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Bioactive growth hormone in older men and women: It's relationship to immune markers and healthspan

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ABSTRACT

Objective: The consequences of age-related decline in the somatotropic axis of humans are complex and remain largely unresolved. We tested the hypothesis that hGH measurements of plasma by bioassay vs immunoassay from samples obtained from free-living, elderly individuals would reveal a dichotomy in GH activities that are correlated with the functional status of the donors, i.e. their healthspan.

Design: Forty-one men and women of advanced age (men: N = 16, age, 80.5 ± 6.5 years; height, 173.1 ± 6.9 cm; body mass, 81.8 ± 13.0 kg) and (women: N = 25, age, 80.7 ± 7.2 years; height, 157.7 ± 6.0 cm; body mass, 68.8 ± 17 kg), were recruited for a cross-sectional study. Participants filled out PROMIS (Patient-Reported Outcomes Measurement Information System, U. S. Department of Health and Human Services) scales, undertook physical performance tests and had fasted blood samples obtained at rest for measurement of hormonal and immunology biomarkers.

Results: When measured by the well-established rat tibial line GH bioassay, one half of the plasma samples (n = 20) contained bioassayable GH (bGH), but the other half (n = 21) failed to mount increases in tibial plate width above saline injected controls. This difference did not correlate with the age, sex or physical functionality of the plasma donor. It also did not correlate with hGH concentrations measured by immunoassay. In those cases in which bGH was detected, various hierarchical regression models predicted that GHRH, c-peptide, VEGF, NPY, IL-4 and T-regulatory lymphocytes were associated with the difference and predicted bGH.

Conclusion: Results from this study suggest that the actions of bGH at the cellular level may be modified by other factors and that this may explain the lack of correlations observed in this study.

1. Introduction

The dichotomy between measurements of growth hormone (GH) concentrations made by bioassay vs those by immunoassay was first reported > 40 years ago [1]. Prior to that time, the bioassay most often used to measure concentrations of GH in plasma and tissue extracts was the rat tibia test that had been developed by Evans many years earlier [2]. As antibody based tests for GH detection became routine in ~1980, use of the more expensive and somewhat cumbersome animal bioassays were largely abandoned and an overwhelming majority of investigators switched to use of more sensitive immunoassay procedures. Differences in blood concentrations between detection methods of immunoassays and bioassays has also been shown for bioactive IGF-I which can be significantly different from immunoreactive and total IGF-I [3,4]. By examining bioactive and immunoreactive GH results can also provide new insights into pituitary function with aging.

Our laboratory group is one of a very few that has continued to investigate the issue of differences (i.e., a dichotomy) between the bGH

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and iGH measurement in human plasma samples. We have consistently found evidence for the discrepancy in the results between the two assays systems, [5–7]. To our knowledge, the possibility that such GH dichotomy exists in plasma samples of elderly individuals is unknown, a fact that provides the rationale for this current report.

Nass et al. [8] report that many studies describe a reduction in GH secretory parameters from 15% to 70% in men and women over 60 years of age compared to young adults; GH levels decrease by 50% every 7 years after mid-puberty. They further argue that attenuation of this axis may lead to frailty by way of a host of physiological correlates that are manifested clinically by slowness, weakness, weight loss, low activity and fatigue. Existing demographic data which document the devastating economic impact of frailty to the economy is not hard to find, and the need to develop strategies to combat it is clear. The possible use for GH therapy in the elderly has been extensively reviewed and there is consensus that this approach is not safe. Nevertheless, in 2009, GH treatment as an anti-aging therapy ranked at the top of the list of health-related internet searches [8].

The evidence to support a link between the somatotropic/IGF axis and healthspan/lifespan is very strong [9,10]. Signaling pathways in this axis are postulated to aid in the regulation of aging and disease. Indeed, suppression of GH signaling in the dwarf mouse leads to extended lifespan, a finding initially reported ~20 years ago [11]. More recently, GH receptor deficient mice have been shown to exhibit decreased risk for cancer and diabetes as well as expressing other health benefits [12,13]. A status report of mechanisms underlying extended lifespan were recently reviewed by Brown-Borg [14]; in general these appear intimately linked to multiple regulatory processes controlling growth, defense mechanisms, inflammation, and metabolic adjustments [9].

In addition to linking defects in the GH/IGF axis to increased longevity in invertebrate and rodent models, the beneficial effects of reduced somatotropic axis activity in humans is also suggested by results from some studies, yet conflicting results and inconsistencies in others, show that the topic is clearly controversial and may indeed even be disease specific [10]. In future investigations aimed at addressing relationships between the complex issues of GH/IGF attenuation, lifespan and healthspan, Milman et al. [10] make the case for employing innovative study approaches, as well as using sex and age stratification designs.

In our current experiment we tested the hypothesis that concentrations of plasma GH, obtained from a group of healthy elderly men and women, would also show features reflecting GH dichotomy. To accomplish this we also measured the concentrations of endocrine and immune system biomarkers in these same samples in order to determine responses under different levels of bGH activity and the associations that might exist to advance our understanding of bGH. Finally, we sought to determine if such association might also be reflected in the physical activity markers of this experimental group.

2. Material and methods

2.1. Experimental design and approach

Forty-one older men and women participants were recruited from the Cornwall Manor Retirement Community in Pennsylvania. Each individual was a free-living resident in that community. A cross-sectional study design was used and consisted of visits for informed consent, screening followed by filling our survey questionnaires and then testing of various physical fitness components of performance. The physical performance tests consisted of standard grip strength, get-up & go, single leg stance, chair-rise, and 8-ft walk. Another day was then used for obtaining a fasted resting blood sample from which endocrine, cytokine and immune variables would be assessed.

Table 1
Characteristics (Mean ± SEM) study participants (men; $n = 16$), (women; $n = 25$), and combined men and women ($n = 41$).

	Men	Women	Men & WOMEN combined
Age (years)	80.5 ± 1.6	80.7 ± 1.4	80.6 ± 1.1
Height (cm)	173.1 ± 1.7	**157.7 ± 1.2***	163.7 ± 1.5
Body mass (kg)	81.8 ± 3.2	**68.8 ± 3.4***	73.9 ± 2.6
BGH signalers (%)	37.5%	56.0%	48.8%
BMI ($kg{\cdot}m^2$)	27.2 ± 0.9	27.7 ± 1.4	27.5 ± 0.9
PROMIS 20 physical function scale	14.7 ± 7.9	16.1 ± 8.0	15.5 ± 7.8

Bold data indicates the women's values that were significantly different from the corresponding values for men.
* $p \leq 0.05$ from corresponding men's value.

