

Activation of Aryl Hydrocarbon Receptor (AhR) by Tranilast, an Anti-allergy Drug, Promotes miR-302 Expression and Cell Reprogramming^{*[5]}

Received for publication, April 7, 2013, and in revised form, June 30, 2013. Published, JBC Papers in Press, July 2, 2013, DOI 10.1074/jbc.M113.475624

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Background: MiR-302 regulates pluripotency genes and helps somatic cell reprogramming.

Results: Tranilast promotes miR-302 expression and somatic cell reprogramming via AhR.

Conclusion: MiR-302 can be up-regulated by AhR ligands to induce pluripotency.

Significance: Activation of AhR facilitates somatic cell reprogramming.

MiR-302 has been shown to regulate pluripotency genes and help somatic cell reprogramming. Thus, promotion of endogenous miR-302 expression could be a desirable way to facilitate cell reprogramming. By using a luciferase reporter system of the miR-302 promoter, we screened and found that an anti-allergy drug, tranilast, could significantly promote miR-302 expression. Further experiments revealed that two aryl hydrocarbon receptor (AhR) binding motifs on the miR-302 promoter are critical and that activation of AhR is required for tranilast-induced miR-302 expression. Consistently, not only tranilast but other AhR agonists promoted miR-302 expression. Furthermore, the activation of AhR facilitated cell reprogramming in a miR-302-dependent way. These results elucidate that miR-302 expression can be regulated by AhR and thus provide a strategy for promoting somatic cell reprogramming by AhR ligands.

Introduction of defined combinations of transcription factors into somatic cells leads to the generation of induced pluripotent stem cells (iPSC)³ (1–3). Enormous efforts have been made to improve the reprogramming efficiency as well as avoid the integration of transgenes into the genome (4–6). Moreover, rationalization of the molecular mechanisms underlying the reprogramming process should also contribute to identify-

ing better strategies of efficient reprogramming (7, 8). Thus far, epigenetic modifiers (9), inhibitors of GSK-3 β (10), TGF- β inhibitors (11) and MEK inhibitors (10), antioxidants (12), longevity-promoting compounds (13) as well as others have been shown to enhance the generation of iPSCs.

MiR-302 is a polycistronic miRNA cluster including miR-302b/c/a/d and miR-367. These miRNAs share the same promoter and are generated from the same primary transcript (14). MiR-302 is highly expressed in embryonic stem cells (ESCs) (15, 16). Recent reports show that pluripotency factors Oct4 and Sox2 bind to the promoter region of miR-302 and tightly regulate miR-302 expression (17, 18). The expression level of miR-302 correlates well with the expression level of Oct4 following induction of ESC differentiation (14, 18). On the other hand, miR-302 functions to regulate cell cycle regulators CDKN1A and RBL2 (19), TGF- β signaling negative regulator Lefty1/2 (20), and pluripotency factors Oct4 and Sox2 (21) and thereby contributes to the maintenance of ESC pluripotency. Recent studies show that ectopic expression of miR-302 can promote somatic cell reprogramming (22, 23). Moreover, introduction of a combination of miR-302 with miR-200c and miR-369 can efficiently induce mouse and human somatic cells to pluripotent stage (24), demonstrating that miR-302 not only plays a critical regulatory role in pluripotency maintenance but also contributes to somatic cell reprogramming.

In light of the pivotal role of miR-302 in the maintenance of pluripotency and the cell reprogramming process, we screened for potential chemicals to manipulate miR-302 expression. We found that tranilast, an anti-allergy drug, can significantly up-regulate miR-302 by activating AhR. Moreover, tranilast and other AhR agonists can promote somatic cell reprogramming in a miR-302-dependent way.

EXPERIMENTAL PROCEDURES

Derivation of Mouse Embryonic Fibroblast (MEF) Cells—MEF cells were isolated from e13.5 embryos heterozygous for the *Oct4::GFP* transgenic allele as described previously (9). Gonads and internal organs were removed when the embryos were processed for isolation of MEF cells. MEF cells were grown in DMEM supplemented with 10% FBS, 2 mM GlutaMAX, 0.1 mM

^{*} This work was supported by the Chinese Academy of Sciences (XDA01010302) and the Ministry of Science and Technology (2011CB910202 and 2009CB940903).

^[5] This article contains supplemental Tables S1–S4.

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³ The abbreviations used are: iPSC, induced pluripotent stem cell; AhR, aryl hydrocarbon receptor; AP, alkaline phosphatase; DMSO, dimethyl sulfoxide; ESC, embryonic stem cell; MiR-302, microRNA-302-367 cluster; MEF, mouse embryonic fibroblast; OSK, Oct4/Sox2/Klf4; LIF, leukemia inhibitory factor; MSR, macrophage scavenger receptor; AhRE, aryl hydrocarbon-responsive element.

nonessential amino acids, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Isolated MEF cells (MEFs) in early passages (up to passage 2) were used for further experiments.

Mouse ESC Culture—Feeder-free E14 stem cells were grown on gelatin-coated dishes and cultured in mouse ESC culture medium consisting of DMEM supplemented with 15% FBS, 2 mM GlutaMAX, 0.1 mM nonessential amino acids, 0.1 mM β -mercaptoethanol, 1000 units/ml LIF, 100 units/ml penicillin, and 100 μ g/ml streptomycin. To detect the role of AhR and tranilast on the maintenance of the pluripotency of E14 stem cells, E14 stem cells were infected with AhR lentivirus or miR-302 antagomirs using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer; the cells were then treated with tranilast and cultured in mouse ESC culture medium with LIF or without LIF for 4 days. For pluripotency gene analysis, cells were harvested for RNA extraction and quantitative real-time PCR. Cells were stained for alkaline phosphatase (AP) activity with an alkaline phosphatase detection kit (Sigma).

Mouse iPSC Generation—Retroviruses were produced by transfection of plat-E cells with pMXs retroviral vectors containing the coding sequences of mouse Oct4, Sox2, and Klf4. MEFs (passages 1 and 2) were seeded at a density of 200,000 cells/well in 6-well plates 18 h before infection. Supernatants containing the three retroviruses were mixed and incubated with MEF cells. Two days post-virus infection, transduced MEF cells were split onto feeders in 96-well plates at a density of 5000 cells/well in mES medium (DMEM supplemented with 15% FBS, 2 mM GlutaMAX, 0.1 mM nonessential amino acids, 0.1 mM β -mercaptoethanol, 1000 units/ml LIF, 100 units/ml penicillin, and 100 μ g/ml streptomycin). At day 6, the culture medium was replaced with KSR medium (knock-out DMEM supplemented with 15% knock-out serum replacement, 2 mM GlutaMAX, 0.1 mM nonessential amino acids, 0.1 mM β -mercaptoethanol, 1000 units/ml LIF, 100 units/ml penicillin, and 100 μ g/ml streptomycin).

Plasmid Construction—The different regions of miR-302 promoter were amplified from mouse genomic DNA using primers listed in supplemental Table S3 and inserted into the pGL3-Basic vector. They were named pGL3-349, pGL3-653, pGL3-872, pGL3-1132, pGL3-2049, pGL3-3344, pGL3-3974, pGL3-4364, and pGL3-4746 according to the length of miR-302 promoter carried. The short hairpin RNAs (shRNA) targeting mouse AhR mRNA and the control shRNA were cloned into the pMKO.1 retroviral vector, which was obtained from Addgene (Cambridge, MA). The sequences for the shRNA targeting mouse AhR and for the control shRNA are shown in supplemental Table S4.

Dual Luciferase Reporter Assay—2 μ g of miR-302 promoter-reporter and 0.1 μ g of *Renilla* luciferase construct were co-transfected into 6 million HEK293 MSR cells; 12 h later, transfected HEK293 MSR cells were split into 96-well plates at a density of 40,000 cells/well, and chemicals were added. Dual-Luciferase activities were measured 24 h later using the Dual-Luciferase reporter assay system according to the instructions of the manufacturer (Promega).

