Effects of excessive energy intake from fructose- vs. high fructose corn syrup (HFCS)- vs. glucose-sweetened beverages on fasting plasma IGF-1 and IGFBP-3 concentrations

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Abstract

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Background: Epidemiological studies indicate that added sugar consumption and plasma concentrations of insulin-like growth factor (IGF-1) are associated with increased cancer risk, particularly for breast and colorectal cancers, but data are less clear for many other cancers (references). Insulin, which stimulates the production and release of IGF-1, is released post-prandially after glucose consumption, but not after fructose consumption. The objective of this study was to investigate whether known differential effects of glucose vs. fructose are linked to differential effects on fasting plasma IGF-1 and IGFBP-3 concentrations.

Methods: In this randomized, crossover, double-blind, controlled feeding study, twenty-four healthy, normal weight to obese men and women were fed identical diets in three eight-day diet phases separated by 20-day washout periods. The only difference between the dietary phases was that subjects consumed beverages sweetened with a different sugar (fructose vs. high-fructose corn syrup vs. glucose) in each phase. Beverages were administered in four servings totaling 25% of each

subject's total estimated energy requirement, and participants were required to drink all of each beverage each day. Solid-foods were also provided, at 125% of estimated energy requirement, and were consumed *ad libitum*. Fasting plasma samples were drawn at the end of each diet phase and tested for concentrations of IGF-1 and IGFBP-3. Our primary outcome measure was the ratio of IGF-1/IGFBP-3. Repeated measures analysis of variance (RM-ANOVA) was used to test for a linear trend in diet effect, as well as for any (non-linear) diet effect.

Results: We observed no significant difference in total energy intake or body weight between the three diet phases. We did not find any differential effect based on diet phase, either linear or non-linear, on any of our outcome measures.

Conclusion: In healthy normal weight to obese men and women, different types of added sugars consumed in the form of sweetened beverages over 8 days each did not differentially affect fasting plasma IGF-1 plasma concentrations.

Background

1. Cancer and Diet

Cancer in the United States

Cancer affects millions of people in the United States and around the world. There are more than 100 types of cancer; the most common are cancers of the breast, prostate, lung, and colorectum. [1, 2] In 2011, there were an estimated 13.4 million people living with cancer in the United States. [3] Based on national data, it is estimated that 460 out of every 100,000 Americans develop cancer each year, and that 174 of every 100,00 Americans die of cancer each year. [3]

The hallmarks of cancer

Biological research has established how cancer develops at a cellular level. Cancerous cell development involves genetic mutations that both enable the growth of cancer cells and disable healthy cells' defense mechanism against this growth. For tumorigenesis to occur, cells must develop several essential traits described in a 2000 review in *Cell* by Hanahan and Weinberg: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these essential traits breaks down a different cellular defense mechanism against cancerous growth. [1,4]

Self-sufficiency in growth signals. For healthy cells to become tumor cells they must reduce their dependence on exogenous growth signals for proliferation. [1,4] They do this in different ways: by producing their own growth factor, overexpressing growth factor receptors, expressing different receptors, altering downstream signal processing, or inducing neighboring cells to release growth signals. This transformation disrupts the cell's homeostatic regulation of growth.[1,4]

Insensitivity to antiproliferative signals. To become cancerous, cells must evade the antigrowth signals that block proliferation in healthy cells. These antigrowth signals operate by forcing cells into a quiescent, or temporarily non-proliferative, state, or by forcing them into a permanent postmitotic state. [1] Normal cells respond to antigrowth signals through the circuitry of the cell division cycle clock, processing the signals through the retinoblastoma protein (pRb). [4,5] In cancerous cells, pRb is disrupted, which alters transcription factors in such a way that the cell is no longer influenced by antigrowth signals. The pRb signaling circuit can be disrupted in different ways, including downregulation of important receptors, mutation of receptors, elimination of transduction proteins through mutation, deletion of genes, inactivation of pRb by hyperphosphorylation, or mutation of the pRb gene. This results in the loss of a tumor suppressing mechanism.[1,4,5]

Evading apoptosis. Tumorous growth depends on cells' ability to resist normal attrition through programmed cell death. [1,4,6,7] Apoptosis in normal cells is

initiated when cellular sensors monitoring the environment indicate the need for programmed death, and effectors then induce death rather than survival. [6,7] Death signals, or pro-apoptotic signals, are conveyed by the FAS ligand, TNF-alpha, the tumor suppressor protein p53, and other tumor suppressors. [4,8] In response to pro-apoptotic signals, the mitochondria release cytochrome C, an apoptosis catalyst, and proteases called caspases carry out apoptosis. [4,6,7] Apoptosis can be disrupted through action by exogenous survival factors such as IGF-1, overexpression of proteins with an anti-apoptotic function such as Bcl-2, and disruption of FAS death signals. [4,6] Cancerous cells acquire traits that allow them to disrupt apoptosis: loss of the p53 or pTEN tumor suppressors, stimulation of survival signaling by IGF-1, IGF-2 or IL-3 or by intracellular signals from Ras, and elimination of the FAS death signal through upregulation of a decoy.[1,7,8]

Limitless replicative potential. Healthy cells have a finite capacity to replicate, and cancerous cells acquire the capacity for unlimited replication. [1] After a certain number of divisions, they reach a state of senescence, in which they are viable but stop proliferating. [4,9] If cells are able to circumvent the senescence barrier, they enter a crisis state, which leads to apoptosis. [4] These barriers to limitless replication are present because with each division of healthy cells, telomeres, protectors on the ends of chromosomes, lose some of their DNA, getting shorter with each cycle. [4,9,10] When the ends of chromosomes are unprotected, they fuse together at their ends, leading to cell death. [4,9,10] For cells to become cancerous they must continue multiplying, and thus must avoid or move beyond both the senescence and crisis states. Cancerous cells acquire the ability to maintain telomere length, in most cases by upregulating the enzyme telomerase, and this allows unlimited replication.[1,4,10]

Sustained angiogenesis. Malignant cells acquire the capacity to promote the growth of blood vessels to supply them with oxygen and nutrients. [1,4] Angiogenesis is normally closely regulated by a complex homeostatic system, but this process is dysregulated when cells become cancerous. [4,11] This dysregulation helps cells to grow explosively and invasively. In healthy tissue, pro- and anti-angiogenic signals are present. [12] Tumors acquire the capacity to promote sustained angiogenesis by shifting the balance of angiogenesis inhibitors and inducers, altering gene transcription to downregulate inhibitors and upregulate inducers.[1,4,12]

Tissue invasion and metastasis. Once they have achieved the first five essential cancerous traits, malignant cells acquire the ability to escape their original locations and to colonize new tissues and organs. [1,4] This requires the disruption of the structures through which cells are attached to their environments, either in cell-cell adhesion or in integrin-created links between cells and the extracellular matrix. These links between healthy cells and their environments provide routes for transmission of regulatory signals. E-cadherin bridges between cells transmit antigrowth signals, and this helps inhibit invasion and metastasis. [4,13,14] These links are disrupted in several ways by cancerous cells. In tumorous growths, CAMS (cell-cell adhesion molecules) become less adhesive, and sometimes repulsive. [1] Integrins are altered to help cancer cells adapt to new microenvironments on their metastatic

journeys.[1] Extracellular protease genes are upregulated and protease inhibitors are downregulated, allowing proteases on the surface of cells to degrade cadherins and pave the way for invasion.[1,4,13,14]

The hallmark capabilities of cancerous cells enumerated by Hanahan and Weinberg are acquired through multiple genetic mutations, each of which is rare. While humans frequently have multiple genetic mutations, they also have efficient genome maintenance and repair systems, so each cell generation's mutation rates are low.[4,15] It has been proposed that the process of tumorigenesis is aided by increased rates of mutation in cancerous cells.[4,15,16] This may be accomplished by increased sensitivity to environmental and endogenous mutagens, and by disabling of genetic surveillance, maintenance and repair systems, including the p53 tumor suppressor protein and other proteins involved in tumor suppression.[4,15,16] Genetic instability helps premalignant cells acquire the hallmark traits of cancer described above.[1,15] These hallmark cancer traits are also fostered by inflammation, which produces abundant growth factors, survival factors, pro-angiogenic factors, and enzymes that modify the extracellular matrix and enable invasion and metastasis.[4,17,18]

Further research has identified additional hallmark traits necessary for tumorigenesis to occur. In a 2011 update to their review, Hanahan and Weinberg add two additional possibilities for emerging traits: reprogramming of energy metabolism to support continuous cell growth and proliferation and evasion of immune destruction.[4] Other researchers have suggested that changes in the "whole organism energy balance," such as caloric excess or caloric restriction, are connected to changes at the level of cellular metabolism.[19]