2.2. Participants

The characteristics of the participants are shown in Table 1. Each subject had the risks and benefits of the investigation explained to them and signed an informed consent document that was part of the approval process for the investigation by the Pennsylvania State University and the Ohio State University Institutional Review Boards for use of human subjects in research. As expected, men were significantly heavier and taller than the women in this study. All participants were free living residents the Cornwall Manor Retirement Community, Cornwall, PA, USA. Subjects were screened so that no confounding variables (e.g., obesity, muscle or joint impairments, impaired mobility, or illness) compromised the tests in this investigation.

2.3. Procedures

On the first day, the study was carefully explained to each participant, including those relating to the risks and benefits of the investigation. For those agreeing to participate, a university approved informed consent document was signed and witnessed. Each participant was then required to complete two surveys; the Patient-Reported Outcomes Measurement Information System (PROMIS™, U. S. Department of Health and Human Services, Washington D.C.) scale for Sleep Disturbance (SD) and Sleep-Related Impairment item scales and the 20-item PROMIS™ physical function scale. Participants were encouraged and allowed to ask questions and clear up any confusion that existed while completing the survey [15–17].

2.4. Physical performance testing

After taking weight and height measurements, each participant was asked to perform the following tests after they had first completed a familiarization and practice trial with the help of an experienced investigator. The best score was used for statistical analyses.

2.4.1. Get-up & Go test

Each participant, seated in the same 43 cm high chair, was asked to rise and walk 10 m before returning to the chair. Two trials were administered with 3–4 min rest between trials. Use of their arms to assist in rising from the chair or for support during the test was permitted. Spotters moved with the participant to assure safety. The task was timed with a stop watch.

2.4.2. Chair raise time

This test was identical to the Get Up and Go test with the following modifications: Each participant was asked to start rise from the chair and return to the seated position 5 times as quickly as possible without the use of any aid. The participant's arms were folded across their chest

to ensure no upper body involvement and full return to the seated position was required. Two trials were used with 3–4 min rest between trials. The time from the first movement until the 5 repetitions were completed constituted a successful test.

2.4.3. Single-leg stance test

Participants was asked to wear flat shoes for this test. Each was asked to shift their weight to the dominant foot and flex their non-dominant leg at the knee to about a 90° angle. Two trials were used with 3–4 min rest between trials. The time they could balance in that position without assistance was recorded. Spotters were used to ensure safety.

2.4.4. 8-Foot walk test

The participant was required to begin at a defined starting point and walk as quickly as they could over a distance of 8 ft. Two trials were used with 3–4 min rest between trials. The time it took for one of their feet to cross a marker on the floor was used for analysis. As before, spotters were nearby and on each side to ensure safety.

2.4.5. Hand grip strength test

Grip strength in each hand was tested using a hand grip dynamometer. The participant stood with feet hip-width apart and held the dynamometer (T.K.K.5401 GRIP-D; DIGITAL GRIP DYNAMOMETER, Niigata City, Japan) in either right or left hand in randomized fashion with respect to their dominant hand. Dominance was defined by which hand was used in writing and throwing a ball the furthest. During the test arms were held at the participant's side with no bend in the elbow. Three attempts, with the best of the three recorded, were used for data analysis. This test is based on our previous work with National Health and Nutrition Examination Survey (NHANES) using an identical test protocol [http://www.cdc.gov/nchs/n(nyfs/manuals.htm) ch.3]. Each subject was tested on the same calibrated dynamometer.

On the second day of testing each participant arrived at the laboratory setting in Cornwall Manor in the morning after an 8 h fast to obtain a resting blood sample for measurements of various biomarkers. Participants were encouraged to drink water and stay hydrated prior to this laboratory visit. Each subject was allowed to rest in a chair for at least 15 min to stabilize hydrostatic pressures. An indwelling Teflon cannula using a 21 gauge butterfly needle was inserted into an antecubital arm vein of the participant kept patent with a normal saline lock. Approximately 45 mL of blood was obtained in appropriate preservative collection vacutainers for whole blood, serum and plasma analyses. Blood was processed by centrifugation and divided into aliquots, and stored at − 80 °C in an ultra-low freezer for later batch analysis.

2.5. GSH growth hormone reduction ELISA procedure

Plasma immunoreactive growth hormone (iGH) concentrations were measured using commercially available mono-clonal antibody ELISA (Calbiotech, Spring Valley, CA, USA). All assays were performed in duplicate and thawed only once prior to assay. To assess potential differences caused by S-S linked GH aggregate variants, samples were also reduced using physiological concentrations of glutathione [GSH (G4251): Sigma-Aldrich, St. Louis, MO, USA]. In brief, 15 uL of 100 mM GSH or 15 uL of dH2O was added to samples (135 uL) in duplicate. After incubation of plasma samples on an orbital plate shaker at room temperature for 18 h, a standard assay procedure was used to measure iGH concentrations under reducing and non-reducing conditions [7]. Minimum detection level of the assay was 0.2 μg L^{-1}. Intra- and inter-assay variances were ≤ 8.2%.

2.6. Bioactive growth hormone

We used the same bGH assay procedure that our group has used over the course of 15 years of work with human plasma [5–7,18] and as originally described by Greenspan and Li [19]. Female Sprague-Dawley rats (Hilltop Labs, Scottsdale, PA), hypophysectomized at 26–28 days of age, were used 2 weeks after surgery. Animals were housed (2 per cage) at the animal care facility at The Pennsylvania State University and handled following guidelines for animal care in accordance with approval of The Pennsylvania State University Institutional Animal Care and Use Committee. Animals and were kept on a 12:12-h light-dark cycle with food and water consumed ad libitum. Animals that weighed < 80 g or > 100 g at the time of sample injection were excluded. Criteria taken as evidence for completeness of hypophysectomy were as follows: failure to gain > 7 g in the 10 days after the operation, deterioration of body tonus, maintenance of infantile ("smooth") hair, and absence of pituitary remnants in the sella turcica at autopsy as determined by visual inspection under magnification. Animals were injected IP once daily for 4 days with either 1) experimental plasma samples, 2) a standard GH preparation (United States Department of Agriculture bovine GH B-1 AFP 5200, 1.4 IU/mg at total doses of 10, 30, or 90 μg), or 3) physiological saline (control). Twenty-four hours after the last injection, the animals were killed, tibial epiphyseal plates were stained, and plate widths were measured in double-blind fashion using an ocular micrometer (10 readings averaged across the plate width for each sample). Additionally, two separate independent readings for determination of the bGH values were accomplished in a blinded fashion by two different investigators. GH responses were expressed in terms of a purified human pituitary preparation (3.0 IU/mg). Assay variance was 7.1% and minimal detection limit of 5 μg L^{-1}. Average tibial widths of animals injected with bovine GH standard were as follows (means ± SD): saline, 149.1 ± 9 μm; 10 μg, 169 ± 12 μm; 30 μg, 192 ± 19 μm; 90 μg, 239 ± 21 μm (line of best fit for standard curve was $r^2 = 0.97$) (see Fig. 1). Our operational definition of a non-responder was an individual that exhibited a very low bGH signal under (500 μg L^{-1}) but above the minimal detection limit as noted above.