Alkaline Phosphatase and Immunofluorescent Staining—Prior to staining, the iPS cells were fixed with 4% paraformaldehyde

for 2 min. The AP staining was performed using alkaline phosphatase kits (Sigma, catalogue No. 85L3R) following the manufacturer's protocol. Images were acquired using Zeiss Observer Z1. For immunofluorescent staining, fixed iPS cells were incubated with primary antibodies against mouse SSEA-1 (Santa Cruz Biotechnology, sc-21702) and rabbit Nanog (Santa Cruz Biotechnology, sc-33760) followed by the appropriate secondary antibodies conjugated to Cy3. Nuclei were counterstained with DAPI. Images were acquired using the Zeiss LSM 710 system.

FACS Analysis—GFP⁺ colonies at day 14 were trypsinized and then analyzed using a FACSCalibur flow cytometer (BD Biosciences). GFP⁺ cells were gated with a control signal from the phycoerythrin channel, and a minimum of 10,000 events were recorded.

Real-time RT-PCR—Total RNAs were extracted from cells using TRIzol reagent according to the manufacturer's instructions (Sigma). For quantitative mRNA and pri-miRNA analysis, RNA was reverse-transcribed using random hexamers and M-MLV reverse transcriptase (Promega). For the real-time quantitative PCR analysis, 2 $\times$ PCR mix (Sigma) and EvaGreen dye (Biotium, Inc.) were used, with a Stratagene Mx3000P PCR machine. The relative expression values were normalized against the inner control. Primer sequences for this section are listed in supplemental Table S3. For mature miRNAs, real-time quantitative PCR was performed either by using TaqMan miRNA assays (Applied Biosystems) or by designed stem-loop primers as described previously (25). The relative expression values were normalized against the inner control, sno-202 (Applied Biosystems).

Western Blot—Cell lysates were extracted, separated on SDS-PAGE, and transferred onto nitrocellulose membrane. Primary antibodies were as follows: rabbit anti-AhR mouse (1:2000, Biomol) and rabbit anti- β -actin (1:1000, Sigma). The membrane was detected by secondary antibody conjugated to a CW800 fluorescent probe (Rockland) using an infrared imaging system (Odyssey, LI-COR).

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed as described previously (17, 26). 2 $\times$ 10⁶ MEF cells were treated with 100 μ M tranilast for 2 h prior to cross-linking for 10 min with 1% formaldehyde. Soluble chromatin was prepared from MEF cells following sonication and centrifugation and then was incubated with anti-AhR antibody (BML-SA210, Biomol), anti-oct4 antibody (Sc-9081, Santa Cruz Biotechnology), or rabbit IgG. The primer sequences used for PCR analysis are listed in supplemental Table S3.

Introduction of miRNA Antagomirs—Mir-302 antagomirs (RiboBio) were transfected into MEF cells at day 2 and day 6 or into E14 stem cells immediately using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer. The oligonucleotide concentration was 50 nM, and the reaction was allowed to proceed for 12 h before the medium was changed.

RESULTS

Tranilast Promotes miR-302 Expression—MiR-302 has been shown to regulate pluripotency-controlled genes and contribute to somatic cell reprogramming (27). Cell reprogramming

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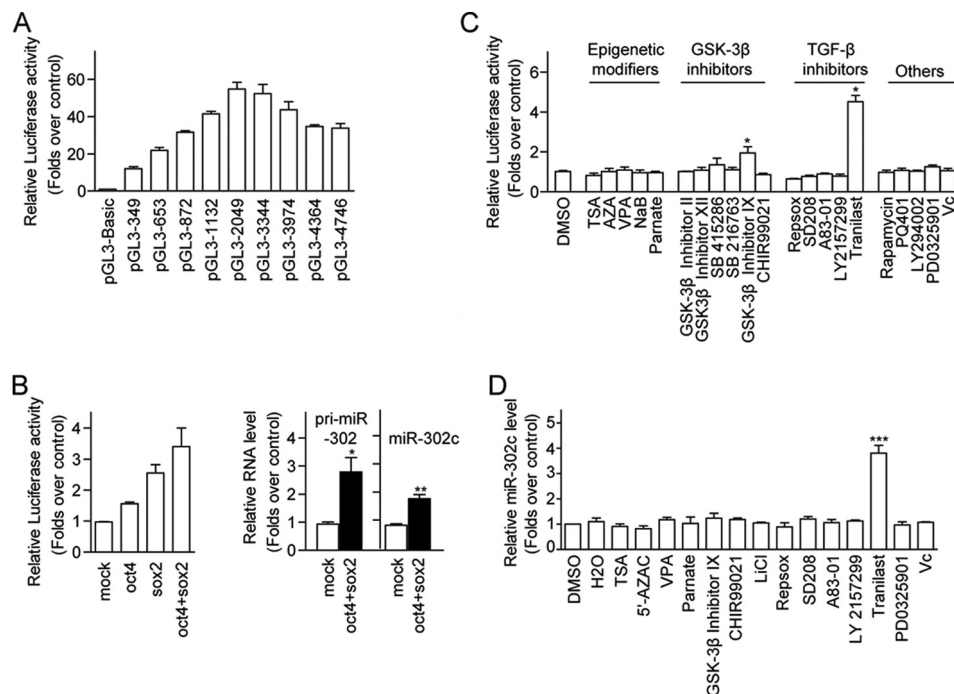


FIGURE 1. Screening chemicals that can promote miR-302 expression. A, luciferase reporter activities with different lengths of miR-302 promoter in mES cells. E14 cells were transfected with luciferase reporter constructs carrying different lengths of miR-302 promoter. 36 h after transfection, the cells were harvested, and the luciferase activities were monitored. The luciferase reporter activities were compared with pGL3-Basic-transfected cells and are shown. B, overexpression of Oct4 and Sox2 increases miR-302 promoter-reporter activities and endogenous miR-302 expression level. HEK293 MSR cells transfected with the miR-302 promoter reporter constructs, along with Oct4 and Sox2, were assayed for luciferase activities. MEF cells were transfected with Oct4 and Sox2, and the levels of endogenous miR-302 precursor (*pri-miR-302*) and mature miR-302c were measured. C, tranilast, but not other reprogramming-promoting chemicals, activated transcription of the miR-302 gene. HEK293 MSR cells transfected with miR-302 promoter-reporter constructs are treated with epigenetic modifiers, GSK-3 β inhibitors, TGF- β inhibitors, and other iPSC-promoting chemicals at concentrations listed in supplemental Table S2. The luciferase reporter activities were compared with DMSO-treated control cells and are shown. D, expression of miR-302c is up-regulated by tranilast in MEF cells. MEF cells were treated with reprogramming-promoting chemicals at concentrations listed in supplemental Table S2 for 24 h, and the miR-302c levels were monitored by real-time quantitative PCR coupled with reverse transcription. The relative miR-302c levels compared with sno-202 snRNA were shown. Data from three independent experiments are shown as means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; versus control.

