14</td>
<td>(0.15)</td>
<td>0.02</td>
<td>(0.79)</td>
<td>-0.04</td>
<td>(0.68)</td>
</tr>
<tr>
<td><strong>IGF2</strong></td>
<td>0.0009</td>
<td>(0.99)</td>
<td>0.116</td>
<td>(0.23)</td>
<td>-0.103</td>
<td>(0.29)</td>
<td>0.20</td>
<td>(0.04)</td>
</tr>
<tr>
<td><strong>Log IGFBP1</strong></td>
<td>-0.17</td>
<td>(0.07)</td>
<td>-0.52</td>
<td>(&lt;0.01)</td>
<td>-0.13</td>
<td>(0.17)</td>
<td>-0.50</td>
<td>(&lt;0.01)</td>
</tr>
<tr>
<td><strong>IGFBP3</strong></td>
<td>0.09</td>
<td>(0.35)</td>
<td>0.05</td>
<td>(0.57)</td>
<td>-0.04</td>
<td>(0.65)</td>
<td>0.11</td>
<td>(0.23)</td>
</tr>
</tbody>
</table>

* Partial Spearman rank correlation is presented for controls only. Values in parentheses represent p values for statistical tests when the correlation test was not equal to 0. All statistical tests were two sided.

†BMI = body mass index
‡WHR= waist to hip ratio
¶HOMA-IR= Homeostatic model assessment of insulin resistance

Levels of insulin growth factor binding protein-1 were significantly lower in obese study subjects, however, no significant differences were found in mean IGFBP-3 levels based on BMI threshold of 30kg/m² (p>0.05). In contrast, serum insulin levels showed a linear association with BMI. Serum insulin levels were significantly higher in obese subjects compared to those with normal weight (10.1 vs. 6.14 μIU/mL, p<0.004).
Table 4. Mean Insulin, IGF-1, and IGFBP-3 concentrations by BMI categories.

<table>
<thead>
<tr>
<th>BMI</th>
<th>Insulin</th>
<th>%</th>
<th>IGF-1</th>
<th>%</th>
<th>IGFBP-3</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-22</td>
<td>1.84</td>
<td>-89%</td>
<td>176</td>
<td>-38.3</td>
<td>3266.5</td>
<td>-32.7</td>
</tr>
<tr>
<td>22.1-24</td>
<td>2.99</td>
<td>-82.1</td>
<td>284.5</td>
<td>---</td>
<td>4850.5</td>
<td>---</td>
</tr>
<tr>
<td>24.1-26</td>
<td>3.92</td>
<td>-76.5</td>
<td>197.5</td>
<td>-30.6</td>
<td>3752.1</td>
<td>-22.6</td>
</tr>
<tr>
<td>26.1-28</td>
<td>5.93</td>
<td>-64.5</td>
<td>215.1</td>
<td>-24.4</td>
<td>3450.1</td>
<td>-28.9</td>
</tr>
<tr>
<td>28.1-30</td>
<td>6.82</td>
<td>-51.2</td>
<td>217.0</td>
<td>-23.7</td>
<td>3484.6</td>
<td>-28.2</td>
</tr>
<tr>
<td>30.1-32</td>
<td>8.80</td>
<td>-47.3</td>
<td>196.5</td>
<td>-30.9</td>
<td>3968</td>
<td>-18.2</td>
</tr>
<tr>
<td>32.1-34</td>
<td>11.2</td>
<td>-32.9</td>
<td>159.3</td>
<td>-44.0</td>
<td>3168.1</td>
<td>-34.7</td>
</tr>
<tr>
<td>34.1-36</td>
<td>11.7</td>
<td>-29.9</td>
<td>161.0</td>
<td>-43.4</td>
<td>3943.5</td>
<td>-18.7</td>
</tr>
<tr>
<td>&gt;36</td>
<td>16.7</td>
<td>----</td>
<td>193.6</td>
<td>-32</td>
<td>3936.3</td>
<td>-18.8</td>
</tr>
</tbody>
</table>

†Percent difference was calculated relative to the category with the highest hormone level.

Serum levels of IGF-1, IGF-2 and IGFBP-3 were significantly lower in Barrett’s cases compared to GERD controls (Table 5). No significant differences were found in the serum levels of IGFBP-1 among the two study groups (p=0.23). Mean insulin levels were higher in case subjects (10.75 μIU/mL [95% CI 9.07, 12.42] for case subjects versus 8.27 μIU/mL [95% CI 7.03, 9.51] for control subjects, p=0.02). Mean HOMA-IR was also higher in case subjects (2.31 [95% CI 1.95, 2.66] for case subjects versus 1.74 [95% CI 1.47-2.01] for control subjects, p=0.01).