2.7. Other hormone analyses

All hormones tested were measured using commercially available ELISA kits (C-Peptide (80-CPTHU-E01.1), IGF-1 (22-IGFHU-E01), Sex Hormone Binding Globulin (11-SHBHU-E01), and Myostatin: Alpco Inc., Salem, NH, USA; Testosterone (TE187S) and Cortisol (CO103S): Calbiotech, Spring Valley, CA, USA; Ghrelin (EZGRT-89K) and NPY (EZHNPY-25K): Millipore Inc., Billerica, MA, USA; Growth Hormone Binding Protein (ELH-GHR): Raybiotech Inc., Norcross, GA, USA). Absorbance was determined using a standard plate reader (Synergy H1, BioTek Winooski, VT, USA) and coefficients of variation were below 15% (average = 6.4 ± 3.18%). Hormone concentrations were calculated per manufacturer recommendations, which included 5-parameter logistic, linear, two-phase decay, and point-by-point standard curve interpolations.

2.8. Blood cell subpopulations and cytokine assays

For analysis of various blood cell populations, 100 μl of heparinized whole blood was incubated with 30 μl of a cocktail of fluorescently labeled monoclonal antibodies directed against lymphocyte cell surface antigens. After a 45 min incubation at room temperature, the red blood cells were lysed with 2 ml of BD Pharm Lyse buffer (BD Biosciences, San Jose, CA) for 15 min, vortexed, incubated for 15 min and centrifuged at 300 g for 5 min. Supernatants were aspirated, and the cell pellets washed by resuspension and centrifugation in 10% PBS, 1% FBS, 1% sodium azide. Cells were analyzed by flow cytometry using a FC500 Beckman Coulter Flow Cytometer, Flow Cytometry Core Facility at The Pennsylvania State University. Lymphocytes were gated with PE-Texas Red CD45 (Beckman Coulter PNIM271OU). The test panel included CD3 + (total T cells), CD4 + (T helper cells), CD8 + (T cytotoxic cells),

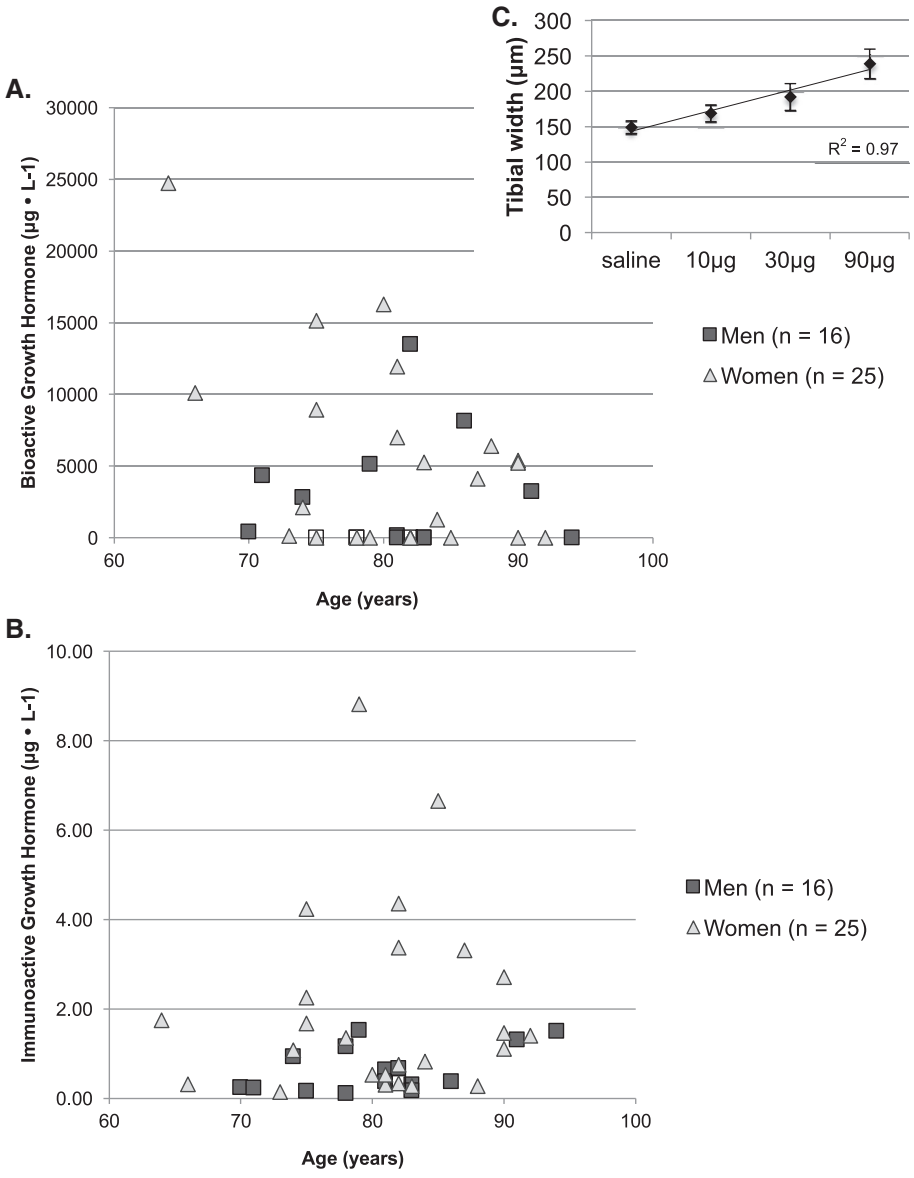

Fig. 1. Individual data for bioactive Growth Hormone (bGH) (A), individual data for immunoreactive Growth Hormone (iGH) (B) plotted over the subject age and (C) average tibial widths and line of best fit of animals injected with bovine GH standard are presented. All data graphically represented were at or above the minimal detection limit of 5 µg L^{-1} for bGH and 0.2 µg L^{-1} for iGH.

CD19 + (B cells), CD56 +, 16 + (NK cells); CD4 + CD25 + $CD27^{low}$ (T activated). These markers were combined with CD69 + to indicate dividing (activated) cells. One 4 ml tube of blood was used to obtain serum which was frozen for later assay of cytokines and other growth factors. We used a 30-plex cytokine assay obtained from Meso Scale Diagnostics LLC (Rockville, MD). Assays were carried out at the Cytokine Core Facility at the Pennsylvania State University. Of the 30 cytokines tested, we only detected values for the following twelve: for TGFβ, IFNϒ, IL-6, TNFα, MCP-1, IL-12p70, IL-17, IL-1α, IL-5, IL-7, VEGF, and eotaxin.