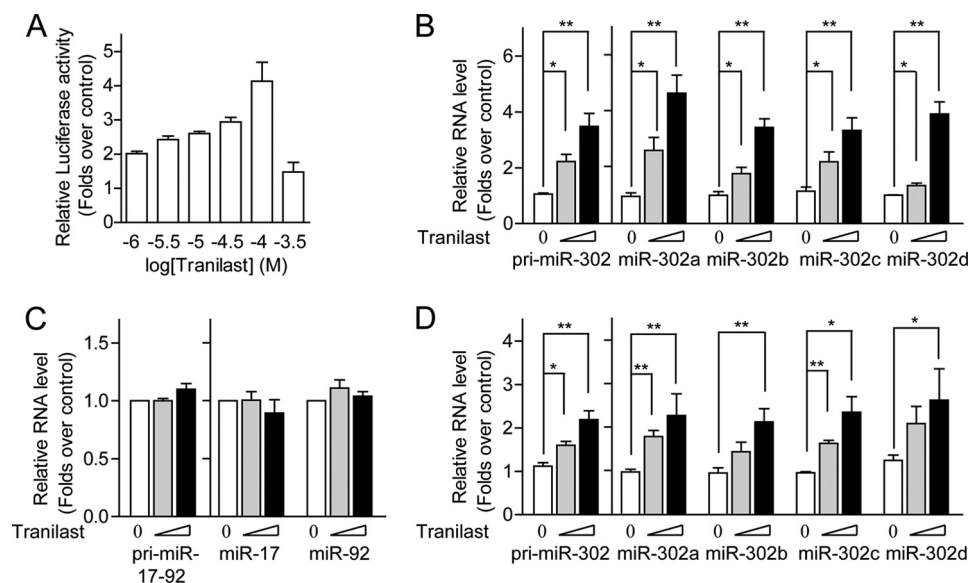


FIGURE 2. Tranilast promotes miR-302 expression in a dose-dependent manner. A and B, activation of miR-302 expression by tranilast is dose-dependent. MEF cells were treated with different concentrations of tranilast (30 and 100 μ M), and the activity of the miR-302 promoter-reporter pGL3-2049 (A) and levels of endogenous miR-302 precursor, pri-miR-302, and mature miR-302 (B) were measured. C, tranilast does not influence endogenous miR-17-92 expression in MEF cells. MEF cells were treated with different concentrations of tranilast (30 and 100 μ M), and the levels of endogenous miR-17-92 were measured. D, tranilast enhances miR-302 expression in E14 cells. E14 cells were treated with different concentrations of tranilast (30 and 100 μ M), and the levels of endogenous miR-302 precursor, pri-miR-302, and mature miR-302 were measured. Data from three independent experiments are shown as means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$; versus DMSO-treated cells.

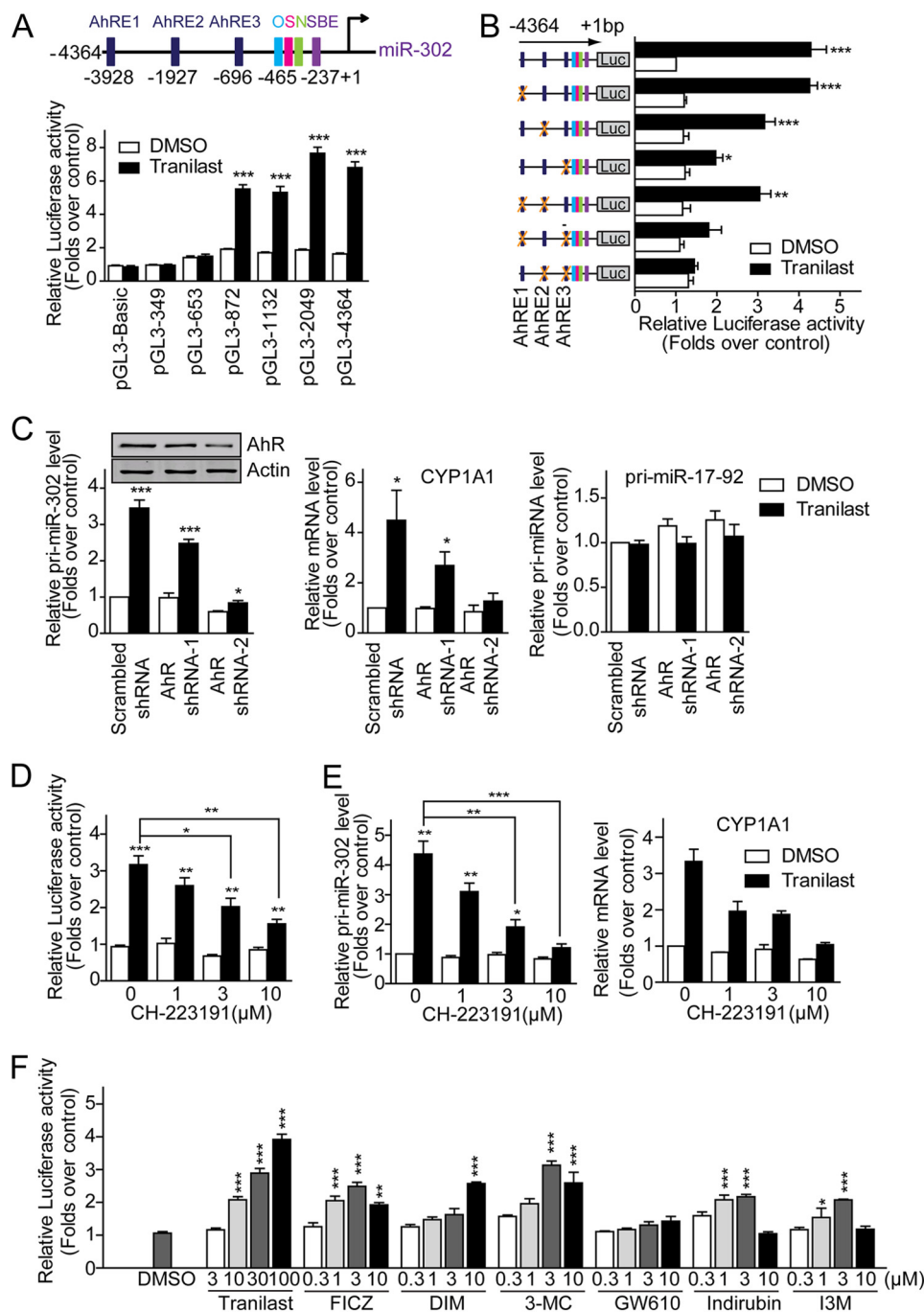


FIGURE 3. Tranilast promotes miR-302 expression via AhR. *A*, luciferase reporter activities with different lengths of miR-302 promoter. *Top*, schematic representation of the promoter region of miR-302. *Bottom*, HEK293 MSR cells transfected with luciferase reporter constructs carrying different lengths of miR-302 promoter. After treatment with tranilast (100 μ M) for 24 h, the cells were harvested and luciferase activities were monitored. *B*, luciferase reporter activities with different mutants of miR-302 promoter. A schematic illustration of mutant constructs of miR-302 promoter-reporter is presented. *C*, knockdown of AhR expression inhibits tranilast-induced miR-302 expression. MEF cells were infected with pseudo retroviruses carrying either scrambled control shRNA or AhR-specific shRNA. 72 h after infection, cellular AhR levels were monitored on Western blots and are shown. Pri-miR-302, Pri-miR-17-92, and CYP1A1 expression levels were assayed in these MEFs treated with tranilast. *D* and *E*, blockage of tranilast-induced miR-302 expression by AhR antagonist. MEF cells were treated with different concentrations of the AhR antagonist CH-223191 in the absence or presence of tranilast, and the activities of miR-302 promoter-reporter pGL3-2049 (*D*) and levels of the endogenous miR-302 precursor, pri-miR-302, and CYP1A1 (*E*) were measured. *F*, AhR agonists up-regulate miR-302 promoter-reporter activities. HEK293 MSR cells transfected with miR-302 promoter-reporter constructs are treated with AhR agonists at the indicated concentrations. The luciferase reporter activities were compared with DMSO-treated control cells and are shown. Data from three independent experiments are shown as means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; versus DMSO-treated cells.

can be promoted by chemicals including histone deacetylation inhibitors, glycogen synthase kinase 3 β (GSK-3 β) inhibitors, TGF- β inhibitors, and others (9, 11, 28, 29). To test whether miR-302 expression is mediated by these reprogramming-re-

lated pathways, we generated a series of luciferase reporter constructs carrying different length of miR-302 promoter. All miR-302 promoter-reporters showed significantly higher activity than the control empty pGL3 vector in mESCs (Fig. 1A). The

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luciferase reporter containing mouse miR-302 promoter spanning base pairs −2049 to +1 (relative to predicted transcription start site) (pGL3-2049) showed the highest activity, which is