Not only do cancerous cells acquire the hallmark traits described above, but normal cells facilitate their development when they are enlisted to contribute to the "tumor microenvironment."[4] This tumor microenvironment has been described as similar in complexity to an organ.[4,20] It has been suggested that tumor initiation may be unavoidable, due to the harmful mutations caused by radiation, oxidative damage, and other factors, but that tumor progression is in many cases alterable.[20] Tissue homeostasis systems protect healthy cells from tumorigenic agents, thereby restraining tumorous growth and allowing most people to live their lives cancer-free.[4,5,7,8,14,20,21] However, when changes in the tissue microenvironment accumulate and are sufficient to cause a breakdown in these homeostatic tissue maintenance systems, the balance is shifted from an anti-cancerous to a pro-cancerous state, and tumor suppressors can be inactivated, allowing tumors to progress.[4,20]

Cancer risk factors and prevention

Epidemiological studies have established associations between lifestyle and environmental factors and many forms of cancer.[22] These factors include cigarette smoking and tobacco use, infections, radiation, immunosuppressive medicine, diet, alcohol, lack of physical activity, obesity, and environmental exposures such as air

pollution, secondhand tobacco smoke, asbestos, arsenic, and pesticides. Specific genetic variants are also risk factors for cancer.[23]

The three most important ways that people can decrease their risk of cancer are avoiding tobacco use, engaging in physical activity and following a healthy dietary pattern. [22] Additionally, high body mass index (BMI) has been associated with cancer risk. [24-27] Evidence indicates that consuming fruits, vegetables and dietary fiber may be protective against many types of cancer. [24,28-32] Epidemiological studies have shown that consumption of large amounts of both red meat and processed meat are associated with increased risk for colon cancer. [24,33,34] Some studies have linked consumption of n-6 polyunsaturated fatty acids with increased cancer risk and consumption of n-3 polyunsaturated fatty acids with decreased cancer risk. [35-38] Many studies have also shown associations between consumption of foods containing specific micronutrients and phytochemicals – including vitamins A, C, E, D, calcium, carotenoids, lycopene and resveratrol -- and decreased cancer risk or better cancer prognosis. [24,25,30,39]

Another dietary factor with putative links to cancer that has been a topic of investigation is added sugar. Evidence indicates that intake of refined grains and sugars is associated with increased cancer risk[40,41] and that dietary sugars may aid tumorigenesis. [42] It is possible that some of the evidence linking sugar and cancer is confounded by other factors, such as low vegetable consumption or low physical activity among individuals who consume high amounts of sugar.

Some studies of the association between sugar intake and cancer risk do take note of and control for relevant potential confounders. In a 2006 prospective, population-based cohort study, Larsson and colleagues found an association between added sugar consumption and pancreatic cancer risk. [40] The study population was composed of 77,797 Swedish men and women with no previous cancer or diabetes diagnoses, who were followed for 7.2 years. [40] The researchers identified 131 incident cases of pancreatic cancer. After adjusting for age, sex, education, smoking, BMI, alcohol, and total energy intake, they found that subjects in the highest tertile of consumption of added sugar had a risk of pancreatic cancer 1.69 times greater than the risk born by participants in the lowest tertile of added sugar consumption (95% CI 0.99 – 2.89). [40] The association was stronger when cases diagnosed during the first two years of follow-up were removed, and the researchers hypothesize that these cases may have resulted from preclinical conditions not identified during screening. In this secondary analysis, subjects in the highest tertile of added sugar consumption had a risk of pancreatic cancer 1.95 times greater than that of the subjects in the lowest tertile of added sugar consumption (95% CI 1.10 – 3.46). [40] Larsson and colleagues adjusted for several potential dietary and lifestyle confounding factors, but these adjustments did not change the calculated risk, and thus they did not include these variables in their published models. [40]

In some studies investigating the association between added sugar intake and cancer risk, the statistical models do not reflect adjustment for potential dietary

confounders such as whole fruit and vegetable consumption. One such study is a 2004 population-based, case-control study in Mexico City investigating the association between carbohydrate intake and breast cancer risk. [41] In this study, composed of 475 cases and 1391 controls, Romieu and colleagues found that for those subjects in the highest quartile of sucrose consumption, compared to those in the lowest quartile, the risk of breast cancer risk was 2.0 times higher (95% CI 1.47 – 2.71). For those in the highest quartile of fructose consumption, compared to those in the lowest quartile, the risk of breast cancer was 1.36 times higher (95% CI 1.00 – 1.86). These risk estimates were calculated after adjustment for total energy intake and potential confounding factors including socioeconomic status, age, age at first birth, and family history of breast cancer. [41] While this study did take insoluble fiber into account, the model did not adjust for several other potential dietary and lifestyle confounders.

Given the mixed epidemiological evidence, it is plausible but not irrefutable that dietary added sugar is associated with cancer. The present study contributes towards a better understanding of one potential mechanism that may link sugar intake and cancer risk. Specifically, our study investigates the effect of different sugars and levels of a cancer risk biomarker that has been implicated in the cancerous process in cancers of the breast, prostate and colon.

2. IGF-1

IGF-I and cancer

Insulin-like growth factor-1 (IGF-1) is a growth hormone implicated in cancerous development. High plasma levels of IGF-1 have been associated with increased risk of several different types of cancer, including cancers of the prostate, colon and breast, [19,24,43-48] and with increased cancer-related mortality. [49] However, some epidemiological studies have found no association between IGF-1 and increased risk of colorectal or breast cancer.[44,45,50]As with the purported association between sugar intake and cancer epidemiological, the evidence suggests that the association between IGF-1 serum concentrations is plausible but confounding cannot be entirely ruled out.

A case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) with 630 cases and 630 controls from 10 European countries found that serum IGF-1 concentrations were weakly positively associated with overall prostate cancer risk, and that the association was somewhat stronger for advanced stage prostate cancer. [24,51] The results were only significant for IGF-1 after adjusting for IGFBP-3, and only for overall risk of disease. The overall prostate cancer risk for the highest vs. the lowest tertile of IGF-1 serum concentrations was 1.35 (95% CI 0.99 – 1.82), and 1.39 (95% CI 1.02 - 1.89) after adjustment for IGFBP-3. The risk of advanced stage prostate cancer for the highest vs. the lowest tertile of IGF-1 serum concentrations was 1.65 (95% CI 0.88 – 3.08). The authors suggest that

the stronger effect size for the association with the risk of advanced stage cancer indicate that IGF-1 serum concentrations may be associated with tumor aggressiveness. [24,51] Because IGFBP-3 regulates IGF-1's bioavailability, [19,44,52] it is plausible that accounting for IGFBP-3 plasma concentration strengthens any association. The researchers in the EPIC study adjusted for several potential lifestyle confounders, and none altered the risk assessment. The analysis would be strengthened by testing for potential confounding by intake of specific foods such as fruits and vegetables. [24,51]

Other studies present slightly stronger evidence for the association between IGF-1 plasma concentrations and cancer risk. In a 2010 study, the Endogenous Hormones and Breast Cancer Collaborative Group analyzed pooled data from 17 prospective studies in 12 countries, to increase the precision of estimated associations of IGF-1 with breast cancer risk. [46] The study was comprised of 4790 cases and 9428 controls. The estimated OR for breast cancer risk in the highest quintile vs. the lowest quintile of IGF-1 plasma concentrations was 1.28 (95% CI 1.14 – 1.44) and was not altered by adjusting for IGFBP-3 plasma concentrations. The association was significant for both premenopausal and postmenopausal women. [46] The researchers adjusted for various potential lifestyle confounders one at a time. None of these adjustments changed the odds ratio by more than 2%, and in all cases it remained significant. [46] This evidence for the association between IGF-1 plasma concentrations and breast cancer risk is compelling. However, as in the EPIC study described above, the analysis would be strengthened by investigating potential confounding by intake of other foods associated with cancer risk, such as fruits and vegetables.

Studies of some hormonal disorders that affect IGF-1 plasma concentrations have contributed support for the potential link between IGF-1 and cancer. Acromegaly, a hormonal disorder leading to elevated levels of growth hormone (GH) and IGF-I, increases the risk of cancer-related mortality. [53,54] Growth hormone receptor deficiency (GHRD) or Laron syndrome, which leads to low IGF-1 levels, is associated with lower cancer risk. [55,56] Animal models have also shown that lower IGF-I levels lead to diminished tumor growth. [26,57-59]

IGF signaling may also be connected to dietary factors that influence cancer risk. For example, changes in whole organism energy balance such as caloric excess or caloric restriction may affect cellular metabolism by changing concentrations of circulating IGF-I. [19]

Identification of the role of IGF-1 in tumor development has led to the development of cancer therapies targeting the IGF-1 receptor signaling pathway. [19,43] The connection between IGF-1 and cancer also makes IGF-1 a cancer risk biomarker, and a useful intermediate endpoint for short- or medium-term studies in which cancer incidence is not a feasible endpoint.