2.9. Statistical analyses

The data in this investigation are presented first as mean ± SEM. Statistical power was determined to be > 0.80 for all measures for the sample sizes for each analysis used in this study at the 0.05 a-level (nQuery Advisor software, Statistical Solutions, Saugus, MA). All data were assessed for violations normality and homogeneity of variance. For those variables that violated assumptions, data were log10-transformed prior to analysis and then rechecked. All data met linear statistics assumptions prior to the linear analyses used. Data were analyzed using a two way ANOVA (sex X groups) for each neuroendocrine, cytokine, or immune response pattern in order to determine any sexual dimorphism or differential signal within sub-groups. Independent *t*-tests were used to determine differences in subject characteristics for men and women. Pearson-product moment correlation coefficients were determined to establish any pairwise relationships within the data base. Hierarchical stepwise regression was used to develop prediction models for presence of bGH in the combined group of older men and women that exhibited a bGH assay signal. For this investigation, statistical significance was set at $P \leq 0.05$.

3. Results

The distribution pattern of plasma bGH and iGH concentrations in each of the 41 participants, as a function of age, is shown in Fig. 1. The widespread variance in bGH values is obvious and reflect the dichotomy of GH measured by these two different assays. Especially note-

Table 2
The values (Mean ± SEM) for bioactive growth hormone (bGH), growth releasing hormone (GRH), growth hormone (iGH), and iGH + glutathione of men (n = 16) and woman (n = 25). Additional subgroups of those that had a bioactive assay signal (bGH) (Men n = 6; Women n = 14) and those that had a very low bioactive assay signal (LbGH) (Men n = 10; Women n = 11) are also presented.

	Men	Women	Men bGH	Women bGH	Men LbGH	Women LbGH
bGH ($\mu g \cdot L^{-1}$)	2360.9 ± 964.2	4966.1 ± 1299.3	6197.6 ± 1649.6	8857.4 ± 1708.4	58.8 ± 41.8	13.6 ± 10.6
GRH ($ng \cdot mL^{-1}$)	3.4 ± 2.8	1.4 ± 0.9	1.0 ± 0.4	2.3 ± 1.5	4.8 ± 4.6	0.2 ± 0.1
iGH ($\mu g \cdot L^{-1}$)	0.6 ± 0.1	**2.0 ± 0.4***	0.8 ± 0.2	1.3 ± 0.3	0.5 ± 0.1	**2.9 ± 0.9***
iGH + glut ($ng \cdot mL^{-1}$)	0.7 ± 0.2	**2.3 ± 0.5***	0.8 ± 0.3	1.5 ± 0.4	0.6 ± 0.2	**3.5 ± 0.9***

Bold data indicates the women's values that were significantly different from the corresponding values for men.
* $p \leq 0.05$ from corresponding men's value.

worthy was the surprising finding that plasma from 21 individuals [men = 10; women = 11] yielded extremely low bGH concentrations, most low bGH concentrations were just at or above the assay sensitivity (5 μg GH). On the other hand, samples from 20 individuals [men = 6; women = 14] generated a tibial width response (i.e. assay signal).

We sought to determine what relationship, if any, these GH bioassay data might reflect peptide concentrations of the GH family that are known to control either release, or end organ activation. In Table 2, and all subsequent tables, we first list the data according to sex for all 41 participants followed by sub-division into 2 groups; viz. group 1 (n = 20) which had higher detectable bGH and group 2 (n = 21) which had a very low bGH response (LbGH). These higher values of bGH are similar to those we have reported in other studies that have tested younger women and men [5,6]. The bGH values shown in Table 2 were not related to participant age.

The concentration range of iGH in this study is on the same order of that reported in many other studies [20,21]. As a group, women had significantly higher iGH than men and interestingly this concentration attained significance in group 2 participants. In previous studies we have found that addition of chemical reducing agent to the plasma sample prior to assay results in significantly higher levels of detectable iGH relative to those in the unreduced sample. This increase is attributed to dissociation of disulfide linked aggregates contained in the sample [22]. These data (Table 2) show increased iGH concentrations in some samples after glutathione reduction, especially in the women of group 2. Concentrations of GHRH, IGF-1 and ghrelin in the 41 plasma samples were similar within their group designations and fail to reveal obvious relationships to bGH concentrations in the same plasma sample.

Tests for the major anabolic and catabolic hormones in the 41 samples revealed no sexual dimorphism (Table 3). Even when these data were transformed (log_{10}) and any outliers redefined, no differences were observed. The lack of differences in testosterone may reflect presence of gonadopause in the older men in our cohort. Concentrations of circulating myostatin in men from group 1 tended to be greater than their female counterparts, but this tendency was not statistically significant most likely due to high variances in the men.

We also tested the 41 plasma samples for concentrations of three peptides, viz. NPY, C-peptide and VEGF, each of which we suspected might be good candidate(s) to help in identifying correlation/association(s) with the bGH results. Of these three candidates only NPY concentrations were significantly different between men and women and, most interestingly, these differences were associated with bGH concentrations of group 1, but not group 2, participants (Table 4). While concentrations of the other two were not significantly different between men and women (in either group 1 or 2), those of C-peptide tended to be higher in men from group 1.

Of cytokines assay detectioned, (Supplemental file 1) 19 were above the lower levels of note, IL-6, which has been shown to increase after GH administration [23], tended to be higher in men than women in both the bGH and iGH groups. The differences were not statistically significant. IL-4 (a mediator of TH2 cells) was near the level of detection, a result which may reflect the decrease in TH cells with age. IP-10 (CXCL-10) is a chemoattractant for T cells, NK cells, monocytes, and was significantly decreased in women with no bioactive growth hormone compared to all the other groups. The other cytokines, chemokines and pro-inflammatory markers were not significantly different among groups.

Table 5 shows the values of the CD25 and regulatory T cells in older men and woman. Women had a significant higher value for the $\%CD45^+ CD4^+ CD25^+$, and in whole blood for $\%CD45^+ CD4^+ CD25^+$ in all cells, and $\%CD45^+ CD4^+ CD25^+ CD127(-)$ in all cells. Women also had a significantly higher values for whole blood in $\%CD45^+ CD4^+ CD25^+$ and %CD45 + CD4 + CD25 + CD127(−) in all cells when compared to men in the same subgroup with a bGH signal. Women in Group 2 with no bGH signal were also significantly higher for whole blood for values of %CD45 + CD4 + CD25 + in all cells compared to men. In this group of healthy aged adults there was not, on average, a significant decrease in the CD4:CD8 ratio. There was however a trend toward an increase in this value in women compared to men both in the group 1 and group 2. There was considerable variability among individuals, a result which can probably be attributed to diet, age, and health status.

Table 3
The values (Mean ± SEM) for testosterone, cortisol, and IGF-I of men (n = 16) and woman (n = 25), Additional subgroups of those that had a bioactive assay signal (bGH) (Men n = 6; Women n = 14) and those that had a very low bioactive assay signal (LbGH) (Men n = 10; Women n = 11) are also presented.