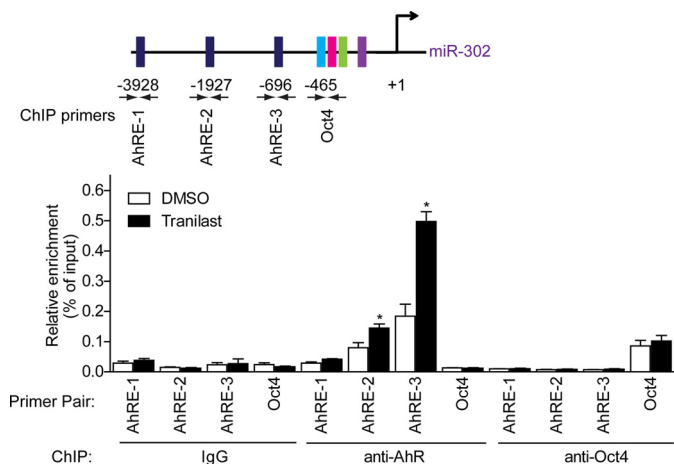


FIGURE 4. Tranilast activates AhR binding to AhRE2 and AhRE3 on the miR-302 promoter. MEF cells were stimulated with tranilast for 2 h and subjected to a ChIP assay using anti-AhR, anti-Oct4, and anti-IgG antibodies. Immunoprecipitated DNA was analyzed by quantitative PCR, and the relative enrichments are presented as a percentage of the input DNA. A schematic illustration of primer pairs used for quantitative PCR is shown. Data from three independent experiments are shown as means $\pm$ S.E. * $p < 0.05$, versus DMSO-treated cells.

~60-fold greater than control empty pGL3 vector. Overexpression of Oct4 and Sox2 further increased the luciferase activity of pGL3-2049 by ~3-fold in HEK293 MSR cells (Fig. 1B). Consistently, we observed that the expression level of endogenous miR-302 in MEF cells was significantly lower than that in ES cells (15, 30) and could be increased in the presence of Oct4 and Sox2 (Fig. 1B). We monitored pGL3-2049 luciferase reporter activities in cells treated with inhibitors of histone deacetylase, GSK-3 β inhibitors, TGF- β inhibitors, and other reported reprogramming-promoting chemicals. We found that the GSK-3 β inhibitor IX increased pGL3-2049 activity ~2 fold, whereas a TGF- β inhibitor, tranilast (*N*-[3,4-dimethoxycinnamoyl]-anthranilic acid; Rizaben®), increased pGL3-2049 activity by ~4–5 fold (Fig. 1C). Consistent with what we observed in the luciferase reporter assay, tranilast increased endogenous miR-302c by ~4-fold in MEF cells (Fig. 1D). Further, tranilast promoted miR-302 promoter-reporter activity and expression of endogenous miR-302 in a dose-dependent manner with a maximum response at 100 μ M in MEF cells (Fig. 2, A and B) as well as in the ES cell line E14 (Fig. 2D). The expression of another pluripotency-related miRNA (miR-17–92) was not altered following treatment with tranilast (Fig. 2C). These data indicate that tranilast can promote the expression of miR-302.

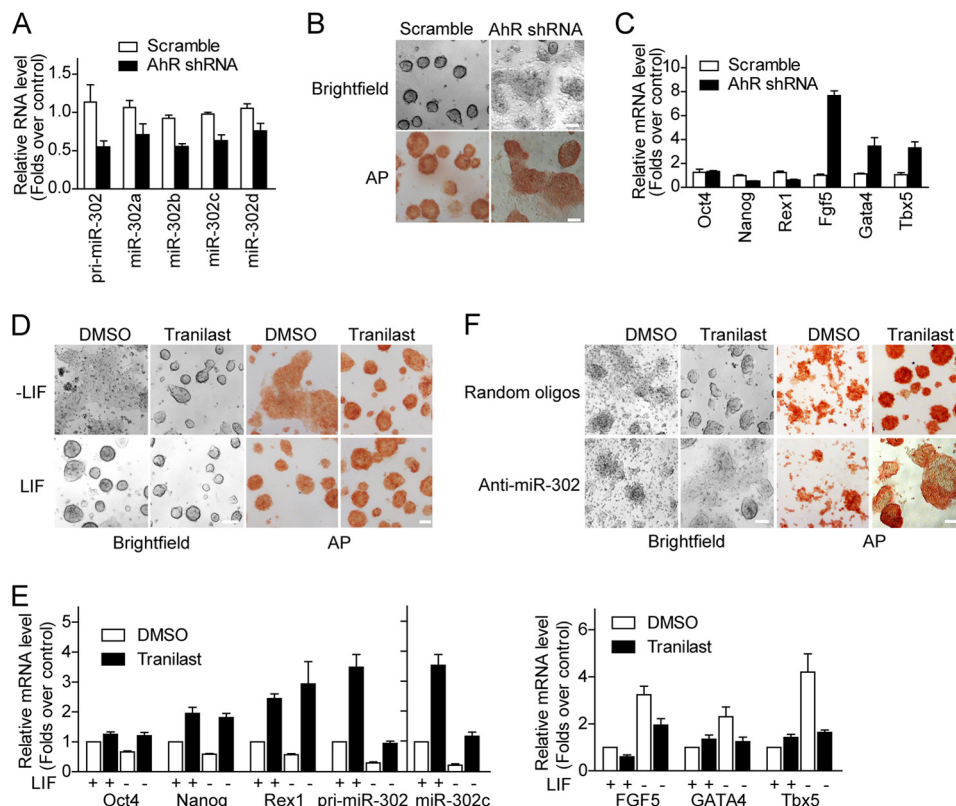


FIGURE 5. Sustaining miR-302 expression by AhR and tranilast supports pluripotency of mouse ES cells. A, knockdown of AhR inhibits miR-302 expression. E14 cells were infected with scrambled control shRNA or AhR-specific shRNA lentivirus for 2 days. Levels of endogenous miR-302 precursor (pri-miR-302) and matured miR-302 were measured. B, silencing AhR results in morphological changes and loss of AP staining in ESCs. E14 cells were infected with the indicated shRNAs and cultured in mouse ESC medium. Cells were stained with the AP staining kit and imaged 4 days after infection. C, AhR silencing leads to down-regulation of pluripotency genes and up-regulation of differentiation markers. E14 cells were infected with the indicated shRNAs and cultured in mouse ESC medium. Cells were harvested for quantitative real-time PCR analysis 4 days after infection. D, representative images of morphology and AP staining in E14 cells, which were cultured for 4 days in mouse ESC medium with or without LIF or tranilast as indicated. E, tranilast promotes pluripotency genes and inhibits differentiation genes. E14 cells cultured as described in D were harvested for quantitative real-time PCR analysis. F, representative images of morphology and AP staining in E14 cells, which were infected with miR-302 antagonomirs and cultured in mouse ESC medium without LIF for 4 days. Data from three independent experiments are shown as means $\pm$ S.E. Scale bars = 100 μ m.