Table 5. Mean serum measurements presented by case status.

<table>
<thead>
<tr>
<th>Mean</th>
<th>Cases (n=86)</th>
<th>Controls (n=108)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 ng/mL [95% CI]</td>
<td>180.4 [166.1-194.7]</td>
<td>204.0 [191.4-216.6]</td>
<td>0.02</td>
</tr>
<tr>
<td>IGF-2, ng/mL [95% CI]</td>
<td>953.7 [898.0-1009.5]</td>
<td>1221.7 [1150.2-1293.2]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IGFBP-1, ng/mL [95% CI]</td>
<td>13.54 [11.35-15.73]</td>
<td>14.3 [11.08-17.44]</td>
<td>0.77</td>
</tr>
<tr>
<td>log[IGFBP-1 [95% CI]</td>
<td>2.33 [2.16-2.50]</td>
<td>2.16 [1.97-2.36]</td>
<td>0.23</td>
</tr>
<tr>
<td>IGFBP-3, ng/mL [95% CI]</td>
<td>3114.1 [2937.8-3290.4]</td>
<td>3696 [3538 – 3854.1]</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Figure 4. Non-linear associations of insulin growth factors with BMI. Values were obtained from 108 GERD controls aged 19-87 years.
Multivariate logistic regression analyses showed a trend for decreased Barrett’s esophagus risk with increasing levels of IGF-1 when adjusted for age, gender and race. The most significant decrease in BE risk for observed in the third quartile of serum IGF-1 (OR=0.19, 95%CI 0.07, 0.49). Increasing levels of IGF-2 and IGFBP-3 also showed a statistically significant decrease in BE risk, with highest reduction in risk seen when top versus bottom quartiles were compared. Odds ratio for top versus bottom quartile of IGF-2 and IGFBP-3 were equal to 0.18 (95% CI 0.07, 0.51) and 0.20 (95%CI 0.07, 0.57), respectively. Logarithm of IGFBP-1 showed that the odds of BE were increased in the third quartile with respect to the bottom quartile (OR=3.47 95% CI 1.41, 8.51). However, this relationship did not hold true in comparison of top vs. bottom quartile (OR=0.82 95%CI 0.33, 2.02). Molar ratio of IGF-1 to IGFBP3 was calculated to estimate the amount of ‘bioavailable’ IGF-1. Mean molar ratio was higher in cases than controls, but this difference was not statistically significant (0.21 [95% CI 0.19, 0.22] for cases versus 0.20 [95% CI 0.19, 0.22] for controls, p=0.52). A statistically significant increase in BE risk was observed in analysis of serum insulin adjusted for differences in age, gender and race. Highest increase in BE risk was observed in the third quartile of insulin resistance (OR=4.13, 95% CI 1.64, 10.44). Risk decreased, but remained statistically significant in the comparisons of top versus bottom quartile of serum insulin (OR=2.59, 95% CI 1.04, 6.42). Similar trend was observed in the analysis of HOMA-IR. Risk of BE was highest in the third quartile (OR 3.20, 95%CI 1.30, 7.89) but then seemed to decrease in the top quartile (OR=1.94, 95%CI 0.80, 4.71).

We also investigated the possibility of any interactions that would alter our estimates of BE risk. We found a significant interaction between BMI and white race. A five unit BMI increase in a white subject increased the risk of BE 1.59 fold compared to a similar increase of BMI in a non-white participant (95% CI 1.03, 1.64). No other statistically significant interactions between baseline covariates or those with serum factors were identified. Interaction between BMI and race
was incorporated to the remainder of multivariate models. The relationship of Barrett’s esophagus was also explored in analyses adjusted for measures of adiposity and the interaction of race and BMI. These are summarized in table 6. Overall, the observed trends were similar to those seen in analyses adjusted for age, gender and race. Increasing levels of IGF-2 and IGFBP-3 showed the most significant association with risk of BE. Associations tended to be most significant in the third quartile. Analyses of IGF-1 were further adjusted for serum levels of IGFBP-3 as well as all other relevant risk factors. Mutual adjustment changed the actual estimates of BE risk, however, the overall trends were preserved compared to multivariate analyses presented in Table 6. Top quartile of IGF-1 showed non-significant decrease in BE risk compared to bottom quartile (OR=0.92, 95%CI 0.29, 2.83). High levels of IGFBP-3 were associated with decreased BE risk (OR=0.15, 95%CI 0.04, 0.51) even after adjusting for serum levels of IGF-1.