	Men	Women	Men bGH	Women bGH	Men LbGH	Women LbGH
Testosterone ($nmol \cdot L^{-1}$)	10.3 ± 3.8	4.5 ± 1.4	4.4 ± 1.0*	6.2 ± 2.5	13.8 ± 5.9	2.4 ± 0.4
Cortisol ($nmol \cdot L^{-1}$)	403.7 ± 63.2	312.8 ± 21.7	286.9 ± 70.1	295.2 ± 31.8	473.8 ± 87.0	335.3 ± 28.4
IGF-1 ($nmol \cdot L^{-1}$)	19.3 ± 1.4	18.2 ± 1.3	21.6 ± 2.3	18.0 ± 2.0	17.8 ± 1.6	18.5 ± 1.7

* $p \leq 0.05$ from corresponding other groupings of men's values.

Table 4
The values (Mean ± SEM) for ghrelin, myostatin, sex hormone-binding globulin (SHBG), neuropeptide-Y, C peptide, and vascular endothelial growth factor (VEGF) in men (n = 16) and woman (n = 25) are presented. Additional subgroups of those that had a high bioactive assay signal (bGH) (Men n = 6; Women n = 14) and those that had very low bioactive assay signal (LbGH) (Men n = 10; Women n = 11) are also presented.

	Men	Women	Men bGH	Women bGH	Men LbGH	Women LbGH
Ghrelin ($ng{\cdot}mL^{-1}$)	16.1 ± 4.5	14.9 ± 3.4	13.3 ± 7.1	14.6 ± 2.9	17.8 ± 6.0	15.3 ± 7.1
Myostatin ($ng{\cdot}mL^{-1}$)	24.1 ± 19.0	8.2 ± 1.3	56.6 ± 50.5	10.0 ± 2.2	4.6 ± 1.1	5.9 ± 0.9
SHBG ($nmol{\cdot}L^{-1}$)	63.5 ± 6.4	71.4 ± 6.5	53.5 ± 8.2	65.7 ± 9.6	69.4 ± 8.8	78.5 ± 8.1
NPY ($pg{\cdot}mL^{-1}$)	39.0 ± 2.7	**49.9 ± 4.1***	39.4 ± 3.4	**55.4 ± 6.5***	38.8 ± 4.0	42.9 ± 3.8
C peptide ($pmol{\cdot}L^{-1}$)	1365.0 ± 213.7	966.5 ± 115.3	1853.4 ± 393.8	1087.0 ± 175.2	1072.0 ± 211.2	813.1 ± 132.4
VEGF ($pg{\cdot}mL^{-1}$)	69.7 ± 11.0	58.3 ± 12.4	60.7 ± 19.0	60.6 ± 21.2	75.1 ± 13.9	55.6 ± 11.7

* $p \leq 0.05$ from corresponding men's value.

Table 6 reflects the values of the B cells of men and woman as well as in the sub-group analyses. There were significant differences of $\%CD45^+CD19^+$ $CD69^-$ B cells between men and women. There was also a significant difference of $\%CD45^+CD8^+$ $CD69^-$ B cells between men and women and this difference was maintained in the group 2 that had no bioactive GH response.

Table 7 shows all 6 physical activity/function tests of the participant, only grip strength was found to be significantly different between men and women. None of these tests were significantly correlated with bGH measures. Furthermore, we failed to find significant correlations between bGH concentrations and quality of sleep reported by the participants (Supplemental file 2).

3.1. Regression analyses

A full simple regression matrix analysis with all of the dependent variables was run to identify any bivariate relationships among related to bGH. Interestingly, we observed only two peptides whose plasma concentrations were significantly inversely correlated with circulating bGH; NPY ($r = -0.51$) and IL-4 ($r = -0.37$).

In order to explore predictive models that might help to explain relationships to bGH, we employed a hierarchical stepwise regression analyses. We found 5 levels of hierarchical models that could explain the variance in the data set of the 20 individuals in group 1 (i.e., those that had measureable quantities of bGH). Each of these 5 models, shown in Table 8, were statistically significant and no multi-collinearity was noted. Model 5 data can account for 86% of the statistical variance.

4. Discussion

Healthspan has been defined as the period of life in which an individual is generally healthy and free from serious disease. We sought to define what relationships, if any, existed between circulating bGH, iGH in this age of the healthspan. Our sample consisted of individuals who are likely to be experiencing an expected decline in activity of their somatotropic axis. Our approach tested circulating metabolic biomarkers, from both the endocrine and immune systems in addition to the physical performance characteristics of each participant. Our results suggest four key findings: First, the dichotomy between circulating bGH vs iGH concentrations in this group of elderly individuals (Fig. 1 and Table 2) was confirmed and is similar to those reported in over 6 previous studies involving testing of much younger participants e.g., [5,6,20,24–27]. To the best of our knowledge, this question has never been addressed in elderly, free-living people. Second, one half of our study group had bGH concentrations, in the 4 ml of plasma sample tested, that were at the sensitivity of the tibial bioassay, which is 5 μg in our hands. Surprisingly, there was no correlation in either sex or age between the two groups that had, or had very low detectable bGH. Third, using a hierarchical regression method, we found 6 potential "biomarkers" that appeared to be associated with bGH measures from group 1 participants (i.e., those that had higher circulating bGH); viz. NPY, IL-4, GHRH, insulin c-peptide, VEGF and regulatory T lymphocytes. How these biomarkers might be related bGH is considered in the discussion below. Fourth, with the exception of grip strength, we found no correlations or predictive indicators between bGH concentrations and performance tests, all of which obviously reflect the physical activity or functional status of the participant. This lack of correlation was surprising since in several studies involving younger people, we and others had found convincing evidence that heavy resistance exercise training or plantar dorsiflexion of increased circulating bGH and was related to strength gains [6,27–29]. Further, results from animal studies reveal that severance of certain muscle afferents (tibial and peritoneal nerves) arising from the lower leg, dramatically reduces bGH concentrations in both the rat pituitary and plasma, but it is not known whether this control mechanism resides at the level of the hypothalamus or pituitary [30]. Interestingly, a complete 2-week bed rest in human volunteers will also produce a similar result, i.e. failure of an exercise induced rise in plasma bGH [28]. Finally, we recently

Table 5
The values (Mean ± SEM) for lymphocytes of men (n = 16) and woman (n = 25) are presented. Additional subgroups of those that had a bioactive signal (bGH) (Men n = 6; Women n = 14) and those that had very low bioactive assay signal (LbGH) (Men n = 10; Women n = 11) are also presented.