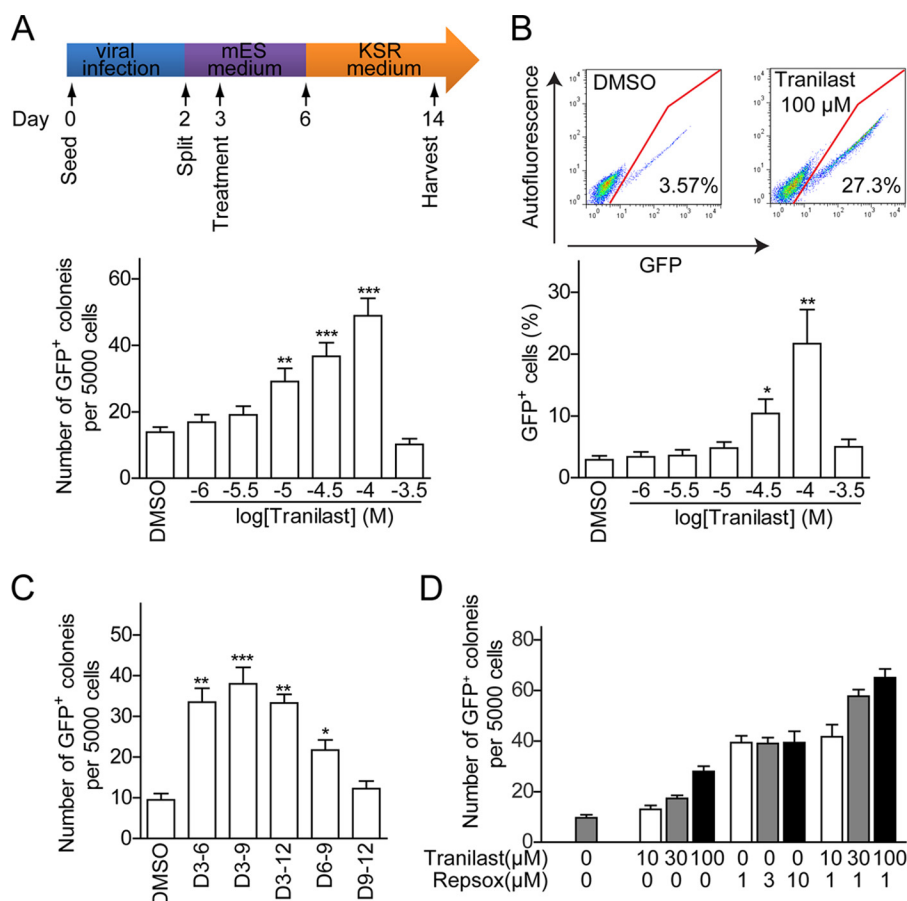


FIGURE 6. Tranilast promotes cell reprogramming. *A* and *B*, tranilast promotes the generation of GFP⁺ colonies from OSK-infected MEFs. *A*, top, schematic representation of *in vitro* cell reprogramming protocol. Bottom, the number of GFP⁺ colonies generated from tranilast-treated, OSK-infected MEFs. *B*, the percentage of GFP⁺ cells was determined by FACS analysis at day 14. Top, representative FACS data demonstrate an increase in the percentage of GFP⁺ cells after treatment of 100 μ M tranilast. Bottom, the percentages of GFP⁺ cells that were induced in OSK-infected MEFs treated with different concentrations of tranilast. *C*, tranilast promotes cell reprogramming at the early stage. OSK-infected MEFs were treated with 100 μ M tranilast for various durations as indicated, and GFP⁺ colonies were counted at day 14. *D*, enhancement of iPSC generation by tranilast and RepSox. OG2 MEF cells infected with OSK plus tranilast or RepSox at the indicated concentrations were added to the culture medium on days 3–9. GFP⁺ colonies were counted at day 14. Data from three independent experiments are shown as means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus DMSO-treated cells.

Tranilast Regulates miR-302 through Binding of AhR to the AhRE Motif—The putative transcription factor-binding elements in the miR-302 promoter region were predicted using the MatInspector promoter analysis tool (31). MatInspector accurately identified the known Oct4, Sox2, Nanog, and Smad binding sites in the miR-302 promoter (Fig. 3A, upper panel). It is of note that the luciferase activities of reporters carrying miR-302 promoter region shorter than 653 bp were not enhanced by tranilast treatment (Fig. 3A), although all truncated miR-302 promoter-reporters showed significantly higher activity than the pGL3-Basic control reporter in ES cells (Fig. 1A), indicating that the promoter region –4364 to –653 bp should be important for tranilast-induced miR-302 expression. We noted that pGL3-653 contains a Smad binding site. It is widely accepted that inhibition of the TGF- β pathway leads to the activation of Smad-controlled gene expression (32). However, the Smad-binding site in the miR-302 promoter did not respond to tranilast treatment (Fig. 3A), suggesting an unknown regulatory mechanism of tranilast other than inhibition of TGF- β pathway. On the other hand, in the promoter region from –4364 to –653 bp, there are three putative AhR binding sites (AhRE1 –3928 to –3904 bp, AhRE2 –1927 to –1903 bp, and AhRE3

–696 to –672 bp). To test whether these three AhREs are responsible for the tranilast-induced miR-302 expression, we further generated a serial mutant reporter constructs. As shown in Fig. 3B, the miR-302 promoter reporter harboring AhRE2 and AhRE3 is as active as the wild type miR-302 promoter reporter following tranilast treatment. Consistently, mutation of either AhRE2 or AhRE3 but not AhRE1 reduced the tranilast-induced luciferase activities. These data suggest that AhRE2 and AhRE3 are essential for the up-regulation of miR-302 expression in response to tranilast treatment.

Studies have shown that tranilast also acts as an agonist of AhR (33). AhR is a basic helix-loop-helix (bHLH) transcription factor. By binding to the AhREs, AhR mediates diverse gene expression and plays an important role in different cellular processes including proliferation, differentiation, cell adhesion, and organ homeostasis (34, 35). Given that tranilast-promoted miR-302 expression depends on AhREs in the miR-302 promoter, we hypothesized that tranilast enhances miR-302 expression through activation of AhR. Indeed, although the endogenous AhR level in MEF cells was knocked down by AhR shRNA retrovirus, tranilast-induced miR-302 expression (Fig. 3C), as well as the AhR downstream gene *CYP1A1*, was signif-

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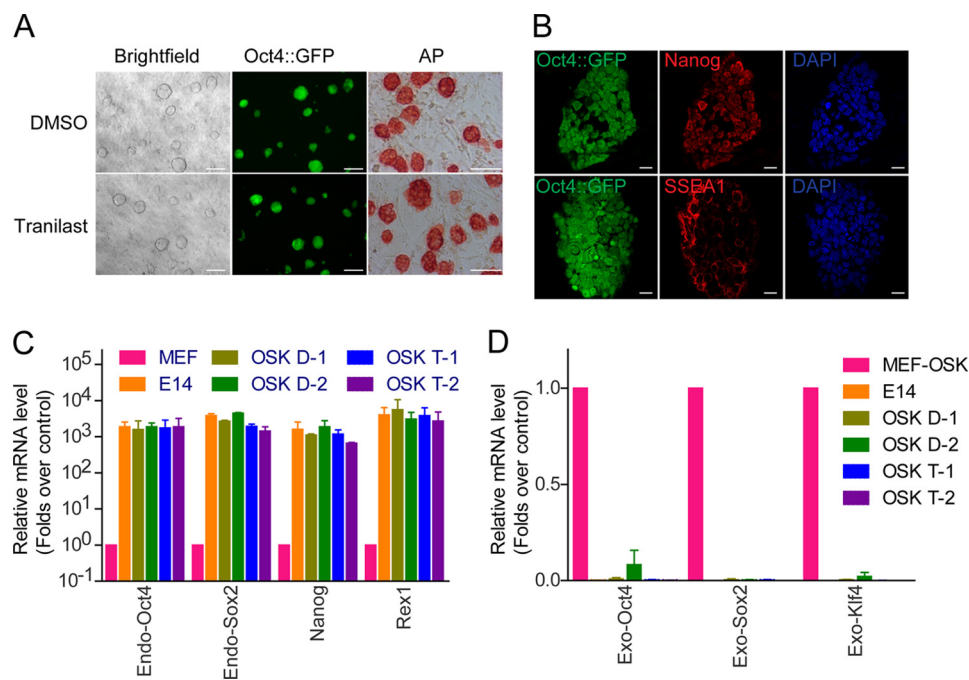


FIGURE 7. Tranilast-promoted iPSCs are pluripotent. *A*, representative pictures of the iPSCs generated in the presence of tranilast. *Left*, phase contrast; *middle*, Oct4::GFP; *right*, AP staining. Scale bars = 100 μ m. *B*, the iPSCs generated in the presence of tranilast express typical pluripotency markers. The representative picture of Oct4::GFP expression and immunofluorescent staining of pluripotency genes SSEA-1 and Nanog are shown. Scale bars = 20 μ m. *C*, the relative gene expression of endogenous pluripotency markers in established iPSC lines. *D*, the silencing of viral transgenes expression. Quantitative PCR analysis of pluripotency genes and exogenous transgenes in two OSK-iPS clones and two OSK-iPS clones generated with tranilast treatment. mES cell line E14, MEF, and OSK-infected MEF at 4 days (MEF-OSK) were used as controls. Data from three independent experiments are shown as means $\pm$ S.E.

icantly reduced. The expression level of pri-miR-17–92 was not changed upon knockdown of AhR. Moreover, in the presence of the AhR antagonist CH-223191, tranilast-mediated miR-302 promoter-reporter activity and expression of endogenous miR-302 (Fig. 3, *D* and *E*), as well as CYP1A1, were significantly blocked. Furthermore, as shown in Fig. 3*F*, all AhR agonists we tested enhanced the miR-302 promoter-reporter activity in a dose-dependent manner. The reported EC₅₀ (half-maximal effective concentration) values of these AhR agonists to activate AhR are listed in supplemental Table S1. These data indicate that activation of AhR positively regulates miR-302 expression.