Basic biology of IGF-1

IGF-1 is produced primarily in the liver in response to stimulation by growth hormone (GH). It is also stimulated by insulin, through insulin's upregulation of GH receptors. [54] IGF-1 travels in the blood bound to the binding protein IGFBP-3, which regulates its bioavailability. [19,44,60] This binding protein regulates IGF-1's bioactivity by limiting its access to its receptor, IGF-1R. [19] When IGF-1 binds to its receptor, the downstream intracellular signaling networks Akt and MAPK are initiated. [19] IGF-1 and its signaling system contribute to the dysregulation of cellular homeostasis that enables tumorigenesis by inhibiting apoptosis and stimulating proliferation.[1,19,27,43,59,61]

IGF-1 has many different effects in the body related to regulation of energy metabolism and growth. However, it appears that the IGF-1 signaling system, which evolved millions of years ago and predates vertebrates, has played a role in other functions, such as regulating cell longevity as a function of nutrient availability.[19,62]In the presence of nutrients, signaling pathways activated by IGF-1 favor protein synthesis and proliferation, while in the presence of nutrient deprivation, the activity of these signaling pathways is reduced, causing an anti-proliferative effect. Hormonal signals, in addition to nutrient depletion, contribute to this effect. [19,57,63,64]

IGF-1 may lead to increased risk of cancer either by initiating early carcinogenesis or by favoring rapid proliferation of existing but clinically undetectable cancerous cells. IGF-1's activity in the cell microenvironment may be an essential component in tipping the apoptosis/survival balance towards survival. IGF-1R activation initiates signaling pathways that favor survival, and in a "high-IGF" environment, survival becomes a more predominant cellular fate. [19]

Inhibition of the FOXO pathway

Both insulin and IGF-1 inhibit the FOXO family of transcription factors, and this inhibition constitutes one possible mechanism by which IGF-1 may contribute to tumorigenesis, as FOXO proteins play a tumor-suppressor role. [65-68] The name "FOXO" is derived from the category "forkhead box proteins," which are present in all eukaryotes and are characterized by a conserved DNA-binding domain. This group contains 19 subclasses ending with different letters of the alphabet. The "O" subclass, known as FOXO, contains the primary FOX transcription factors found in mammals. [65,68,69]

FOXO proteins translate extracellular signals into activation of several target genes that affect numerous cellular functions including cell proliferation, transformation, and differentiation. Activity of FOXO transcription factors is also linked to longevity. The extracellular signals that affect FOXO proteins are initiated by insulin, growth factors such as IGF-1, cytokines, and oxidative stress. In response to these signals, FOXO proteins undergo post-translational modifications including phosphorylation, acetylation, ubiquitination, glycosylation, and methylation. These modifications regulate FOXO proteins by altering their levels, their subcellular location, their DNA binding activity, and their transcriptional activity.[65,69]

Among the signaling cascades that phosphorylate FOXO proteins are AKT and MAPK, both of which are initiated when IGF-1 binds to its receptor. [65,70] FOXO serves as a substrate for these enzymes, and phosphorylation changes FOXO's structure, making it possible for the chaperone protein 14-3-3 to bind to it and thereby regulate it. This binding by a chaperone protein has the effect of increasing the export of FOXO proteins from the nucleus, decreasing their entry into the nucleus, and decreasing their DNA-binding activity through conformational changes. Phosphorylation of FOXO proteins by AKT is also an important step in making it possible for them to undergo ubiquitination, a post-translational modification that leads to degradation through the ubiquitin-proteasome pathway. [65,67,69,70] These causal chains indicate that signaling cascades initiated by IGF-1 inhibit FOXO transcription activity.

In the absence of inhibitory signals such as those initiated by insulin and IGF-1, FOXO proteins move into the nucleus and up-regulate genes that promote stress resistance, cell cycle arrest, apoptosis, DNA repair, and inhibition of angiogenesis. [68,69,71,72] Given their role in these cell processes, FOXO proteins are considered tumor suppressors. Many types of evidence provide support for this understanding. Anti-oxidant molecules that inhibit the AKT pathway, including sulforaphane, resveratrol and epigallocatechin gallate, enhance FOXO transcription actions promoting apoptosis and cell cycle arrest. [65] In mouse studies, acute deletion of FOXO induced over-proliferation of endothelial cells and premature death. [65] Deregulation and chromosomal deletion of FOXO proteins have been found in leukemia and in tumors of the breast and prostate. Low levels of FOXO proteins are associated with Hodgkin lymphomas, and ectopic expression of FOXO proteins in Hodgkin lymphoma culture cells has reduced cell proliferation and promoted cell cycle arrest. Increased levels of FOXO in the nucleus have been negatively associated with tumor size. [65] Down-regulation of FOXO is associated with poor prognosis and chemotherapy resistance in patients with liver cancer. [65]

As evidence linking FOXO transcription factors and tumor suppression has accumulated, researchers have begun attempting to develop cancer therapies that work by increasing or restoring FOXO activity. Under consideration are drugs that block pathways involved in exporting FOXO proteins from the nucleus and into the cytoplasm, where it is degraded. One drug being developed would act as a specific inhibitor of the AKT pathway.[65]

In addition to suppressing tumor growth, FOXO transcription factors also coordinate the adaptive response to stress, a capability associated with lifespan extension. [62,68,70] Extracellular stress signals, such as those communicating that nutrient intake is low, trigger the movement of FOXO proteins into the nucleus, where they promote an adaptive response via changes in gene expression. This includes up-regulating genes involved in DNA repair or free radical scavenging enzymes such as Mn superoxide dismutase (MnSOD) and catalase. [69] Studies in worms and flies have shown that activation of FOXO proteins increases longevity. When mutations are created on the worm FOXO orthologue DAF-16, longevity

decreases.[68,69,73] These properties have also been observed in some mammalian FOXO proteins. By contrast, mice with decreased expression of insulin receptors and IGF-1 receptors have longer lifespans and are more resistant to oxidative stress.[69]

As dietary restriction, by reducing circulating concentrations of insulin and IGF-1, will keep FOXO factors active, and thus help the cell defend against stressors such as lipid or oxygen radicals, FOXO and IGF-1 signaling may constitute a possible explanation for the association between dietary restriction and increased lifespan that has been observed in many different species.[62] FOXO's influence on longevity may also be tied to its tumor-suppressor role. Cancer onset is age-dependent, and it has been suggested that FOXO factors may constitute a molecular link between longevity and tumorigenesis.[65,69]

Taken together, the FOXO family of transcription factors plays crucial roles in the maintenance of cellular health by coordinating normal cell function, the defense against external stressors, DNA repair, and apoptosis. Activation of FOXO transcription factors is strongly linked to longevity. Thus, the inhibition of FOXO transcription factors may be one mechanism by which IGF-1 promotes tumorigenesis.

3. Insulin

Insulin, a hormone produced and released by the pancreas in response to a rise in blood glucose, plays a major role in glucose homeostasis. Insulin stimulates glucose uptake by the body's cells and the synthesis of triglycerides, glycogen, and protein. Insulin also regulates food intake, and is considered one of the two main adiposity signals (with leptin) that regulate body fat mass in the long term.[54,74,75]

Insulin stimulates the production and release of IGF-1 by upregulating growth hormone receptors.[19,43] Insulin may also indirectly increase plasma levels of IGF-1 by decreasing IGFBP-3, leaving more IGF-1 unbound and thus bioavailable.[43] When insulin resistance develops, the pancreas must produce extra insulin to maintain glucose tolerance, and this leads to compensatory hyperinsulinemia. IGF-1 levels rise in response to hyperinsulinemia.[19,43,74]

Observational studies have found associations between type 2 diabetes and increased mortality from several types of cancer, including cancers of the breast, colon, pancreas, liver and bladder.[48,76-79] The increased cancer mortality may be caused by hyperglycemia, by the hyperinsulinemia that tends to proceed the development of glucose intolerance, by elevated IGF-1 levels, or by the interaction between insulin and IGF-1 signaling pathways.[43,80,81] Evidence suggests that this interaction may contribute to tumorigenesis.[43] It appears that insulin may play an independent role in promoting abnormal growth, as well as interacting with IGF-1 signaling in ways that contribute to tumor development. Low insulin levels, like low IGF-1 levels, are associated with decreased cancer incidence.[43]

Obesity is also associated with increased cancer risk and increased cancer mortality in observational studies, and this may be partly related to obesity-related insulin resistance leading to hyperinsulinemia.[19,26,27,48]

4. Effects of different carbohydrates on insulin and IGF-1 plasma concentrations

Different carbohydrates have different effects on plasma insulin, and may also have different effects on plasma IGF-1 concentrations. Glucose ingestion acutely stimulates insulin release, and plasma glucose is consequently taken up by cells in the liver or the periphery (muscle, fat tissue) and used for energy or stored. In contrast, fructose is taken up by the liver from the portal vein, and does not acutely trigger insulin release. [82-85] Human feeding studies have shown that glucose is associated with post-prandial plasma glucose- and insulin-peaks, while fructose is not. [83,86] Because glucose and fructose differ in their acute effects on insulin plasma concentrations, they may also acutely differ in their effects on plasma IGF-1 concentrations.

