SUPPLEMENTARY INFORMATION

Alveolar macrophages from overweight/obese asthmatic subjects demonstrate a pro-inflammatory phenotype

Njira L. Lugogo[1], John W. Hollingsworth[1,2], Druhan L. Howell[3], Loretta G. Que[1], Dave Francisco[1], Tony D. Church[1], Erin N. Potts-Kant[1], Jennifer L. Ingram[1], Ying Wang[1], Sin-Ho Jung[4], Monica Kraft[1]

[1] Department of Medicine, Duke University Medical Center

[2] Department of Immunology, Duke University Medical Center

[3] Departments of Medicine and Pediatrics, University of South Alabama College of Medicine

[4] Departments of Biostatistics and Bioinformatics, Duke University Medical Center

Corresponding Author: Njira Lugogo, M.D., Instructor in Medicine, Duke University Medical Center, PO Box 2629, Durham, NC 27710, Phone: (919) 613-5707, Fax: (919) 668-0494, Email: njira.lugogo@duke.edu

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Methods:

We initially choose the dose of leptin based on prior publications assessing the response of peritoneal macrophages to leptin in the presence and absence of LPS. Loffreda *et al.* reported a lack of response to leptin in peritoneal macrophages in the absence of LPS. However, pre-stimulation with 500ng/ml of leptin resulted in a robust pro-inflammatory response to LPS[(1)]. Secondly, we performed dose response experiments and have included the dose response curves below. We determined that the most robust response was occurred at the 250ng/ml dose (**Figure E1**).

The response to LPS was modest but not uncommon when human cells are studied. There is limited data in the literature on the expected fold-change response to LPS in human lung macrophages. Responses in mice are more robust than demonstrated in our cohort. We choose 90 minutes based on prior experiments by Loffreda *et al*. assessing innate immune responses in peritoneal macrophages to leptin and LPS. The paper demonstrated responses to LPS at 90 minutes. We performed time course experiments in macrophages obtained from overweight asthmatics (*data not shown*) and noted a significant response. Therefore, we chose this time point for the studies in the larger cohort. The physiologic relevance of the results in this pilot study are unclear at this time, although we believe that the differential response noted in macrophages from obese asthmatic subjects raises intriguing questions that should be studied more extensively in the future.

Results:

Effect of obesity and asthma on adipokine levels in the BALF

Previous work has shown increased levels of leptin in both serum and BALF from obese subjects[2, 3]. These data support prior publications by Holguin *et al.* However, our cohort of subjects is different as the asthmatics in this cohort exhibited both reversibility and methacholine positivity but had mild asthma that was *not* treated with inhaled corticosteroids. Inhaled corticosteroids have the potential to alter the inflammatory milieu in the lung. We also identified that obesity had an effect on BALF leptin levels with increased levels in obese subjects, when compared to lean controls ($p=0.013$). We thereafter aimed to determine the correlation between leptin and obesity as measured by BMI. Linear regression analysis revealed that log leptin level was significantly correlated with body mass index when all subjects were included ($r^2=0.34$, $p<0.0001$) (**Figure E2A**). This correlation was more closely associated within subjects with asthma ($r^2=0.47$, $p<0.0001$). We were interested in the effect of gender and therefore we compared the association of BALF log leptin levels and BMI in female and male subjects. We observed a significant correlation between leptin and BMI in both females ($r^2=0.44$, $p<0.0001$) and males ($r^2=0.20$, $p=0.04$), although the correlation was stronger in females **(Figure E2B and E2C).** Interestingly, we observed no differences in mean BMI between male and female subjects (26.2 ± 0.88 versus 28.78 ± 1.16, $p=0.15$ respectively). However, the range of BMI values appeared quite different between males and females (21.32-38.87 in males and 18.0-60.9 in females). Together, these findings support that BALF leptin levels are associated with BMI.