	Men	Women	Men bGH	Women bGH	Men LbGH	Women LbGH
Lymphocyte						
$\%CD45^+$	29.6 ± 1.7	27.7 ± 2.0	29.0 ± 2.8	28.9 ± 2.9	29.9 ± 2.3	26.3 ± 2.7
$\%CD45^+CD4^+CD25^+$	10.1 ± 1.3	**27.8 ± 3.9***	12.7 ± 2.8	**28.7 ± 5.7***	8.5 ± 1.2	26.7 ± 5.4
$\%CD45^+CD4^+CD25^+CD127(-)$	87.1 ± 6.1	94.0 ± 3.8	89.3 ± 5.0	98.1 ± 0.6	85.8 ± 9.5	88.9 ± 8.6
Whole blood						
$\%CD45^+CD4^+CD25^+$ in all cells	3.0 ± 0.4	**8.0 ± 1.3***	3.8 ± 1.0	**8.5 ± 1.8***	2.5 ± 0.4	**7.5 ± 2.0***
$\%CD45^+CD4^+CD25^+CD127(-)$ in all cells	2.7 ± 0.4	**7.4 ± 1.3***	3.3 ± 0.8	**8.2 ± 1.7***	2.4 ± 0.4	6.4 ± 2.0

* $p \leq 0.05$ from corresponding men's value.

Table 6
The values (Mean ± SEM) percentages of B cells, T_H, and T_C men (n = 16) and woman (n = 25), bioactive growth hormone (BGH) men and women. Additional subgroups of those that had a bioactive signal (bGH) (Men n = 6; Women n = 14) and those that had a very low signal bioactive assay signal (LbGH) (Men n = 10; Women n = 11) are also presented.

	Men	Women	Men bGH	Women bGH	Men LbGH	Women LbGH
Lymphocyte						
%$CD45^+$	27.5 ± 2.1	31.2 ± 2.7	25.3 ± 2.7	33.0 ± 4.5	28.8 ± 3.0	28.8 ± 2.1
$CD19^+$ $CD69^+$ dividing B cells	1.2 ± 0.4	1.9 ± 0.8	2.1 ± 1.0	2.1 ± 1.3	0.7 ± 0.3	1.7 ± 1.0
$^+CD19^+$ $CD69^-$ non-dividing B cells	6.9 ± 1.0	**18.8 ± 4.4***	6.7 ± 1.2	17.7 ± 6.3	7.0 ± 1.4	20.2 ± 6.2
$CD4^+$ $CD69^+$ dividing T_H cells	0.7 ± 0.3	0.7 ± 0.2	1.2 ± 0.7	0.6 ± 0.2	0.4 ± 0.1	0.8 ± 0.4
$CD4^+$ $CD69^-$ non-dividing T_H cells	45.4 ± 2.9	43.5 ± 3.0	43.2 ± 4.9	40.1 ± 4.5	46.8 ± 3.8	47.7 ± 3.6
$CD8^+$ $CD69^+$ dividing T_C cells	2.3 ± 1.5	1.6 ± 0.6	4.7 ± 3.9	2.1 ± 1.0	0.9 ± 0.1	0.9 ± 0.3
$CD8^+$ $CD69^-$ non-dividing T_C cells	25.5 ± 2.1	**18.9 ± 1.8***	23.9 ± 2.5	20.4 ± 2.5	26.4 ± 3.1	**17.0 ± 2.7***
%$CD45^+CD3^-$ $CD56^+CD16^{+\%total}$ gate NK cells	0.09 ± 0.1	0.04 ± 0.007	0.02 ± 0.005	0.04 ± 0.008	0.14 ± 0.1	0.04 ± 0.01

* $p \leq 0.05$ from corresponding men's value.

Table 7
This table (Mean ± SD) reflects the values of the grip (right and left), stand, chair rise, walk, and get up and go of men (n = 16) and woman (n = 25). Additional subgroups of those that had a bioactive assay signal (bGH) (Men n = 6; Women n = 14) and those that had very low bioactive assay signal (LbGH) (Men n = 10; Women n = 11) are also presented.

	Men	Women	Men bGH	Women bGH	Men LbGH	Women LbGH
R Grip Best (kg)	32.2 ± 2.3	**20.8 ± 1.0***	33.5 ± 5.0	21.6 ± 1.4	31.4 ± 2.4	**19.9 ± 1.4***
L Grip Best (kg)	29.6 ± 3.3	**20.0 ± 1.1***	34.5 ± 5.6	**19.8 ± 1.5***	26.6 ± 4.0	20.4 ± 1.6
Stand Best (sec)	10.0 ± 3.7	14.5 ± 3.6	9.6 ± 6.3	18.4 ± 5.0	10.2 ± 4.9	9.5 ± 4.9
Chair Rise (sec)	15.4 ± 0.9	16.5 ± 1.6	14.2 ± 1.3	15.3 ± 2.0	16.1 ± 1.1	18.0 ± 2.7
Walk Best (sec)	2.5 ± 0.2	2.8 ± 0.2	2.7 ± 0.4	2.8 ± 0.3	2.4 ± 0.1	2.8 ± 0.3
Get up and Go (sec)	16.4 ± 1.2	18.8 ± 2.4	18.7 ± 3.0	16.6 ± 1.8	15.0 ± 0.8	21.5 ± 4.9

* $p \leq 0.05$ from corresponding men's value.

Table 8
The additive models for a hierarchical step-wise regression analysis for people who have bGH signal for men and women combined is presented below (n = 20).

Model	R	R square	Adjusted R square	Std. error of estimate	F	Significance
1	0.688[a]	0.474	0.441	4445.8	14.42	0.002
2	0.856[b]	0.733	0.697	3271.9	20.58	0.000
3	0.901[c]	0.811	0.771	2848.5	20.03	0.000
4	0.899[d]	0.808	0.783	2770.1	31.65	0.000
5	0.940[e]	0.884	0.859	2235.1	35.45	0.000

[a] (1). Predictors: (Constant), GHR.
[b] (2). Predictors: (Constant), GHR, C_PEPTIDE.
[c] (3). Predictors: (Constant), GHR, C_PEPTIDE, VEGF.
[d] (4). Predictors: (Constant), C_PEPTIDE, VEGF.
[e] (5). Predictors: (Constant), C_PEPTIDE, VEGF, Whole blood_$CD45^+$ $CD4^+$ $CD25^+$ CD127(1) all cells.

reported that an exercise-induced response rise in plasma bGH occurred in 30 years old, but not 60 year old women participating in the same study group [18]. Based on these reports we initially hypothesized that analogous control mechanisms (indirectly reflected by physical activity, such as gait speed, and other health-span status markers of each participant) in our study group (mean age = ~81 years), might reasonably be expected to reflect differences in concentrations of circulating bGH. Our data clearly indicate that this is not the case.

What is bGH? The provocative and prescient review by Lewis in 1992 [31] emphasized that pituitary GH is not a single substance. Approximately 10% of the dry weight of the pituitary is GH, an amount that is ~800 × that of any other anterior pituitary hormone. In his report, Lewis argued for the need to address the question of GH heterogeneity from the standpoint of both physiology and chemistry. The large number of iGH variants in human pituitary extracts, and their structures have been reviewed [5], but much less is known in terms of the physiological response(s) that such variants might evoke. The working hypothesis by Lewis, and others at that time was that "intact" 22 kDa GH acted as a prohormone … "from which metabolically active peptides are produced by proteolytic processing" [32].