While glucose is hypothesized to increase IGF-1 plasma concentrations by being directly glycemic and insulinemic, fructose may increase IGF-1 by leading to insulin resistance and hyperinsulinemia. Glucose metabolism via phosphofructokinase is regulated by feedback inhibition from hepatic energy status. Metabolism of fructose via fructokinase is not similarly regulated, so fructose metabolism continues even when hepatic energy stores are high. Thus, when large amounts of fructose are consumed, more substrate is available for triglyceride production, and this production increases. Fructose is largely taken up by the liver from the portal vein, and unregulated fructose metabolism occurs in the liver. This results in increased *de novo* lipogenesis, which leads to the buildup of hepatic lipids and inhibits fatty acid oxidation. [83,84,87] Excess hepatic lipid deposition impairs hepatic insulin sensitivity, which ultimately raises hepatic glucose production, elevates insulin secretion, and may impair systemic glucose tolerance. [83] Fructose may also increase *de novo* lipogenesis by activating sterol receptor element binding protein-1c (SREBP-1c). [83] These proposed mechanisms by which fructose elevates hepatic lipid levels are biologically plausible, but there is no conclusive experimental evidence that they occur as just described. The one relevant study did not resolve the question of whether elevated hepatic lipids were due to fructose or increased energy or the combination of both.[83]

Thus, it may be that both fructose and glucose independently cause insulin and thus IGF-1 to rise, but they may do so on different time scales, with glucose acting faster than fructose in this regard. This is because glucose acts directly by elevating plasma insulin acutely, and fructose acts indirectly by leading to insulin resistance and hyperinsulinemia, a process that would take longer and that eventually may also affect plasma IGF-1 concentrations. [83,84] Both processes would

be expected to be exacerbated when glucose and fructose are consumed in caloric excess, as they were in this study.

The above explanations describe the ways in which glucose and fructose metabolism differ in their effects on insulin and possibly also IGF-1 concentrations in the short and long term. However, it is unclear what constitutes "long term" and "short term" in this context. It is not clear what duration of fructose consumption for humans will lead to elevation of IGF-1 levels (if at all), or how these levels will compare to those resulting from an equivalent duration and amount of glucose consumption. One previous study did show a change in IGF-1 levels after 7-days of a protein-based intervention,[88] but most dietary intervention studies showing changes in IGF-1 have a duration of 7 weeks or more.[89-91] Furthermore, while we know that glucose and fructose have differential effects on insulin levels in the post-prandial state, it is not clear whether those differences persist in the fasting state, particularly in response to an eight-day intervention as conducted here.

5. Public Health Impact of thesis work: Implications of increasing fructose consumption

Fructose is a growing part of the American diet, consumed in sucrose, high fructose corn syrup, and honey.[92] Fructose has also been implicated in the rise of obesity and metabolic syndrome.[83] Rodent studies have established that chronic high fructose consumption is associated with hepatic and peripheral insulin resistance, obesity, type 2 diabetes, and high blood pressure in these animals.[92] These connections are so well established with respect to animals that feeding highly fructose-enriched diets to laboratory animals has become a common technique for reliably producing metabolic disease when necessary for experiments.[83] However, these connections are less well established with respect to humans. Evidence from human studies indicates that fructose, at least when consumed in excess, as part of a hypercaloric diet, and in beverage form, is associated with several individual components contributing to obesity and metabolic syndrome, including visceral adiposity, *de novo* lipogenesis, dyslipidemia, and reduced insulin sensitivity, thus possibly increasing the risk for type 2 diabetes and cardiovascular disease.[83,84,92-94] However, these adverse metabolic consequences have not resulted in most studies in which fructose is consumed in solid form, as part of a eucaloric diet.[95-101] Some studies also suggest that fructose-sweetened beverages may not have adverse consequences for healthy, normal weight individuals, but may exacerbate adverse metabolic conditions in obese individuals.[85]

In studies of humans, fructose has also been found to alter neurological satiety signals and to produce leptin resistance.[84,87,102] The differential satiety response to fructose compared to glucose may be linked to the fact that fructose, unlike glucose, does not cross the blood-brain border.[84] These effects have been implicated in the development of chronic diseases and conditions such as obesity and metabolic syndrome. However, some human studies have found that fructose,

even in excess and in beverage form, did not increase food intake or attenuate the satiety response.[94,103,104]

While both fructose and glucose have been suggested as contributors to metabolic disease, results from controlled feeding studies comparing fructose and glucose suggest that fructose causes more damaging metabolic and endocrine consequences.[83] In human feeding studies, fructose consumption elevated plasma triglycerides, reduced plasma insulin and leptin, and attenuated postprandial depression of ghrelin, compared to glucose consumption.[84,85] In one study, overweight and obese men and women consumed 25% of their energy requirements as glucose- or fructose-sweetened beverages for 10 weeks. The two groups experienced comparable weight gain as a result. However, only subjects consuming glucose-sweetened beverages experienced a rise in fasting triglyceride levels. Similarly, only subjects consuming fructose-sweetened beverages experienced increased hepatic *de novo* lipogenesis, postprandial and nocturnal triglycerides, and visceral adiposity. Furthermore, only subjects consuming fructose-sweetened beverages had increased fasting plasma glucose and insulin levels and decreased insulin sensitivity.[94] This suggests that these two monosaccharides have different effects when consumed for 10 weeks in isocaloric amounts, and these effects may be relevant to IGF-1 concentrations. These results do not tell us anything about high fructose corn syrup, which contains both fructose and glucose, and could have a synergistic effect or an intermediate effect. Our study adds to this literature by including high fructose corn syrup, by investigating whether differential effects of different sugars affect plasma concentrations of IGF-1, and by determining whether this occurs in the short term, after eight days of excessive consumption of these different sugars.

The link between long-term excessive fructose consumption and insulin resistance and the hypothesized link between hyperinsulinemia and elevation of IGF-1 levels raise the question of whether long-term excessive fructose consumption is a risk factor for elevated IGF-1 concentrations, and ultimately potentially for cancer.

6. Objective of thesis: Fructose, IGF-1, cancer and the DASI study

Our objective is to compare the effects of short term (8 days) intake of excessive fructose, glucose, and high fructose corn syrup on plasma IGF-1 and IGFBP-3 concentrations, as measured by the ratio of IGF-1 to IGFBP-3. We hypothesized that acute, excessive fructose consumption would lead to less insulin release than acute, excessive glucose consumption, and would thus lead to a lower ratio of plasma IGF-1 to plasma IGFBP-3. Achieving this predicted result would suggest that in the short term, glucose leads to higher levels of this cancer biomarker, and that it is only when excessive fructose consumption goes on for long enough to produce insulin resistance that it leads to elevation of IGF-1. If long-term excessive fructose consumption is a risk factor for cancer, it is important to

determine the duration, form, and amount of fructose that produces tumor-promoting effects. This study sheds some light on that question by examining whether eight days of excessive fructose consumption on top of a typical American diet raises the ratio of IGF-1 to IGFBP-3 compared to eight days of excessive glucose consumption and eight days of excessive HFCS consumption. This project also has the secondary benefit of helping to refine our understanding of IGF-1 as a biomarker.