**Immunohistochemistry results**

Optimized immunohistochemistry protocol was applied to 86 paraffin imbedded sections of Barrett’s esophagus. Staining intensity in the areas of special intestinal metaplasia was compared relative to surrounding normal esophageal tissue. Staining for ki-67 was predominantly nuclear, whereas phosphorylated m-TOR, p-Akt and p-IRS antibodies displayed predominantly cytoplasmic staining pattern. Staining intensity was ranked as strong/intense vs. weak by an expert pathologist who was blinded to anthropometric measurements and serum results. Of the 86 Barrett’s cases analyzed in this study, 31 (36%) were considered to have intense staining for pIRS-1. Intense staining of p-mTOR was also observed in 33/86 (38%) of cases. Most cases stained only weakly for p-Akt (58%). Phosphorylated ki-67 was explored as a marker of proliferation; 46% of cases showed an intense reaction with this antibody. High levels of serum insulin or HOMA-IR did not correlate with intense staining of pIRS-1 \(r=-0.01 \) (\(p=0.93\)) and \(r=-0.04 \) (\(p=0.71\),
respectively). Tissues derived from obese persons (BMI>30) did not tend to show intense staining of pIRS-1 ($p_{trend}=0.61$). High serum levels of IGF-1, IGF-2, and IGFBP-3 did not correlate with intense immunohistostaining of pIRS-1 ($p_{trend}>0.20$ for all tested correlations). Intense staining of pIRS-1 correlated moderately with intense staining of p-mTOR, p-Akt, and p-ki-67 (all $p$ values <0.001). Interestingly, subjects in the highest quartiles of serum insulin were more likely to show intense staining for p-mTOR ($p_{trend}=0.13$).

**Discussion**

In our analysis of 86 cases of BE and 108 GERD controls, we found that high serum levels of IGF-2 and IGFBP-3 were associated with decreased risk of Barrett’s esophagus. High serum insulin and HOMA-IR were associated with increased BE risk, however, this association was somewhat attenuated after adjustment for gender, race, and measures of adiposity. Observed associations were most significant in the third quartile of serum measurements. These findings support the proposed ‘insulin- cancer’ hypothesis. In contradiction, increasing levels of serum IGF-1 were associated with decreased BE risk and unbound IGF-1, as assessed by molar ratio of IGF-1 and IGFBP-3, was not significantly associated with case status.

Postulating a relationship between obesity and IGF-1 is reasonable given that obesity stimulates growth hormone secretion, which is the major determinant of serum concentrations of IGF-1. IGF-1 is also important in tumorigenesis due to its role in the regulation of differentiation, cell size, and organization of the cellular cytoskeleton. Activation of the insulin receptor triggers intracellular signaling cascades in the extracellular signal regulated kinase (erk) and phosphotidylinositol 3-kinase (PI3K) pathways, making insulin signaling both mitogenic and anti-apoptotic. Our finding regarding the lack of association between IGF-1 and obesity associated
Table 6. Estimates of BE risk from multivariate analysis.

<table>
<thead>
<tr>
<th></th>
<th>Quartiles</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1†</td>
<td>2</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>&lt;3.9</td>
<td>4-8.39</td>
</tr>
<tr>
<td>Case/control OR (95% CI)</td>
<td>12/36</td>
<td>19/30</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.26(0.47, 3.37)</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>&lt;0.82</td>
<td>0.83-1.72</td>
</tr>
<tr>
<td>Case/control OR (95% CI)</td>
<td>15/33</td>
<td>14/34</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.52(0.19, 1.42)</td>
</tr>
<tr>
<td><strong>IGF-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>&lt;153</td>
<td>154-182</td>
</tr>
<tr>
<td>Case/control OR (95% CI)</td>
<td>28/19</td>
<td>29/21</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.88(0.35, 2.17)</td>
</tr>
<tr>
<td><strong>IGF-2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>&lt;887</td>
<td>888-1058</td>
</tr>
<tr>
<td>Case/control OR (95% CI)</td>
<td>29/19</td>
<td>35/14</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.64(0.60, 4.48)</td>
</tr>
<tr>
<td><strong>Log(IGFBP-1)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>&lt;1.67</td>
<td>1.67-2.39</td>
</tr>
<tr>
<td>Case/control OR (95% CI)</td>
<td>17/31</td>
<td>17/32</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.07(0.40, 2.87)</td>
</tr>
<tr>
<td><strong>IGFBP-3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>&lt;2975</td>
<td>2975-3409</td>
</tr>
<tr>
<td>Case/control OR (95% CI)</td>
<td>30/18</td>
<td>31/18</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.31(0.50, 3.48)</td>
</tr>
<tr>
<td><strong>IGF1/IGFBP3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>molar ratio</td>
<td>&lt;0.165</td>
<td>0.166-0.203</td>
</tr>
<tr>
<td>Case/control OR (95% CI)</td>
<td>18/29</td>
<td>35/33</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.75(0.75, 4.10)</td>
</tr>
</tbody>
</table>