To test whether AhR was directly recruited to AhREs upon tranilast treatment, we then performed a ChIP assay. DNA fragments associated with immunopurified AhR or Oct4 were analyzed by quantitative PCR (Fig. 4*A*). A considerable amount of endogenous Oct4 and AhR was bound to the promoter region of miR-302. The presence of AhR on AhRE2 and AhRE3 but not AhRE1 was significantly enhanced following tranilast treatment. On the other hand, the occupancy of Oct4 on miR-302 had not been altered by tranilast treatment. Together, these results demonstrated that binding of activated AhR to the AhREs on miR-302 promoter is required for tranilast-induced miR-302 expression.

Sustaining miR-302 Expression by AhR and Tranilast Supports Pluripotency of Mouse ES Cells—It is known that expression of miR-302 activates pluripotent genes and contributes to the maintenance of pluripotency (36); thus we tested whether AhR mediates ES cell pluripotency through miR-302. We knocked down AhR expression in mouse E14 cells using AhR shRNA lentivirus. As shown in Fig. 5*A*, the expression of pri-

mary and mature miR-302 was significantly reduced following AhR shRNA lentivirus infection. Four days after infection, the self-renewal status of these cells was determined by morphology and the AP staining assay as well as the expression of pluripotency markers and differentiation genes. We found a reduction of AhR impaired the colony morphology with decreased AP activity (Fig. 5*B*), reduced pluripotency gene (*Nanog* and *Rex1*) expression, and increased the level of differentiation markers (*Fgf5*, *Gata4*, and *Tbx5*) (Fig. 5*C*). These data suggest that knockdown of AhR leads to reduced miR-302 expression and ESC pluripotency.

LIF is an essential supplement in culture medium for E14 stem cells and maintains the pluripotency of E14 cells. Under this normal culture condition, E14 cells treated with tranilast showed similar colony morphology and AP activity but higher pluripotency gene expression levels compared with that of mock-treated cells (Fig. 5, *D* and *E*). When cultured without LIF, E14 cells lost colony morphology with decreased AP activity, reduced pluripotency gene expression, and an increased level of differentiation markers (Fig. 5, *D* and *E*). Interestingly, in the presence of tranilast, E14 cell cultured in the absence of LIF showed colony morphology, higher AP activity, and expression of both pluripotency genes and miR-302. These data indicate that tranilast can help to maintain pluripotency of mouse ES cells.

Furthermore, blocking the function of miR-302 by miR-302 antagonists abolished the pluripotency-supporting function of tranilast on E14 cells under differentiation condition (Fig. 5*F*). Together, these results indicate that tranilast sustains miR-302 expression and supports mouse ES cell pluripotency.

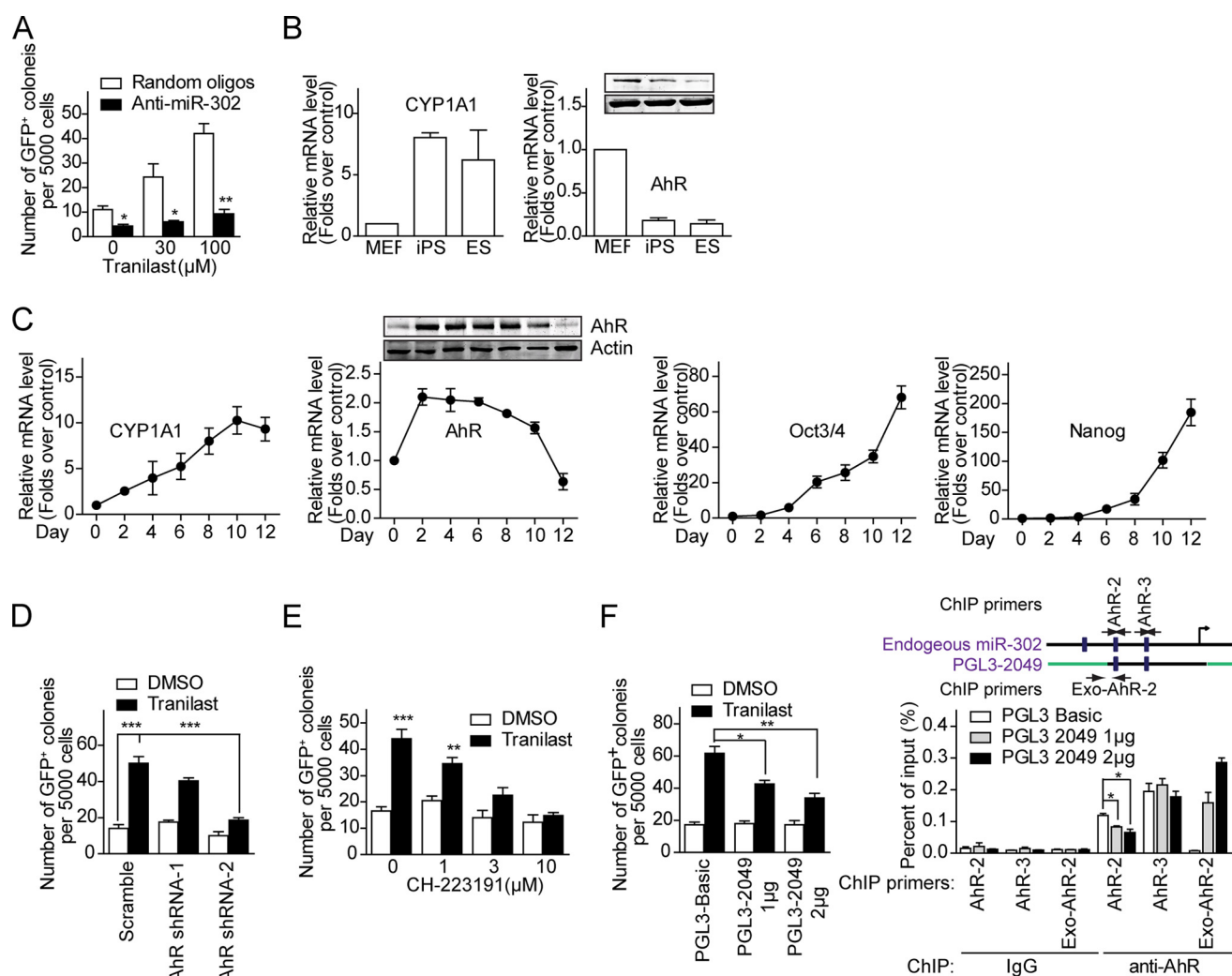


FIGURE 8. Impairment of AhR-mediated miR-302 expression inhibits tranilast-promoted cell reprogramming. *A*, blockage of tranilast-promoted cell reprogramming by miR-302 antagonists. Antagonists were transfected into OSK-infected MEF cells at day 2 and day 6. Tranilast was added to the culture medium on days 3–9. *B*, AhR is activated in ES cells and iPS cells. Quantitative RT-PCR and Western blot analysis of the AhR downstream gene *CYP1A1* and AhR expression in MEFs, iPS cells, and ES cells. *C*, quantitative RT-PCR and Western blot analysis of *CYP1A1*, AhR, Oct4, and Nanog expression on the indicated days in OSK-transduced MEF cells. *D*, knockdown of AhR reduces tranilast-promoted iPS cell generation. MEF cells were infected with OSK together with scrambled control shRNA or AhR-specific shRNA. Tranilast was added to the culture medium on days 3–9. *E*, blockage of tranilast-induced cell reprogramming by AhR antagonist. Tranilast and AhR antagonist CH-223191 at the indicated concentrations were added to the culture medium of OSK-infected MEFs on days 3–9. *F*, interruption of AhR binding to the promoter of miR-302 blocks tranilast-induced cell reprogramming. MEF cells were infected with OSK together with pGL3-Basic and pGL3-2049. Tranilast was added to the culture medium on days 3–9. *Left*, the number of GFP⁺ colonies generated from tranilast-treated, OSK-infected MEFs is shown. *Right*, occupation of AhR on miR-302 promoters presented on either pGL3-2049 or miR-302 gene. MEF cells at day 3 were subjected to ChIP assay using anti-AhR and anti-IgG antibodies. Immunoprecipitated DNA was analyzed by quantitative PCR, and the relative enrichments are presented as a percentage of the input DNA. A schematic illustration of primer pairs used for quantitative PCR is shown. Data from three independent experiments are shown as means ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; versus control.