Short Introduction

Based on national data, there were an estimated 13.4 million American adults and children who had cancer in 2011, and it is estimated that 40% of adults in the U.S. will develop cancer in their lifetime.[3]

Biological research has produced a better understanding of how cancer develops at a cellular level. Cancerous development involves genetic mutations that both enable the uncontrolled growth of cancer cells and disable healthy cells' defense mechanisms against this growth. [1,4] In a healthy person, the tissue microenvironment maintains homeostatic systems that create antitumorigenic signals. [20] However, when enough changes in the tissue microenvironment occur, the balance is shifted from an anti-cancerous to a pro-cancerous state.[4,20]

Epidemiological studies have established associations between specific lifestyle and environmental factors and many forms of cancer. [23] Although different kinds of cancer have different pathophysiology, people can generally decrease their risk of cancer by avoiding tobacco use, engaging in physical activity, and eating a healthy diet. [20,22,105] Additionally, obesity has been associated with cancer risk. [24-26] With respect to diet, research shows that consumption of fruits, vegetables, and dietary fiber are protective against cancer, and that consumption of large amounts of red meat and processed meat are risk factors for cancer. [23,24,30-33,48,106] More recently, studies have shown that n-3 polyunsaturated fatty acids are protective against cancer and that n-6 polyunsaturated fatty acids are associated with higher cancer risk. [38,107,108] Many studies have also shown associations between consumption of foods containing specific micronutrients and phytochemicals and decreased cancer risk or better cancer prognosis. [24,25,30,39]

Another dietary factor with putative links to cancer that has been a topic of investigation is sugar. Several cohort studies indicate that intake of refined grains and sugars is associated with cancer risk[30,40-42,48] This study adds to our understanding of the links between diet and cancer by investigating the association between different sugars and levels of a cancer biomarker, insulin-like growth factor 1 (IGF-1), that has been implicated in the cancerous process, as described next.

The hormone IGF-1 is produced and secreted by the liver in response to stimulation by growth hormone, and has many effects on energy metabolism and growth. IGF-1 production is also stimulated by insulin, through insulin's up-regulation of growth hormone receptors. [54] Plasma levels of IGF-1 are associated with increased risk of several different types of cancer, including cancers of the prostate, colon and breast, [19,24,43-48,52] and evidence indicates that the IGF-1 signaling system contributes to tumorigenesis. [1,19,26,43,59,61,109] IGF-1 circulates in the blood bound to the binding protein IGFBP-3, which regulates its bioavailability. The ratio of IGF-1 to IGFBP-3 is more strongly associated with cancer risk than are absolute levels of IGF-1.

Insulin, a hormone produced and released by the pancreas in response to a rise in blood glucose, is the primary means by which the body maintains glucose

homeostasis. Insulin stimulates cellular glucose uptake and the synthesis of triglycerides, glycogen, and protein. The release of insulin also simulates the release of IGF-1. [19,43] When the body becomes insulin-resistant, the pancreas must produce extra insulin to maintain glucose homeostasis, and this leads to hyperinsulinemia. IGF-1 levels also rise in response to hyperinsulinemia. [19,43] Evidence suggests that the interaction between insulin and IGF-1 signaling, in addition to the independent effects of IGF-1 and insulin, may contribute to tumorigenesis. [43]

Ingestion of glucose acutely stimulates insulin release, and the glucose is consequently taken up by cells and used for energy or stored. In contrast, fructose is taken up by the liver from the portal vein and does not acutely stimulate insulin release. [82,83] Because glucose and fructose differ in their acute effects on insulin concentrations, they may also differ in their acute effects on IGF-1 concentrations. Chronic, excessive fructose consumption, however, leads to insulin resistance through a series of events involving the buildup of hepatic lipids promoted by fructose metabolism. This, in turn, leads to a compensatory elevation of insulin and possibly to elevation of plasma concentration of IGF-1. [83]

Our double-blinded, randomized crossover controlled feeding study compared the effects of eight days of excessive glucose, fructose, and high fructose corn syrup intake on the ratio of plasma levels of IGF-1 to plasma levels of IGFBP-3. Participants consumed beverages containing these sweeteners in addition to eating a typical American diet, which was prepared and provided to participants by study staff and was identical for all three arms of the study.

The overall goal of this project was to investigate the relationship between three common beverage sweeteners and the insulin-like growth factor system. This possible association has not yet been directly addressed in any study. The **specific aim** of the proposed study was to compare the effects of short-term (8 days) excessive intake of beverages sweetened with fructose, high fructose corn syrup (HFCS), and glucose on the ratio of fasting plasma concentrations of IGF-1 to fasting plasma concentrations of IGFBP-3. Our **hypothesis** was as follows:

We hypothesized that the ratio of plasma IGF-1 concentrations to plasma IGFBP-3 concentrations would be highest among participants consuming glucose sweetened beverages, followed by the ratio for participants consuming HFCS sweetened beverages, with participants consuming fructose sweetened beverages having the smallest ratio between IGF-1 concentrations and IGFBP-3 concentrations.

Methods

This study is an ancillary study based on the DASI (Diet and Systemic Inflammation) study; we obtained funds to have stored specimens measured for new analytes that were not proposed in the parent study. The DASI study is a crossover, randomized, controlled, feeding study with three eight-day dietary periods. The study was double-blinded with respect to the type of beverage administered in each diet phase, which was the only part of the intervention that differed between diet phases.

Study subjects

Volunteers were recruited using flyers and advertisements, and were screened for potential eligibility in a phone interview and then more thoroughly during a clinic visit to the Fred Hutchinson Cancer Research Center (FHCRC). To be invited for the visit to the center, potential participants had to be 18-65 years old, have a BMI between 20 and 40 kg/m^2, be weight stable to within 10 pounds for 6 months prior to the study, be within 10 pounds of their lifetime maximum weight, and be able and willing to provide informed consent and follow all study protocols, including eating only the provided foods and beverages during the three eight-day arms and attending six clinic visits at the FHCRC. Potential participants were excluded if they had a self-reported history of chronic inflammatory, autoimmune or metabolic disease; had fructose malabsorption or intolerance, phenylketonuria, or malabsorption syndromes; took medications likely to interfere with study endpoints (such as insulin); had present or recent anemia; were currently pregnant or pregnant within the past year; or were not willing or able to eat the provided food. At the in-person screening visit, potential participants were weighed and measured, provided fasting blood samples for tests including plasma glucose, completed a Blair physical activity questionnaire, and underwent a breath hydrogen test after drinking a fructose-sweetened beverage in order to exclude fructose malabsorbers. Recruitment goals (n = 24) were met. A block randomization procedure was used, with blocking on sex and BMI group (normal weight vs. overweight/obese).

Study protocol

The controlled feeding portion of the study was completed in early April 2014. The twenty-four participants were fed an identical diet in all three dietary periods and drank three drinks per day sweetened with either fructose, glucose, or high fructose corn syrup (HFCS).

Designed to be generic sugar-sweetened beverages, the study beverages were non-carbonated and flavored with Kool-aid. The beverages were prepared by mixing water and Kool-aid according to the package instructions and then adding the designated sweetener. All beverages were prepared to contain 12 grams of the designated sugar per 100 grams of beverage. Each subject received a daily volume of sweetened beverages providing 25% of that person's daily caloric requirements, i.e. the amount of sugar consumed was identical in each of the three dietary phases.

The rationale for providing sugar sweetened beverages providing 25% of each person's total estimated energy requirement is to ensure comparability with previous work in this area.[84,94]

Aspartame was included in both the glucose and HFCS beverages in amounts sufficient to ensure that these beverages matched the sweetness of the fructose beverage. The concentration of aspartame in the glucose- and HFCS-sweetened beverages was determined by consulting a panel of volunteers, who were asked which of the trial glucose- and HFCS-sweetened beverages had the same sweetness as the fructose-sweetened beverage.[103] The HFCS was made up of 55% fructose, 41% glucose, and 4% higher saccharides.[103]

Participants drank beverages sweetened with one sweetener for each eight-day period, and were then switched to a different sweetener for each subsequent arm of the study, in random order. The order in which each participant received their beverages was not known to participants or to any investigators or staff who had contact with them. The three eight-day study periods were separated by 20-day washout periods. Study participants received all their meals, including the sweetened drinks, from the Human Nutrition Lab at the FHCRC. Food was provided in the form of three meals per day with a composition similar to that of the average American diet in terms of macronutrient composition (50% carbohydrates, 34% fat, and 16% protein).[110]

Subjects received food providing 125% of their estimated caloric needs for each day, as calculated by the Mifflin formula, and the volume of sweetened beverages provided for each day was adjusted to provide 25% of each person's total daily caloric requirement. The amount of added sugar provided in the beverages was consistent across the 3 diets, because during all three diets daily beverages provided 25% of estimated caloric needs. The only difference between the diet periods was the type of sugar used to sweeten the beverages. Subjects were asked to drink all of the beverages each day (i.e., beverages were mandatory), and to eat as much of the solid food as they needed to feel satiated (*ad libitum* consumption).

Participants were instructed to return all uneaten food to study staff. All foods provided were weighed before study participants received them, and then weighed again after containers were returned. Participants were also asked to maintain a food record, documenting how much of each food they ate. Accuracy was assessed by comparing food records with recorded weights of foods returned. Finally, study staff interviewed participants during clinic visits and when possible at food pick-up, to determine their level of compliance and to encourage them to disclose any off-study foods they had eaten so that daily calorie consumption totals could be adjusted.