Do native, physiologically active bGH variants reside in the pituitary, or are they generated when GH reaches its target receptors? Both possibilities are likely. With respect to the rat tibia bioassay for hGH, the multi-center collaborative study by Bristow and Jeffcoate [33] showed that pretreatment of 22 kDa rhGH with subtilisin (a serine protease) prior to activity testing in different types of GH assays [including a) the rat tibial line bioassay, b) an in-vitro receptor bioassay, immunoassay, c) an HPLC reverse phase assay or a size exclusion assay], resulted, (relative to untreated control samples) in *increased* tibial plate widths of hypophysectomized rats using the tibial assay. However, this same treatment resulted in *degraded* activity in all in vitro assays. Where might "physiological activation" of bGH occur? The data of Schepper et al. [34] indicated that the 22 kDa form of hGH is cleaved by preparations of rabbit liver plasma membranes,(but not microsomal membranes), in the large disulfide loop to generate a two chain variant of hGH. This two chain variant is ~2 × as active in the tibial assay as the intact form [35].

A bGH form that is undetectable by routine antibody measurements was reported by our group in 2000 [36]. We described the isolation of a unique "GH" – like species, present in the human postmortem pituitary gland, which has no hGH activity by immunoassay, but is active in the rat tibial assay. This peptide, purified by free flow electrophoresis and anion exchange chromatography was 3374 kDa [mass spectrometry] and had an isoelectric point of 5.1. Although it was blocked at the N terminus, the peptide had a string of middle hydrophobic residues [9–25] which bore sequence similarity to insulin c-peptide [36]. Our finding was later confirmed by Grindeland (personal communication).

Size exclusion column chromatography has also been used to study bGH variant forms in post exercise human plasma. We reported that ~30–40% of the bGH activity recovered in human plasma emerges from the column as either dimeric (~44 kDa) or higher molecular weight aggregated, oligomeric species [7,18]. Although the volume of plasma sample collected in our current experiment was insufficient to use this gel filtration separation technique, we found that treatment of the plasma samples under reducing conditions (GSH pre-treatment) did,

in fact, result in increased immune- reactivity of samples from the women, but not men, and only in Group 2 individuals (Table 2). We have attributed similar increases to the presence of disulfide linked forms of hGH in human plasma samples [5,7]. Why individuals in group 2, but not 1, showed this interesting response difference warrants further investigation. In summary, some evidence suggesting that variant forms of GH may account for bGH assay differences between participants in group 1 vs 2 appears feasible and offers a rationale for further testing.

Additional insight as to what may account for GH activity dichotomy comes from our cellular and subcellular studies with rat pituitary tissue. We have reported that 2 sub-populations of pituitary somatotrophs that can be separated by density gradient centrifugation. One of these produces and releases GH with higher tibial line bioactivity than the other [37,38]. Whether the dichotomy in human GH measurements we report herein might also be attributed to somatotroph sub-population heterogeneity in the human pituitary gland is of course unknown.

Precisely how 5 biomolecules in the group 1 plasma samples (GHRH, c-peptide, VEGF, NPY, IL-4, and regulatory T lymphocytes) might help account for bGH detectability in this population sub-group is unknown. Evidence for possible mechanisms may be gleaned from the literature. In this section, we briefly summarize the more important possibilities.

The data in Fig. 1 could be explained by expression patterns of NPY and its receptor subtypes (predominantly Y1R and Y2R), in hypothalamic and pituitary tissue. A recent review indicated that this neuropeptide plays a role in extending lifespan by down-regulating often suggested hallmarks of aging; viz. loss of proteostasis, stem cell exhaustion, altered intercellular communication, deregulated nutrient sensing, cellular senescence, and mitochondrial dysfunction [39]. Further, a genome-wide search for human genes involved in the up-regulation of fat free body mass (i.e. mainly skeletal muscle and bone mass) was found to include candidate gene regions associated with phenotypes identified as NPY, IGF-I and GHRH receptor genes [40]. We speculate that the association between bGH and NPY in women from group 1 is strong and may reflect a categorical longer lifespan prediction. Modification of NPY circuits in the hypothalamic arcuate nucleus of the Y2R knockout mouse control bone formation [41]. These knockout mice a) have increased trabecular bone (2 ×) distal to the epiphyseal growth plate that extends several mm proximally, b) have increased osteoblast activity and c) increased bone mineralization and formation. Because no changes in circulating Ca, anterior pituitary hormones (including GH) or reproductive hormones in this (or other studies aimed at modifying NPY circuits), the general thinking has been that NPY/Y2R does not modulate bone formation by humoral factors, but rather by central regulatory circuits (including GH, cited in Baldock et al. [41] (See their refs 24 and 26). The study by Huang et al. [42] showed that genetic modifications of NPY, and its receptor subtypes, modulate pulsatile GH secretion in complex ways which reveal that GH output is “tightly coupled” to food intake via hypothalamic mechanisms. NPY neurons, long known to stimulate somatostatin release and thereby suppress GH release [43], might do so via a NPY receptor subtype. In fact, the data of Huang et al. [42] show that body length measurements (nose to rump) of the Y2R knockout mice are significantly shorter than their wild-type littermates. Body lengths of the Y1R nor NPY knockout mice were not different from their wild-type control littermates.

Mechanism studies such as those involving genetic modifications in mouse model systems are obviously not possible in human studies. However, a recent anatomical study of post-mortem human hypothalamic tissue offers evidence that although most of the GHRH neuronal elements received contacting somatostatin axonal varicosities in the arcuate nucleus, GHRH axonal varicosities were only rarely contacted by somatostatin perikayra. The finding of such juxtaposition was postulated to offer an additional mechanism of GH release control over the one that uses the traditional release mechanism of hypothalamic peptides directly into the hypophysial portal system. If these juxtapositional elements are eventually found to be influenced directly by changing activities of NPY receptor subtypes, then the finding that ~one half of the individuals in our study did not have detectable bGH might be explained by such a mechanism. On the other hand, those individuals that had detectable bGH in their plasma might have active GHRH neuronal involvement, not over-ridden by NPY, that would account for the results of model 1 (Table 8) in our hierarchical regression analysis.

A recent report shows that NPY expression in human pituitary adenomas was detected in 34 of 57 cases while NPY mRNA expression was found in all 57. Furthermore, mRNA expression of Y1R and Y2R was found across all subcategories of adenoma and expression was positively correlated with Y2R with “remarkable statistical significance” [33].