<u>Association between atopy and obesity status</u>

To determine the effects of obesity on asthma phenotype, we obtained BALF and performed differential cell counts and then assessed the effects of obesity, asthma and the

interaction of these factors on BALF differentials (**Table E1**). Overall, 85% of the BALF cells were macrophages in both normal and asthma subjects. The numbers of lymphocytes, neutrophils, and eosinophils were not significantly altered by the presence of either asthma or obesity. In this cohort, 13% of lean asthma subjects and 15% of obese asthma subjects had greater than 2% eosinophils in BALF. Only 3.8% of the normal subjects had greater than 2% eosinophils (*data not shown*). The presence of greater than 2% eosinophils is suggestive of an allergic or Th2 driven phenotype. Notably, 100% of lean asthma subjects and 88% of obese asthma subjects had reported a history of atopy. The prevalence of eosinophilic asthma, as defined by > 2% BALF eosinophils, was low in both obese and lean asthmatics. We report a high incidence of patient reported allergic rhinitis in our cohort, a finding that is similar to prior publications[4, 5]. The use of serum IgE may have altered the percentage of subjects thought to have allergic disease. Dixon *et al.* demonstrated a significant correlation between change in airways hyperresponsiveness following weight loss and non-atopic status as defined by a low serum IgE but no changes in inflammatory markers with weight loss[5]. Shore *et al.* demonstrated increased allergic responses that were augmented with leptin treatment resulting in increased BALF IL-5 levels and increased serum IgE[6]. We identified differences in inflammation that have not previously been characterized. Previous observations could be resultant from differences in baseline asthma therapies. This is the first publication that includes a cohort of subjects that is *not* receiving inhaled corticosteroid therapy that could alter the inflammatory milieu in the lung. Our study suggests that leptin may play a role in both non-allergic and allergic responses in the lung but that differences in leptin levels in BALF may not be the differentiating factor between the two proposed obese asthma phenotypes: the early-onset

atopic phenotype and an adult onset non-atopic phenotype[4]. However, our *ex vivo* macrophage studies were not powered to determine whether macrophage responses would differ based on the presence of atopy.

Functional consequences of leptin and LPS on lung macrophages

The macrophage response was reported as fold change in comparison to un-stimulated cells obtained from the same subject. We were interested in determining if constitutive levels of cytokines were significantly different between groups. Therefore, we performed a one-way ANOVA comparing baseline levels of cytokines obtained from the four groups of subjects and detected significant differences in the baseline levels of IL-5 ($p=0.008$ with lean and obese asthmatics and obese normal subjects demonstrating higher levels of IL-5 than lean normal subjects). Additionally, baseline levels of IL-10 were significantly higher in obese normal subjects than the other three groups ($p=0.02$). We did not observe lower levels of baseline cytokine production from obese asthmatics and therefore do not attribute the increased fold change in cytokine levels to a larger difference in comparison to baseline levels but rather a more robust response to stimulation.

For the *ex vivo* experiments assessing functional response of macrophages to leptin and LPS, we determined if comparisons of absolute levels of various cytokines would be superior to analyzing fold changes. To determine if there were significant differences in results obtained we analyzed the data based on absolute levels of cytokines in the supernatant (not normalized to baseline levels) and noted no significant differences between the four groups in levels of cytokines produced post leptin and LPS stimulation. There is a wide range of baseline cytokine levels that may account for the lack of observed

response when cytokine production is not corrected for the baseline variability. For instance, baseline TNF-a levels range from 103.2 to 921.03 pg/ml. We believe correction for the constitutive levels of cytokines most accurately reflects the biological response to leptin and LPS. Therefore, we have analyzed the data as expressed by fold change defined as post-exposure level/baseline level of each cytokine involved.

Lastly, we performed analyses to determine the effects of weight status, asthma status and the interaction of these two factors on the difference in cytokine levels following exposure to leptin, LPS and leptin-LPS. The presence of asthma had a significant impact on TNF-α production in response to leptin (**Table E2-A**). Additionally, asthma status had an effect on IL-5 and IL-8 levels in response to LPS and leptin-LPS treatment (**Table E2-B**). The interaction between obesity and asthma resulted in significant alterations in TNF-α, IFN-γ, IL-8 and IL-10 levels in response to LPS (**Table E2-B**). Similarly, the effects of leptin pre-treatment followed by LPS exposure was altered by an obesity*asthma interaction with higher fold change levels of TNF-α, IL-8 and IL-6 (**Table E2-C**). These findings support the assertion that obesity and asthma intersect to create unique inflammatory responses in the lungs.

Table E1: BALF Differential Cell Counts: Values are reported in median [IQR].