Lab tests

Participants came to the FHCRC for a clinic visit on Day 1 and Day 9 of each study period and provided fasting blood samples. At each clinic visit, 43 mL of blood

were collected in chilled EDTA tubes and placed on ice and spun immediately. The plasma was then separated, aliquoted and frozen at -70 degrees Celsius. ELISA assays were conducted by a blinded member of the Kratz laboratory to measure the concentrations of IGF-1 and IGFBP-3 in fasting plasma collected at the end of each 8-day dietary period. Plasma concentrations of IGFBP-3 and IGF-1 were measured by ELISA (R&D Systems, Minneapolis, MN) in the Kratz Laboratory at FHCRC.

These assays were performed in triplicate in the Kratz Laboratory at Fred Hutchinson Cancer Research Center. Intra- and inter-assay CVs were 5.2% and 0.8% for IGF-1, and 24.0% and 8.3% for IGFBP-3, respectively. The relatively high intra-assay CV for IGFBP-3 was found to be due to a consistent and linear drift in IGFBP-3 concentrations within each plate, from left to right and from top to bottom, likely related to the time it took to add all chemicals to each well. All three samples from each participant were run on the same plate, and in close vicinity to one another. Further, the order in which samples from each participant were analyzed within each plate was random. Thus, samples from each participant were unlikely to be unduly affected by this drift. However, to remove the small effect the systematic drift may have had on the IGFBP-3 data, we normalized the data within each plate using data from identical standards that were run twice in triplicate, before and after the unknown plasma samples run on each plate.

Self-reported physical activity

Physical activity was not part of the primary or secondary analyses for this study, but it was used to conduct sensitivity analyses. Subjects self-reported physical activity by completing the Blair Physical Activity Questionnaire during their screening visit, and this information was used to determine each subject's estimated total energy expenditure. Subjects then filled out the Blair Physical Activity Questionnaire on day 9 of each diet period to report their physical activity during that diet phase.[103]

Statistical Analysis

The statistical analysis was done with the Statistical Package for the Social Sciences for Macintosh (versions 16.0 and 20.0; IBM Corporation). We analyzed the distribution of variables by checking histograms and normal plots of the data, and we tested normality with the Shapiro-Wilk test. We performed this test for IGF-1, IGFBP-3, and the ratio of IGF-1 to IGFBP-3 at the end of each 8-day period. We log(10)-transformed IGF-1 and the ratio of IGF-1 to IGFBP-3, because they were not normally distributed prior to statistical analysis. IGFBP-3 was normally distributed and thus was not log(10)-transformed for our statistical analysis.

Our primary endpoint was the ratio of IGF-1 to IGFBP-3 concentrations in fasting plasma. Our statistical analyses assessed whether the type of sugar used in the sweetened beverages (fructose vs. HFCS vs. glucose) differentially affected the IGF-1/IGFBP-3-ratio. For our primary analysis, we used repeated measures analysis of variance (RM-ANOVA) to test for a linear trend, to

determine whether there was a linear trend in diet effects from glucose- to HFCS- to fructose-sweetened beverages. This primary analysis follows from our hypothesis that the ratio of IGF-1/IGFBP-3 would be highest for glucose, followed by HFCS, followed by fructose. For our secondary analysis, also using RM-ANOVA, we tested for *any* diet effect, to determine whether there was any non-linear difference in diet effects. This could be the case if, for example, glucose and fructose had a synergistic effect, resulting in the strongest effect being produced by HFCS, which we consider the intermediate exposure, as it is composed of 55% fructose and 45% glucose. We used the same two-pronged analytic approach to determine whether diet explained variation in IGF-1 or in IGFBP-3. The level of significance was set to $p < 0.05$ for all analyses.

In additional secondary analyses, we assessed whether the effect of the diets on the IGF-1/IGFBP-3-ratio, IGF-1, and IGFBP-3 differed depending on adiposity (normal weight vs. overweight/obese), age (above vs. below the median age of 36), or sex by re-running the RM-ANOVA tests for linear trend stratified for these co-variates (one at a time). This stratified analysis adding each of these variables as a between-subjects factor allowed us to assess the impact of diet phase on the endpoints, adjusted for adiposity, age, or sex, and to test whether the effect of diet differed between normal weight vs. overweight/obese, younger vs. older, and male vs. female participants. The latter test that assessed whether diet effect varied over each of our secondary variables was a test for multiplicative interaction. We called both of these analyses (adjustment and interaction) 'stratified' analyses,' because they entailed dividing participants into strata defined by the secondary variables, although this is not a common term used in this context.

Finally, we conducted two types of sensitivity analysis. First, we re-ran the primary analysis after removing subjects who (a) were sick with a minor illness (e.g., cold) during any part of the study (n=6), or (b) who had widely discrepant energy intakes (defined as greater than 10% variability in energy intake) during any two diet phases [n=5). Second, we conducted linear regression analyses to adjust for physical activity and for total energy intake (one at a time).

Table of variables

Independent variables	Dependent variables	Possible covariates	Possible effect modifiers
Type of sugar consumed by each participant for each study arm (fructose, HFCS, or glucose).	Ratio of IGF-1 to IGFBP-3 (primary), IGF-1 and IGFBP3 (secondary) for each participant at the end of each eight day study arm	Adiposity (normal weight vs. overweight/obese), age (below vs. above median), and sex	Adiposity (normal weight vs. overweight/obese), age (below vs. above median), and sex

Results

We enrolled 25 subjects into the study, of which 24 completed the entire study *per protocol*. One participant dropped out after completing one study diet period. Subject characteristics for the 24 participants who were included in our analysis are shown in **Table 1**. Half of subjects were normal weight and half were overweight or obese. Fifteen subjects were men and nine were women. Subjects' ages ranged from 19 to 60 years. Subjects' total energy intakes during the three diet periods were higher than their estimated total energy requirements, but did not differ significantly between the three diet phases. [103] There was no significant change in body weight over time, nor was there a difference in the change in weight between the three diet phases. [103]

In our primary analysis, we found that there was no significant linear trend between diet periods for IGF-1, IGFBP-3, or the ratio of IGF-1 to IGFBP-3 (**Table 2**); the sugars did not affect our outcome measures in a graduated fashion from highest to lowest amount of glucose. In our secondary analysis, we found that there was no significant diet effect of any kind for IGF-1, IGFBP-3, or the ratio of IGF-1 to IGFBP-3. Thus, we did not find any effect, linear or non-linear, between type of sugar consumed in the form of a beverage and any of our outcome measures.

In our secondary analyses, stratified by adiposity category (normal weight vs. overweight/obese), we still did not detect a diet effect for any endpoint (**Table 3**). However, we detected a diet by adiposity category interaction in the test for linear trend for IGFBP-3 ($p=0.035$). Specifically, we detected a linear trend for higher IGFBP-3 plasma concentrations when individuals consumed fructose-sweetened beverages, intermediate IGFBP-3 plasma concentrations for HFCS-sweetened beverages, and lower IGFBP-3 plasma concentrations for glucose-sweetened beverages in normal weight individuals ($p=0.029$ in post hoc testing), but not in overweight/obese individuals ($p=0.627$ in post hoc testing). No diet by adiposity interaction was found for IGF-1 or the ratio of IGF-1 to IGFBP-3.

Stratifying the analysis by sex did not affect any outcome measure differently (**Table 4**), nor did we detect an interaction between diet group and sex for any endpoint.

In analyses stratified by age category (below median age vs. above median age of 36) we observed a linear trend in diet effect for IGF-1 ($p=0.034$) (**Table 5**). We also detected an interaction between diet group and age in both the test for linear trend for IGFBP-3 ($p=0.014$) and the test for any diet effect for IGFBP-3 ($p=0.022$). Specifically, we detected a linear trend for higher IGFBP-3 plasma concentrations when individuals consumed fructose- followed by HFCS- followed by glucose-sweetened beverages in individuals younger than the median age ($p=0.023$ in post hoc testing), but not individuals older than the median age ($p=0.405$ in post hoc testing). We also detected a significant non-linear difference in diet effects in IGFBP-3 plasma concentrations for individuals younger than the median age

(p=0.021 in post hoc testing) but not individuals older than the median age (p= 0.730 in post hoc testing).

In sensitivity analyses, excluding individuals who reported a minor illness (e.g., a cold) at any of the three clinic visits, or who had widely discrepant energy intake (>10% between any two periods), we also did not detect an effect of diet on any outcome measure. In further sensitivity analyses, adjustment for physical activity or total energy intake did not change the relationship between diet group and any of our outcome measures.