*Conditional logistic regression analyses were adjusted for age, gender, race, WHR, BMI and the interaction between gender and BMI. CI=Confidence interval. †Reference category. ‡All p values are two-sided.

condition such as Barrett’s esophagus is similar to other studies that have also reported on the lack of association between IGF-1, measures of adiposity and cancer. In a case-control study of colorectal cancer risk, Kaaks et al39 showed that high serum C-peptide but not IGF-1 was
associated with increased colorectal cancer risk. High serum C-peptide increased colorectal cancer risk approximately 3-fold (RR=2.92, 95%CI 1.26, 6.75) but high IGF-1 resulted in non-significant increase in colorectal cancer risk (RR=1.88, 95%CI 0.72, 4.91). Results of Kaaks’ study as well as our results differ from those obtained by the prospective assessment of colorectal cancer risk in the Physician’s Health Study. High serum levels of IGF-1 were associated with increased risk of colorectal cancer (RR=2.51, 95%CI 1.15, 5.46, p=0.02), even after controlling for serum levels of IGFBP-3. Differences in the estimates of colorectal risk due to increased IGF-1 are somewhat interesting given similarity of study design between Kaaks’ and Physician’s Health study. One should not forget the important differences among these studies. Physicians’ Health Study included only men in the age range of 40-84 years. Rudolf Kaaks’ study utilized case-control design nested in a cohort of younger women aged 30-65 years. Men are known to have higher levels of IGF-1 and these levels further vary with age. Could these discordant findings be explained by baseline differences between study populations as well as complex interactions between age, gender, and IGF-1?

It is also not entirely clear why insulin, but not IGF-1, would result in increased Barrett’s and colorectal cancer risk. Growth hormone is the key regulator of IGF-1 synthesis. Insulin enhances IGF-1 synthesis by stimulating the number of growth hormone receptors. Insulin also increases IGF-1 bioavailability by inhibiting the synthesis of insulin growth factor binding proteins. Obesity generally stimulates growth hormone synthesis, but it can also lead to growth hormone inhibition and subsequent decrease in IGF-1 levels. Our observation of non-linear association between IGF-1 and BMI reflected the presence of this negative feedback mechanism due to excess weight. IGF-1 levels peaked in the BMI range of 22-24kg/m² and subsequently declined. This was in sharp contrast to insulin, which maintained linear associations with BMI. A 5 unit increase in
BMI was associated with 7mIU increase in serum insulin. The fact that obesity was associated with decreased serum concentrations of IGF-1 but increased serum insulin levels could perhaps explain why serum insulin was a stronger risk factor for esophageal metaplasia compared to serum IGF-1.

Current literature also debates whether it is hyperinsulinemia or insulin resistance that actually leads to carcinogenesis. Hyperinsulinemia is common in early insulin resistance, whereas late disease is characterized by hypoinsulinemia reflective of beta cell failure. Postprandial insulin and C-peptide measurements which are reflective of beta cell function have been associated with increased colon cancer risk. In a study by Yang et al (2004) chronic insulin therapy was associated with significantly increased risk of CRC with 21% incremental risk with each year of exogenous insulin administration. In a rat model of colon cancer utilizing 10-h euglycemic clamp, insulin increased epithelial cell proliferation in a dose dependent manner, however, the addition of hyperglycemia did not further increase proliferation. Based on these results the authors proposed that hyperinsulinemia, rather than insulin resistance, is the key component of increasing cellular proliferation. Underlying mechanism is unknown and could perhaps be mediated through IGF-1. Epidemiologic and basic science evidence points toward hyperinsulinemia rather than insulin resistance as a risk factor for neoplasia, however, more studies are needed for better understanding. Our study considered fasting insulin and HOMA-IR in relation to BE risk simultaneously. Fasting insulin is still reflective of insulin resistance and therefore we were unable to satisfactorily delineate the relative contribution of each individual factor to risk of Barrett’s esophagus.