			Normal Subjects		Asthma Subjects	
BAL Differential cell counts	**P-values**		**Lean *(n=17)***	**Overweight/ Obese *(n=17)***	**Lean *(n=12)***	**Overweight/ Obese *(n = 20)***
Macrophages (%)	O	0.68	94.27 [90.6, 96.4]	94.5 [79.38, 96.82]	87.18 [76.28, 97.59]	91.71 [79.13, 95.43]
	A	0.24				
	O-A	0.59				
Lymphocytes (%)	O	0.16	1.87 [0, 3.73]	0.16 [0, 4.66]	2.11 [0, 3.16]	0.19 [0,0.5]
	A	0.38				
	O-A	0.68				
Eosinophils (%)	O	0.68	0.51 [0.11, 1.29]	0.24 [0, 0.48]	0.36 [0, 1.78]	0.5 [0, 2.2]
	A	**0.026**				
	O-A	0.27				
Neutrophils (%)	O	0.78	1.05 [0.49, 2.98]	1.21 [0.49, 4.15]	0.97 [0.37, 8.17]	2.43 [0.95, 6.87]
	A	0.09				
	O-A	0.9				

Table E2: Macrophage Supernatant Cytokine Levels. Independent factors include weight (O), asthma (A) and the interaction term (O-A) on the difference between post-exposure and baseline cytokine level in response to leptin (**A**), LPS (**B**) and leptin-LPS (**C**). Cytokine levels are reported as fold change/negative control. P-values indicating significance are highlighted ($p<0.05$).

Supernatant Cytokine Level in Response to Leptin	**P-values**		**Normal Subjects**		**Asthma Subjects**	
			Lean (n=5) Fold Change	**Overweight/ obese (n=4) Fold Change**	**Lean (n=5) Fold Change**	**Overweight/ obese (n=8) Fold Change**
IL-5	**O**	0.83	0.75±0.11	1.02±0.08	1.31±0.18	1.34±0.11
	A	0.07				
	O-A	0.55				
TNF-α	**O**	0.57	0.83±0.1	0.96±0.09	1.26±0.27	2.21±0.46
	A	**0.02**				

	O-A	0.39				
IFN-γ	**O**	0.94	0.76±0.17	0.97±0.16	1.01±0.03	1.21±0.07
	A	0.63				
	O-A	0.71				
IL-8	**O**	0.89	1.04±0.07	0.86±0.45	1.05±0.26	1.82±0.12
	A	0.16				
	O-A	0.09				
IL-10	**O**	0.06	0.68±0.08	1.04±0.14	1.01±0.14	1.53±0.25
	A	0.08				
	O-A	0.08				
IL-6	**O**	0.88	0.98±0.06	1.58±0.64	1.04±0.15	1.21±0.22
	A	0.86				
	O-A	0.77				

Supernatant Cytokine Level in Response to LPS	**P-values**		**Normal Subjects**		**Asthma Subjects**	
			Lean (n=5) Fold Change	**Overweight/ obese (n=4) Fold Change**	**Lean (n=5) Fold Change**	**Overweight/ obese (n=8) Fold Change**
IL-5	**O**	0.94	0.84±0.06	0.87±0.07	1.38±0.24	1.72±0.23
	A	**0.002**				
	O-A	0.51				
TNF-α	**O**	0.56	2.65±0.81	1.27±0.31	2.13±0.77	4.13±1.06
	A	0.21				
	O-A	**0.03**				
IFN-γ	**O**	0.14	1.19±0.34	0.79±0.11	1.04±0.10	1.59±0.30
	A	0.59				

	O-A	**0.048**				
IL-8	**O**	0.92	1.26±0.06	0.57±0.07	1.94±0.71	3.52±0.60
	A	**0.011**				
	O-A	**0.024**				
IL-10	**O**	0.06	1.85±0.48	0.89±0.13	1.56±0.52	1.77±0.21
	A	**0.042**				
	O-A	**0.034**				
IL-6	**O**	0.30	1.95±0.51	1.14±0.30	1.94±0.97	2.62±0.44
	A	0.09				
	O-A	0.09				

Supernatant Cytokine Level in Response to Leptin 250+ LPS	**P-values**		**Normal Subjects**		**Asthma Subjects**	
			Lean (n=5) Fold Change	**Overweight/ obese (n=4) Fold Change**	**Lean (n=5) Fold Change**	**Overweight/ obese (n=8) Fold Change**
IL-5	**O**	0.38	0.66±0.11	0.94±0.17	1.46±0.31	2.29±0.36
	A	**0.002**				
	O-A	0.33				
TNF-α	**O**	**0.02**	2.66±0.96	0.54±0.24	2.08±0.68	5.15±1.05
	A	0.17				
	O-A	**0.003**				
IFN-γ	**O**	0.33	1.19±0.34	0.79±0.11	1.05±0.14	1.99±0.40
	A	0.37				
	O-A	0.09				
IL-8	**O**	0.63	1.04±0.07	0.86±0.45	1.05±0.26	1.82±0.12

	A	**0.015**				
	O-A	**0.035**				
IL-10	**O**	0.14	0.68±0.08	1.04±0.13	1.01±0.14	1.53±0.26
	A	0.11				
	O-A	0.10				
IL-6	**O**	0.26	1.82±0.58	0.62±0.32	1.55±0.60	3.72±0.97
	A	**0.048**				
	O-A	**0.041**				

Figure Legends:

Figure E1: Dose Response Curves to Leptin and LPS in Overweight/obese Asthmatic Subjects.