Discussion

We achieved a null result for our primary analysis, which tested our hypothesis that the ratio of IGF-1 to IGFBP-3 would be highest for glucose-sweetened beverages, followed by HFCS-sweetened beverages, with fructose-sweetened beverages producing the lowest ratio. Neither IGF-1, IGFBP-3, nor the ratio of IGF-1 to IGFBP-3 was affected by diet in this study, either in terms of a linear trend in diet effects or in terms of a non-linear difference in diet effects.

It is unclear why this occurred, but the length of our study is one possible explanation. It may be that eight days was not long enough to produce a sufficient change in these biomarkers. Most previous controlled feeding studies with IGF-1 as an endpoint had a longer duration than our study, and do not indicate what is the shortest possible length of time sufficient for a dietary intervention to measurably alter fasting IGF-1 or IGFBP-3 plasma concentrations. In a 2012 study, Runchey and colleagues used two 28-day high- and low-glycemic index diets, and found that 28 days of the low-glycemic index diet led to a decrease of 4% in fasting IGF-1 plasma concentrations and a 4% decrease in the ratio of IGF-1 to IGFBP-3, compared to 28 days of the high-glycemic index diet. [111] They also found that the glycemic index of the test breakfast did not acutely affect post-prandial IGF-1 or IGFBP-3 concentrations.[111]

In a 2012 study, Young and colleagues measured fasting IGF-1, IGFBP-3, and other endpoints in response to three 8-week diets: a low-fat (LF) diet, a high-fat (HF) diet, and a low-fat/high-omega-3 (LFn3) diet. [112] They found that 8 weeks of the LF diet increased IGFBP-3 and decreased the IGF-1/IGFBP-3 ratio, while 8 weeks of the LFn3 diet increased both circulating IGF-1 and IGFBP-3, and increased the ratio. [112] The researchers cited two previous studies of much longer duration that measured IGF-1 as an endpoint – one of 12 months and one of four years – and proposed that perhaps the changes they had observed in their 8-week study were transitory changes that may not have persisted in the long term.[112]

In addition to the two studies just described, many other studies using dietary interventions with IGF-1 as an endpoint had a longer duration than our study. These include a study measuring the effect of a 7-week high-protein diet vs. a 7-week low-protein diet on calcium excretion and IGF-1, [91] a study investigating the effect of 3 months of drinking two alcoholic drinks per day vs. 3 months of drinking

no alcoholic drinks on IGF-1, [90] and a study investigating the effect of 2 years of a whey protein supplement drink vs. a placebo drink on IGF-1. [89] All three of these studies produced changes in IGF-1 fasting plasma concentrations resulting from the dietary interventions.

There are fewer examples of dietary intervention studies using IGF-1 as an endpoint that had a duration shorter than or equal to the duration of our study. One such study is a 2009 study in which Hoppe and colleagues measured the effects of 7 days of drinking a high-whey milk-like drink vs. 7 days of drinking a high-casein milk-like drink on the serum IGF-1 concentrations of 8-year-old boys. [88] The researchers found that fasting serum IGF-1 concentrations increased by 15% after 7 days of the casein drink and did not change after 7 days of the whey drink. [88] This study indicates that IGF-1 may be alterable through a dietary intervention lasting less than 8 days, but the evidence that supports this possibility was produced in a protein-based trial rather than a carbohydrate-based trial, and may not be applicable to our study. It therefore remains unclear whether, in our study, a longer duration would have lead to differences in plasma concentrations of IGF-1 or IGFBP-3.

Another factor that may provide insight into whether our study diet periods were long enough to produce a change in the endpoints is the half-life of IGF-1 and IGFBP-3. The half-life of IGFBP-3 is 30 – 90 minutes, and IGFBP-3 controls the half-life of IGF-1. [113,114] The half-life of free IGF-1 is 10-12 minutes. [114,115] Given these data, it is very plausible that these proteins could turn over in the blood during the course of each of our 8-day study phases.

It is possible that the timing of our blood draws influenced our results. Blood draws for this study were taken at the end of each 8-day diet phase, in the fasting state, and thus did not measure the acute, post-prandial effects of each sweetener. It may be that taking post-prandial blood samples would have changed our results. Previous research on post-prandial IGF-1 response has been mixed, and it has been suggested that IGF-1 concentrations do not change based on acute, post-prandial events. [111,116] In a 2012 study, Runchey and colleagues found that concentrations of IGF-1 and IGFBP-3 changed very little during the four hours immediately following test meals. However, they did observe a significant decline in IGF-1 concentrations during the first hour following a mixed meal, suggesting that IGF-1 and IGFBP-3 concentrations are acutely affected by food intake. [111] In a 2005 study, Brand-Miller and colleagues found that IGFBP-3 declined in the four hours after both of two test meals, but declined more after a low-glycemic index meal compared to the high-glycemic index meal. In this study, IGF-1 did not change appreciably in the four hours after the test meals. [117] Given these results, it is possible that diurnal plasma concentrations of IGF-1 and IGFBP-3 may have varied between the three diet phases.

Our secondary analyses adjusting for adiposity category, sex, and age category produced some non-null results. In testing for effect modification by adiposity category, we detected a linear trend in diet effects for IGFBP-3 plasma

concentrations in normal weight individuals, but not overweight/obese individuals. In minimizing variability caused by age category, we detected a linear trend in the effect of diet group on IGF-1 plasma concentrations. In testing for effect modification by age group, we detected both a linear trend in diet effects for IGFBP-3 plasma concentrations and a significant non-linear difference in diet effects for IGFBP-3 plasma concentrations, in both cases for individuals lower than the median age, but not for individuals higher than the median age. All of these significant findings in our secondary analyses are of questionable clinical relevance, given the overall null findings in our primary analysis. In our stratification by age category in particular (**Table 5**), the effect size was very small, and HFCS was not intermediate, both of which further diminish the clinical relevance of these non-null results. Furthermore, while it is known that adiposity or age affect plasma concentrations of IGF-1 or IGFBP-3, [118-122] it is unclear biologically why adiposity or age should affect the response of plasma IGF-1 and IGFBP-3 to dietary sugars. Given that we did not correct for multiple testing, it is possible that these non-null findings in our secondary analyses adjusting for adiposity category and age category may be false positive.

To summarize, in the primary analysis we found that diet phase did not affect any of our study endpoints, and we thus accept the null hypothesis of no difference in effect IGF-1, IGFBP-3, or the ratio of IGF-1/IGFBP-3 based on type of sweetener. It is unclear whether a differential effect on our outcome measures based on type of sweetener would be produced in a study of longer duration, or if we had studied postprandial / diurnal plasma concentrations of IGF-1 and IGFBP-3 while participants were drinking the sweetened beverages. The significant results we achieved in our secondary analyses may reflect false positive findings, and are of questionable clinical relevance given the small effect size, and thus should not be interpreted as indicative of differential effects of fructose vs. glucose on IGF-1 and IGFBP-3.

Study strengths and weaknesses

Our study has several strengths that make these results relevant to the general population and useful for guiding future studies. First, we excluded potential participants who had fructose malabsorption syndrome, thus ensuring that fructose was indeed being metabolized by our study participants. [123,124] Second, the macronutrient composition of the study diet closely approximated that of the typical American diet, and the sweetened drinks included high fructose corn syrup, the form in which excess carbohydrates are typically consumed. These components of the study protocol make our results relevant to Americans following common dietary patterns. Third, the study was a double-blind crossover study, so each participant served as his or her own control.

The fourth and final strength was the strict control we exerted over the intervention. Our study was a controlled feeding study, in which participants received all foods from a research kitchen. While the study participants were free-living, and thus complete adherence could not be ensured, several methods were

used to minimize noncompliance and inaccuracies in reporting. Study participants were given 125% of their required calories for each day in the form of solid food, so they theoretically had more than enough to eat and were not driven by hunger to consume non-study foods. They were asked to keep logs of everything they ate, and these logs were checked against weigh-backs of returned study foods, both to validate the amounts eaten and to monitor participants' compliance. Study staff encouraged participants to report non-study foods eaten and conducted in-person interviews to monitor attitude towards the study and points of confusion. Most of the subjects kept accurate records and appeared to be conscientious about following study protocol. For example, participants reported having drunk a cup of herbal tea (including the brand name and flavor) or adding a single-serving packet of hot sauce to a dish. On average, 0.08% of energy was consumed as non-study food items, ranging from 0.015 to 0.41% of subject's total calorie intake across all three diet periods. While controlled feeding studies are not perfect, they produce a greater likelihood of accurate results than studies in which the intervention consists of dietary guidance, and participants must purchase and prepare their own food.