Tranilast Promotes Cell Reprogramming through AhR-mediated miR-302 Expression—Studies show that expression of miR-302 facilitates somatic cell reprogramming (21–24). We then tested whether tranilast could promote iPS cells generation through miR-302. Two days after the retroviral transduction of three vectors that express Oct4, Sox2, and Klf4 (OSK), primary MEFs derived from OG2^{+/−}/ROSA26^{+/−} (OG2) mice (containing a transgenic Oct4-GFP reporter) were seeded onto feeder cells in 96-well plates in mouse ESC culture medium; this was designated as day 2. These MEFs were treated with different concentration of tranilast from day 3 to day 9 as indicated in Fig. 6A, upper panel. At day 14 post-infection, we observed the appearance of morphological ES-like and GFP⁺ colonies. Tranilast treatment increased the number of GFP⁺ colonies in a

dose-dependent manner. The treatment with 100 μM tranilast increased the number of GFP⁺ colonies by ~3-fold (Fig. 6A). FACS analysis of OSK-infected OG2 MEFs at day 14 post-infection displayed a >7-fold increase of GFP⁺ cells in samples treated with 100 μM tranilast (Fig. 6B). Furthermore, we found that the treatment of MEFs with tranilast following day 3 post-infection exhibited prominent reprogramming-enhancing effects (Fig. 6C), suggesting that tranilast promotes the reprogramming process at the initial stage. Interestingly, a combined treatment using tranilast with the iPS-promoting TGF-β inhibitor RepSox led to an ~2-fold increase in the reprogramming efficiency of the OSK-infected MEFs compared with tranilast or RepSox treatment alone (Fig. 6D). This synergistic effect of tranilast with RepSox on cell reprogramming suggests that

Tranilast Promotes iPSCs via AhR-mediated miR-302 Expression

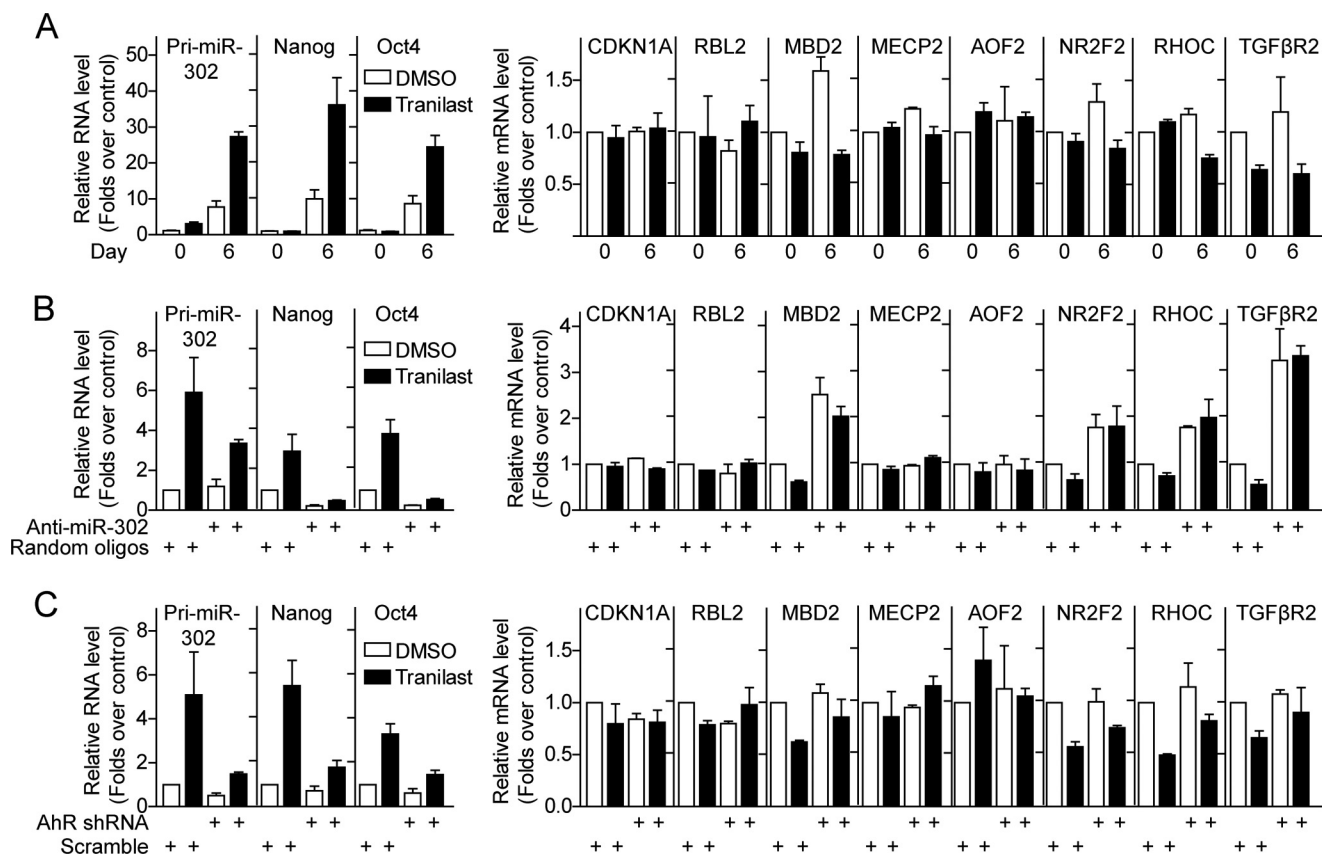


FIGURE 9. Tranilast down-regulates miR-302 target genes during reprogramming process. A, tranilast treatment up-regulates pluripotent genes and down-regulates miR-302 target genes during reprogramming process. Quantitative RT-PCR analysis of expression of pluripotent genes and miR-302 target genes at day 6 after infection with OSK or uninfected MEFs treated with tranilast. B and C, knockdown of miR-302 or AhR diminishes tranilast-mediated expression of pluripotent genes and miR-302 target genes. Quantitative RT-PCR analysis of cells harvested 6 days after infection with OSK together with miR-302 antagonists (B) or AhR shRNA (C). Data from three independent experiments are shown as means $\pm$ S.E.

although tranilast shows an inhibitory effect on TGF- β signaling (37), it is less possible that tranilast promotes reprogramming through inhibition of the TGF- β pathway. Further, we found that tranilast also promoted single-factor Oct4-induced reprogramming in the presence of VPA, CHIR99021, parnate, and RepSox by $\sim$ 2-fold (data not shown). Together, these results indicate that tranilast promotes somatic cell reprogramming.

To further evaluate the pluripotency of the iPSCs generated by tranilast treatment, we picked colonies randomly from the culture of tranilast-treated OSK-infected MEFs and established multiple iPSC lines. All of these lines were morphologically indistinguishable from mouse ES cells, and they stained positive for alkaline phosphatase (Fig. 7A). A randomly chosen clone was then subjected to further immunofluorescent analysis. These cells expressed high levels of the pluripotency markers SSEA1 and Nanog (Fig. 7B). The endogenous expression of Oct4, Sox2, and Nanog, as well as the silencing of exogenous retroviral factors expression, was verified by real-time quantitative PCR (Fig. 7, C and D). These results suggest that tranilast treatment does not compromise iPSC pluripotency.