Figure E2: Correlation between BALF Log Normalized Leptin Levels and BMI.

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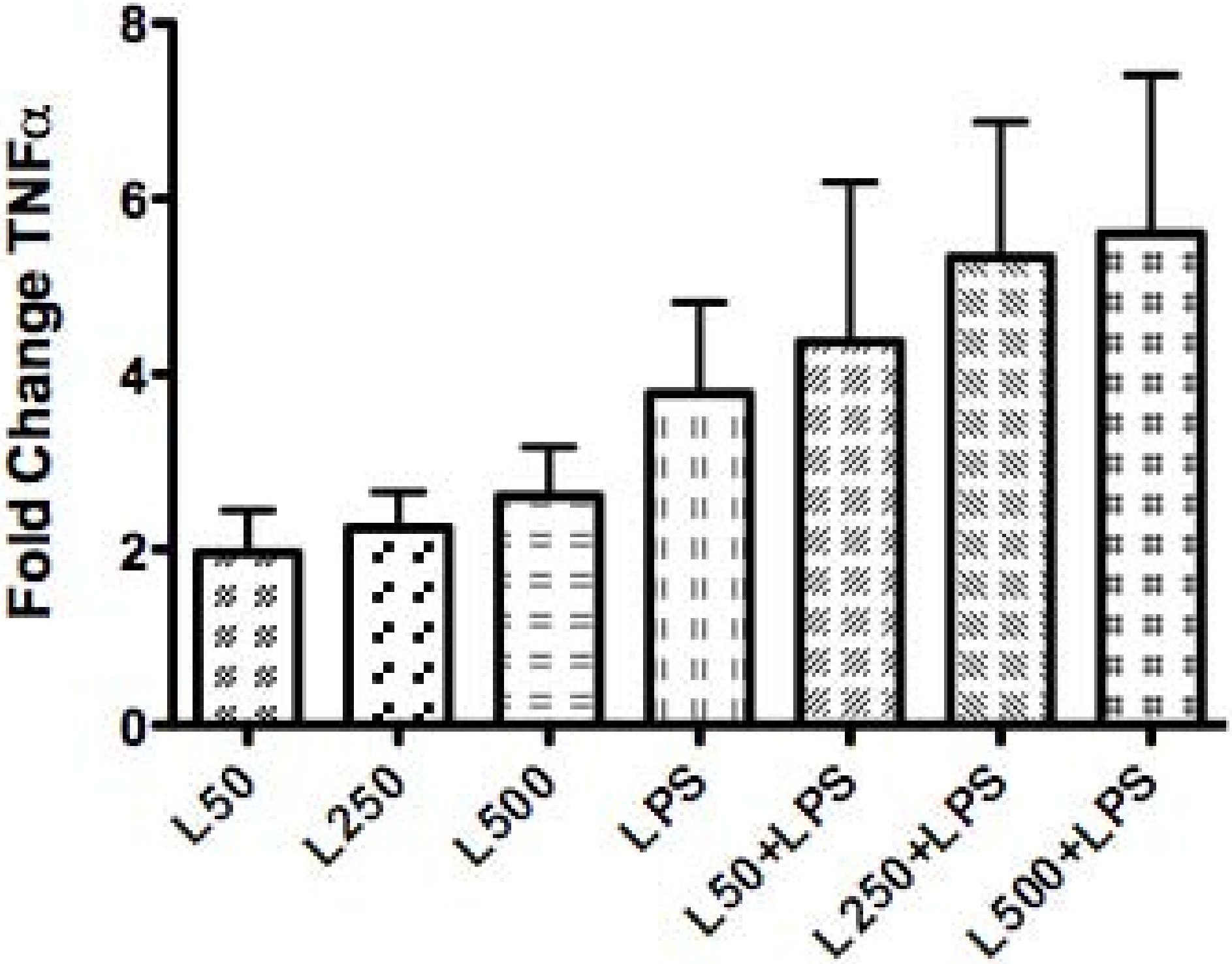
Fold Change TNFα
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6
4
2
0
L50
L250
L500
LPS
L50+LPS
L250+LPS