Our study also had several potential weaknesses that may have influenced our results. First, our assays were only performed on Day 9 of each diet period, and not on Day 1 of each diet period. Thus, we measured the concentrations of IGF-1 and IGFBP-3 after each diet, but not the change in these endpoints that occurred during each diet. We do not believe that comparing the change in outcome measures from Day 1 to Day 9 of each diet period would have improved our results, because Day 1 blood draws were taken at the end of each 20-day washout period, a time during which we had no control over what participants were eating.

A second potential weakness in our study is that, while our crossover study design allowed us to use each participant as his or her own control, additional information would have been provided by including a study period in which participants consumed no sweetened beverage or in which they consumed beverages sweetened with non-nutritive (zero-calories) sweeteners, as was done in a pilot study our group conducted. In the pilot study, subjects in the glucose and fructose study phases consumed an average of 371-455 additional kcals per day compared to the aspartame phase, and thus, adding a phase with a non-caloric sweetener would have introduced a significant difference in energy intake between diet periods. Any difference found between the control diet group in which participants did not consume sugar-sweetened beverages and any sugar-sweetened beverage group could therefore be due to the excessive consumption of the sugar, the excessive energy intake, or both. Thus, while we were able to compare the relative effect of fructose- vs. HFCS- vs. glucose-sweetened beverages on the endpoints, our study design did not allow us to assess whether the consumption of sugar-sweetened beverages changes IGF-1 and IGFBP-3 concentrations from baseline.

Third, while controlled feeding studies produce better study adherence than studies in which subjects consume foods that they purchase and prepare

themselves, we cannot know for certain what level of compliance was achieved. Additionally, our due diligence in checking participant logs against food weigh-back records indicated that a small number of study subjects were likely not entirely compliant on several study days, and thus, we may have achieved more accurate results if we had excluded those participants' data from our analyses. However, as we did not even find a trend for a diet effect in our primary analysis, it is unlikely that minor non-compliance had a substantial effect on our results.

A potential fourth weakness is that we did not adjust for multiple testing in our secondary analyses. Thus, we cannot exclude the possibility that we achieved false positives in these analyses due to the large number of tests.

The fifth weakness is, as indicated above, that it is possible that eight days was not long enough to measure a difference in IGF-1 or IGFBP-3 between diet periods, and that our study would have been more informative if the duration had been longer. As discussed above, the only carbohydrate-based intervention that we know of that produced a change in IGF-1 plasma concentrations was composed of 2 28-day dietary interventions (high glycemic and low glycemic), [111] and the only dietary intervention trial we know of that produced a change in IGF-1 in fewer than 8 days was a protein-based intervention. [88] Additionally, the plasma samples for our study were drawn when participants were fasting, and it is unclear whether taking post-prandial samples would have improved our results.[111,116,117]

Despite these weaknesses, the results we produced are useful for understanding the effect of diet on these cancer biomarkers. Our primary conclusion is that in the short term, different sugars consumed as beverages do not differentially affect IGF-1, IGFBP-3, or the ratio of IGF-1/IGFBP-3. It is unclear whether a differential effect would appear in a longer-term study.

Tables

Table 1. Characteristics of Study B participants at baseline[1].

	All subjects	Normal weight	Overweight/obese
Sex (female/male)	9/15	3/9	6/6
Age (y)	36 ± 12	33 ± 11	39 ± 12
BMI (kg/m2)	27.4 ± 4.8	23.7 ± 1.0	31.0 ± 4.3
Fasting plasma glucose (mg/dL)	92 ± 10	87 ± 10	96 ± 8
Physical activity (MET-h/wk)	68.7 ± 45.9	81 ± 54.5	56.3 ± 33.1
Estimated total kcal requirement (kcal/d)	2,560 ± 370	2,610 ± 380	2,510 ± 370

[1]Values are mean ± standard deviation.

Table 2. IGF-1, IGFBP-3, and the ratio of IGF-1 to IGFBP-3 during each dietary period [1].

	Fructose	**HFCS**	**Glucose**	**RM-ANOVA**	
				p-value (linear trend)	**p-value (any diet effect)**
IGF-1 (ng/mL)	124 (55 - 198)	127 (63 - 219)	124 (63 - 237)	0.851	0.601
IGFBP-3 (ng/mL)	1940 ± 419	1977 ± 414	1882 ± 367	0.153	0.101
Ratio of IGF-1 to IGFBP-3	0.064 (0.041 – 0.086)	0.061 (0.045 – 0.084)	0.067 (0.041 – 0.115)	0.524	0.147

[1] n=24, values are means ± standard deviations, or medians (min - max) for non-normally distributed data.

Table 3. IGF-1, IGFBP-3, and the ratio of IGF-1 to IGFBP-3 during each dietary period, separately for participants that were normal weight vs. overweight or obese [1].

	Fructose	**HFCS**	**Glucose**	**RM-ANOVA**			
				Linear trend		**Any diet effect**	
				p-value (diet)	p-value (diet x adiposity)	p-value (diet)	p-value (diet x adiposity)
IGF-1 (ng/mL)							
Normal weight (n=12)	124 (95 – 198)	128 (87 – 219)	133 (86 – 237)	0.854	0.841	0.611	0.777
Overweight/obese (n=12)	122 (55 – 146)	126 (63 – 141)	114 (63 – 155)				
IGFBP-3 (ng/mL)							
Normal weight (n=12)	2003 ± 297	1983 ± 279	1865 ± 246	0.123	0.035	0.094	0.166
Overweight/obese (n=12)	1877 ± 520	1971 ± 530	1900 ± 469				

Ratio of IGF-1 to IGFBP-3							
Normal weight (n=12)	.067 (.052 - .086)	.062 (.045 - .084)	.069 (.042 - .115)	0.512	0.132	0.146	0.321
Overweight/obese (n=12)	.065 (.041 - .080)	.059 .045 - .076)	.060 (.041 - .100)				

[1] Values are means ± standard deviations, or medians (min - max) if non-normally distributed data.

Table 4. IGF-1, IGFBP-3, and the ratio of IGF-1 to IGFBP-3 during each dietary period, separately for men and women [1].

	Fructose	HFCS	Glucose	RM-ANOVA			
				Linear trend		Any diet effect	
				p-value (diet)	p-value (diet x sex)	p-value (diet)	p-value (diet x sex)
IGF-1 (ng/mL)							
Women (n=9)	130 (55 – 198)	130 (63 – 219)	136 (63 – 237)	0.850	0.964	0.760	0.641
Men (n=15)	123 (95 – 148)	124 (87 – 137)	123 (90 – 167)				
IGFBP-3 (ng/mL)							
Women (n=9)	1998 ± 559	1984 ± 495	1974 ± 431	0.225	0.521	0.222	0.319
Men (n=15)	1905 ± 327	1974 ± 377	1828 ± 326				

Ratio of IGF-1 to IGFBP-3							
Women (n=9)	0.07 (0.04 – 0.08)	0.06 (0.05 – 0.08)	.063 (.041 - .115)	0.670	0.458	0.328	0.201
Men (n=15)	0.07 (0.05 – 0.09)	0.06 (0.05 – 0.08)	0.07 (0.05 – 0.12)				

[1] Values are means ± standard deviations, or medians (min - max) if non-normally distributed data.

Table 5. IGF-1, IGFBP-3, and the ratio of IGF-1 to IGFBP-3 during each dietary period, separately for participants who were younger than the median age (group 1) vs. older than the median age (group 2).[1] The median age was 36 (27, 43) years.

	Fructose	**HFCS**	**Glucose**	**RM-ANOVA**			
				Linear trend		**Any diet effect**	
				p-value (diet)	p-value (diet x age group)	p-value (diet)	p-value (diet x age group)
IGF-1 (ng/mL)							
Age group 1 (younger than median) (n = 12)	135 (100 – 198)	135 (91 – 219)	133 (105 – 237)	0.034	0.970	0.614	0.990
Age group 2 (older than median) n = 12	106 (55 – 145)	107 (63 – 141)	109 (63 – 139)				
IGFBP-3 (ng/mL)							
Age group 1 (n = 12)	2089 ± 440	2144 ± 432	1939 ± 422	0.111	0.014	0.076	0.022
Age group 2 (n = 12)	1791 ± 354	1811 ± 334	1825 ± 309				

Ratio of IGF-1 to IGFBP-3							
Age group 1 (n = 12)	0.07 (0.04 – 0.09)	0.06 (0.05 – 0.08)	0.07 (0.04 – 0.12)	0.511	0.125	0.136	0.146
Age group 2 (n = 12)	0.06 (0.04 – 0.07)	0.06 (0.05 – 0.08)	0.06 (0.04 – 0.07)				

[1] Values are means ± standard deviations, or medians (min - max) if non-normally distributed data.

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