The association between bGH and VEGF in group 1 plasma samples was surprising and one which we speculate might be related to hGH amyloid. As shown in a recent report [44], small segments of the 191 amino acid hGH monomer (residues 72–82, by Tango analysis) as well as 4 fibrillation segments (each 6–10 residues, by Zipper DB software) are maintained as B aggregation “hot spots”, even while allowing the helical character of the entire GH molecule to be maintained. Furthermore, Hohman et al. [45] found that elevated VEGF in cerebral spinal fluid was associated with a neuro-protective effect in individuals showing enhanced Alzheimer disease biomarkers. Other literature associates lower VEGF levels in relation to brain aging outcomes, but only in amyloid positive people (see ref. 13 in Hohman et al.). Both of these neuroimaging reports, taken together with new information about GH and its folding configuration(s), all point to fruitful areas for new study.

C-peptide is one of the new biomarkers that predicts health span success. In a recent review, c-Peptide had a Hazard ratios for all-cause mortality of 1.42, $p < 0.001$; cancer-mortality 1.62, $p < 0.009$; and CVD-mortality of 1.31, $p = 0.033$; all data which make the case for c-peptide measures as a primary biomarker for health-span [46]. C-peptide also has been used as a predictor of diabetes [47]. The finding that c-peptide was found to play a significant role in statistical models that predict bGH levels in group 1 individuals (Table 10) is considered highly relevant and worthy of further study. In that context, we also emphasize that the 3374 kDa peptide we isolated from the postmortem human pituitary had sequence similarity to C-peptide. This peptide was un-detectable by GH immunoassay (27). Unpublished evidence also indicates that this pituitary peptide is associated with a secretory granule fraction. We point out that similarities in processing events between insulin in the pancreatic beta cell granule and of GH the pituitary somatotroph granule [44] offers yet another rationale for further testing of C-peptide/bGH associations in human plasma samples. We speculate that differential secretion patterns of this pituitary peptide might account for the differences between bGH levels in plasma between group 1 and 2 individuals.

The association between GHRH and bGH may be related to a novel physiological mechanism, recently reviewed by Fridyland et al. [48]. These authors point out that *in addition* to the well documented neuroendocrine action of GHRH, literature clearly establishes a role for GHRH and its receptor in other tissues and cell targets, such as the pancreatic beta cell, in modulating glucose metabolism. Because GHRH undergoes rapid enzymatic degradation in the blood, they further suggest that extra-hypothalamic GHRH may be produced via autocrine/paracrine mechanisms.

Many factors that we tested yielded results that failed to offer additional insight into the major hypothesis under test. Ghrelin-stimulated release of iGH from the pituitary is postulated reflect physiological regulation of different tissue systems. For example, ghrelin has been associated with a) impaired thermogenesis during aging, especially in regard to obesity and diabetes [49]; b) insulin resistance [50,51]; c) thymus function and aging [51] and promotion of

slow wave sleep [52]. Our data failed to reveal significant associations in ghrelin concentration differences between individuals in group 1 vs 2, nor differences in their sleep quality.

Addition of a chemical reducing agent, such as mercaptoethanol or glutathione, to a sample containing a mixture of GH isoforms, offers a simple way to assess the presence of disulfide linked GH aggregates in that sample, since that treatment results in increased GH immunodetectability. We have often used this test while evaluating effects of resistance exercise on release of aggregates from the pituitary gland during and after an exercise regimen [5,7] in an effort to understand complex relationships between iGH and bGH in plasma. Our most recent study showed that an acute resistance exercise stimulated greater release of iGH dimeric forms in plasma from ~22 year old women vs those from ~22 year old men [22]. The results we now report (Table 2) also show that iGH concentrations in plasma of elderly subjects are not only sexually dimorphic, but that women have significantly more iGH after chemical reduction of the sample. Interestingly, this result is specific for women in group 2, but not group 1 (Table 2). Sporadic reports indicate that the hGH covalent dimer has very low activity in the rat weight gain assay [35] as well as in the tibial line assay [33]. Whether the GH aggregates in group 2 women reflect their non-functional action in terms of bone growth obviously requires more research.

Further, our tests sought to find out if any obvious associations between GH, steroid regulators of muscle mass, cytokines, lymphocytes, and dimorphism existed. Our regression analyses failed to indicate such associations. In conclusion, we believe that failure to find obvious relationships between muscle/steroid variables, tested in the context of bGH, tends to favor an alternate physiology, viz. stronger associations with brain-pituitary interactions already discussed. Additionally, with the exception of IL-4 and regulatory T lymphocytes, a majority of the immunology-related measured failed to reveal differences in plasma levels of these markers in samples from group 1 vs group 2.

A recent, and provocative report [8] indicates that elements of the GHRH/GH/IGF-1 axis may have bearing on issues of immune-senescence in the context of health-span and function in the elderly. They point out that pituitary involvement in thymus function, (suspected as long ago as 1930 when it was found that hypophysectomy lead to thymic involution and loss of thymopoiesis), may be a factor linking the endocrine and immune systems. By 1970 it had been shown that administration of GH antisera could also induce thymus atrophy. More recent data from animal models suggest that GH supplementation will improve thymic cellularity and T cell proliferation of the dwarf mouse. In cultured human thymic epithelial cells, Bodart et al. [53] demonstrate a “strong relationship” between IGF-1 and IL-7 and suggest that a) acute stressors may be considered as discriminators of susceptibility to frailty and further b) that GH may counteract deleterious immunosuppressive effects of steroids. IGF-1, a mediator of GH, maintains normal thymopoiesis and T lymphocyte function [23]. CD4 T helper cells decrease during aging, a change which in turn decreases the CD4:CD8 ratio. Such a loss in T helper cell function was postulated to explain frailty observed in the elderly population. However in our current study we found no sub-group differences in CD4:CD8 ratios (Table 6) between men and women or between individuals in groups 1 and 2.

5. Conclusion

In conclusion, the results of this study show that antibody based measurements of circulating hGH may not necessarily reflect their full biological activity, a result similar to that we have reported previously when testing plasma donors of younger people. Even though one half of the participants (n = 21) had detectable plasma iGH in the expected range, those same samples, upon injection into the young hypophysectomized rat, failed to produce widening of the growth plate in the tibial bioassay. On the other hand, 20 individuals had GH levels that were able to mount variable, but detectable, growth responses in that same bioassay. These differences were not related to either gender, age or physical activity status.

Hormone and cytokine analyses showed that two markers, viz. NPY and IL-4, were significantly (and negatively) correlated with the presence of plasma bGH in the sample. However, when the entire data set was analyzed by hierarchical stepwise regression methodology, several models emerged which collectively show that GHRH, c-peptide, VEGF and T regulatory lymphocytes can account for 86% of the shared variance predicting bGH. We believe that this molecular diversity reflects the influence of different tissue systems on specific, complex cellular and subcellular events within a heterogeneous population of pituitary somatotrophs. In turn, such events may regulate processing and release of GH variants known to be present within the GH family of molecules. Whether any of these molecules have the potential to serve as biomarkers for circulating bGH obviously sets an important experimental basis for further investigation.

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Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state this:

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