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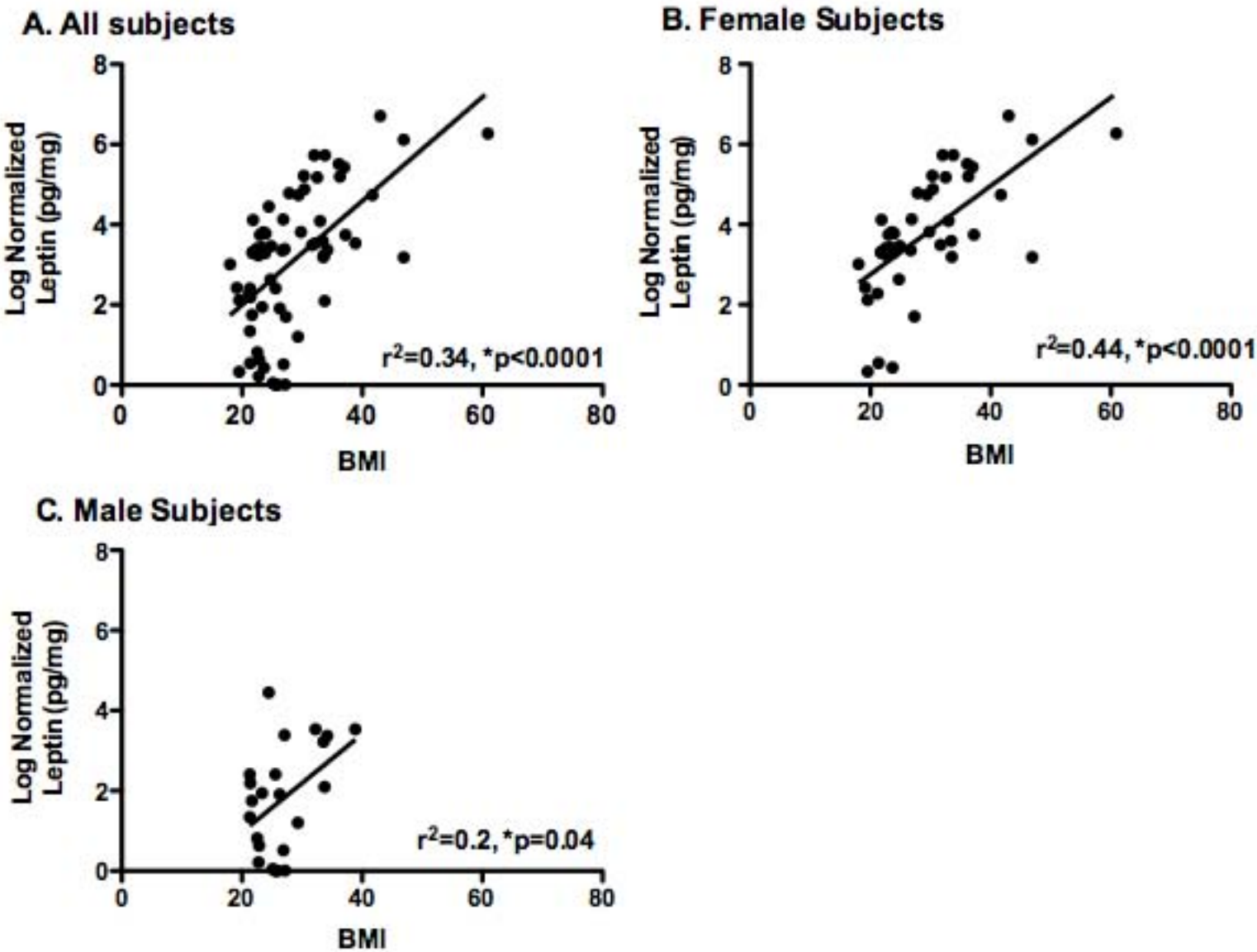
A. All subjects
Log Normalized Leptin (pg/mg)
BMI
r²=0.34, *p<0.0001
B. Female Subjects
Log Normalized Leptin (pg/mg)
BMI
r²=0.44, *p<0.0001
C. Male Subjects
Log Normalized Leptin (pg/mg)
BMI
r²=0.2, *p=0.04