This study utilized staining of the p-IRS1, rather than insulin or IGF-1 receptors. This decision was based on our preliminary studies which showed nonspecific activation of the insulin/IGF-1 receptor by EGF and platelet derived growth factor (PDGF). This non-specificity of
the anti-IR and anti-IGF-1R antibodies was further confirmed by Western blot analysis. Activation of the p-IRS1 is an important stepping stone to activation of mTOR and Akt both of which participate in pathways important to cellular proliferation. All samples with areas of special intestinal neoplasia showed a positive immunohistochemical reaction with pIRS1, yet only 36% of samples showed intense staining of the pIRS. Immunohistochemical staining specificity of the anti-IR and anti-IGF1R antibody has been shown in cell culture models of prostate cancer, however, it remains to be shown in tissue biopsy samples. Looking at activation of the p-IRS1 therefore represents a very reasonable surrogate measure of activation of insulin signaling pathway intracellularly.

We did not observe a significant correlation of intense staining of pIRS-1 and high serum insulin levels. This could have occurred due to difficulties with assessing insulin availability at the tissue level. Interstitial insulin levels correlate with serum insulin levels but are significantly lower. Interstitial insulin levels have been measured in research settings, but even those measurements do not represent insulin levels in the tissues themselves. Moreover, responsiveness to equivalent levels of interstitial insulin differs between lean and obese subjects, suggesting that the actions of insulin are dictated by the complex milieu of molecular changes that accompany obesity. Tissue levels of the IGF binding proteins are most likely varied as well. The lack of correlation between serum markers and pIRS activation does not refute their role in insulin signaling, it simply highlights our inability to accurately estimate tissue activity of these growth factors in living organisms.

The Insulin – IGF molecular pathway links excess weight with cancer in a way that is plausible and easily understood. However, this approach may be oversimplified. The simple version of the ‘insulin-cancer’ hypothesis should rather be viewed in the larger context of
molecular pathways that govern carcinogenesis overall. These systems are not mutually exclusive; interactions between IGF-1 and other growth factor pathways have been previously described. Data from breast cancer cell lines suggests that combined treatment of breast cancer cell lines with IGF-1 and leptin enhances cellular proliferation and that combined treatment with these factors synergistically induced epidermal growth factor receptor (EGFR) activation. A study by Carvalheira et al (2005) addressing metabolic aspects of insulin signaling showed that insulin alone produced molecular activation of pIRS/PI 3-kinase/Akt pathway, however, insulin in combination with leptin activated pIRS/PI 3-kinase signaling with no effect on Akt. Leptin and insulin signaling therefore converged at the level of pIRS/PI 3-kinase but diverged at the level of Akt. Combined treatment with leptin and insulin also led to quantitative potentialization of molecular signaling through the Janus tyrosine kinase protein (JAK) family. JAK protein family is required for optimal activation of the Src-kinase cascade, the Ras-MAP kinase pathway, PI3K-AKT pathway and STAT signaling. Aberrations in JAK kinase activity that lead to alterations of above pathways could cause irregularities in proliferation, differentiation and ultimately lead to tumorigenesis. Above studies highlight the complex interactions of insulin signaling that need to be integrated into future studies of esophageal adenocarcinoma. Insulin signaling should be examined in the broader context of other growth factor pathways.

There are several limitations to this study that need to be acknowledged. The results presented here are preliminary as we did not reach the projected sample size of 120 BE cases and 120 GERD controls. This sample size was calculated based on expected exposure frequencies of 0.3 and our interest in identifying factors that increase BE risk two to four fold. We were unable to provide adequate matching of cases and controls raising the possibility that residual differences between the two study groups affected our estimates of BE risk. Our serum measurements of
insulin had intra- and inter-assay coefficients of variation larger than quoted in other studies. This may have contributed to increased variation in laboratory factors and could have provided further confounding. Further, levels of insulin growth factors were not measured prospectively. The associations of IGFBP-3 and BE risk observed in our study were so significant and consistent with results of prospective studies, that we do not believe that a prospective design would significantly alter our conclusions.

Overall, we have seen that increasing levels of insulin increased BE risk, whereas increasing serum levels of IGF-2 and IGFBP3 were associated with decreased BE risk. The clinical implications and the relevance of this finding are unclear. A major drawback of our study is that we have failed to provide evidence that would support the link between circulating levels of IGFs and insulin receptor mediated activation of downstream proliferative factors at the tissue level. Future, larger studies, should explore the relationship of circulating IGFs with tissue activation of IGF-1R/pIRS across the spectrum of esophageal carcinogenesis. If this pathway is indeed active, progression of esophageal cancer could be slowed by interventions aimed at reducing obesity and insulin resistance, as well as pharmacological strategies that reduce IGF-1R signaling through IGF1R antibodies or IGF-1R tyrosine kinase inhibitors. Addition of these interventions and drugs to the armamentarium of therapies for esophageal cancer could better the prognosis of patients diagnosed with this deadly disease.
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