To test whether the promotion of iPSC generation by tranilast depends on miR-302, OSK-infected OG2 MEF cells were treated with miR-302 antagonists and examined for reprogramming efficiency. As shown in Fig. 8A, antagonist-mediated silencing of endogenous miR-302 decreased OSK-infected OG2 MEF reprogramming and abolished the reprogramming-

enhancing effect of tranilast, demonstrating a requirement for miR-302 in the cell reprogramming progress.

We have shown that tranilast treatment led to binding of AhR to AhREs in miR-302 promoter and thereby enhanced expression of miR-302. To assess the involvement of AhR involved in the cell reprogramming process, we examined the expression levels of AhR and the AhR downstream gene *CYP1A1* in MEFs, iPS cells, and mES cells. As shown in Fig. 8B, although AhR was expressed at low level in iPS cells and mES cells compared with MEFs, the high expression level of *CYP1A1* in iPS cells and mES cells suggested an activation of AhR-mediated gene expression in iPS cells and mES cells. To further clarify the activation of AhR during reprogramming, we examined the expression levels of *CYP1A1* and AhR at different time point during reprogramming (Fig. 8C). The dynamic increase of the pluripotent genes *Oct4* and *Nanog* indicated successful reprogramming. We found the expression level of AhR was increased at day 2 post-virus infection and then gradually decreased by half by day 12 compared with MEFs. Interestingly, *CYP1A1* gradually increased with the reprogramming process, which suggested that AhR was activated during the reprogramming process and that activation of AhR may facilitate cell reprogramming.

We then tested whether the reprogramming-enhancing effect of tranilast was due to its activation of miR-302 expression by AhR. OSK-infected OG2 MEFs were treated with AhR

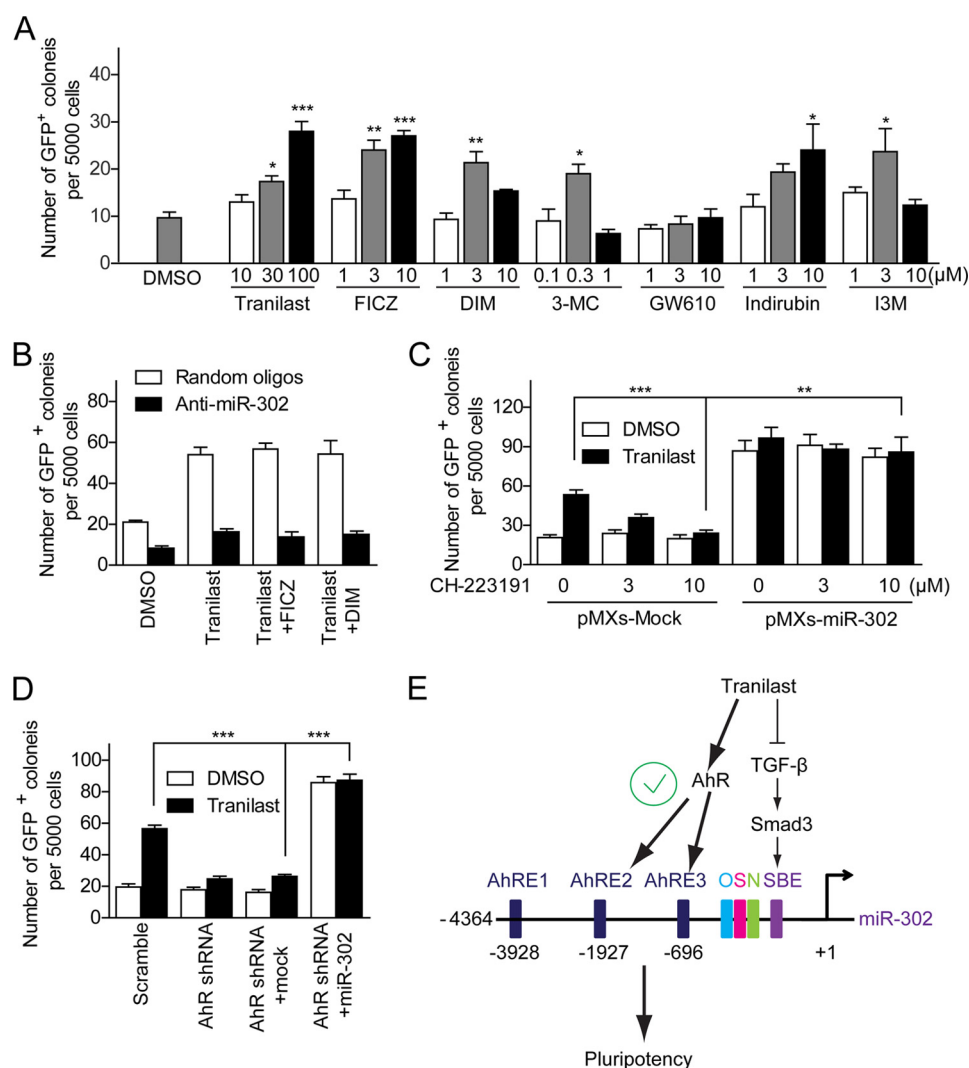


FIGURE 10. Activation of AhR-mediated miR-302 expression promoted cell reprogramming. A, AhR agonists promote cell reprogramming. OSK-infected MEF cells were treated with AhR agonists at the indicated concentrations from days 3–9. B, AhR agonists cannot rescue the blockage of cell reprogramming by miR-302 antagonists. Antagonists were transfected into OSK-infected MEF cells at day 2 and day 6. Tranilast and AhR agonists were added to the culture medium on days 3–9. C, supplementing miR-302 expression with retrovirus abolishes the blockage of tranilast-promoted cell reprogramming by AhR knock-down. MEF cells were infected with OSK together with the indicated shRNA and pMXs-miR-302 retrovirus. Tranilast was added to the culture medium on days 3–9. D, supplementing miR-302 expression with retrovirus rescues the blockage of tranilast-promoted cell reprogramming by AhR antagonist. MEF cells were infected with OSK together with pMXs-miR-302 retrovirus. Tranilast and AhR antagonist CH-223191 at the indicated concentrations were added to the culture medium on days 3–9. E, schematic diagram shows that tranilast facilitates cell reprogramming through AhR-mediated miR-302 expression. Data from three independent experiments are shown as means $\pm$ S.E. *, $p < 0.05$, ***, $p < 0.001$; versus control.

shRNA or different doses of the AhR antagonist CH-223191 in the absence or presence of tranilast from day 3 to day 9 post-infection, and the number of GFP⁺ colonies with ES cell-like morphology was counted at day 14. As shown in Fig. 8, D and E, we observed a robust inhibition of the appearance of tranilast-promoted GFP⁺ colonies by AhR shRNA or the AhR antagonist CH-223191, demonstrating that expression and activation of AhR is required for tranilast-promoted cell reprogramming. Further, the tranilast-promoted cell reprogramming could be reduced significantly in the presence of the miR-302 promoter reporter pGL3-2049 (Fig. 8F, left panel), because the ectopic exogenous miR-302 promoter on the reporter constructs competed with the endogenous miR-302 promoter for AhR binding as shown in the ChIP experiment (Fig. 8F, right panel).

Tranilast treatment not only leads to activation of miR-302 expression but also increases expression of the pluripotent genes *Oct4* and *Nanog* during cell reprogramming (Fig. 9A). The miR-302 target genes including *MBD2*, *NF2R2*, *RHOC*, and *TGFβ2R* were also down-regulated by tranilast treatment in these OSK-infected MEFs (Fig. 9A). In the presence of miR-302 antagonists or AhR shRNA, tranilast could not increase expression of pluripotent genes *Oct4* and *Nanog* or down-regulate miR-302 target genes (Fig. 9, B and C), indicating that tranilast mediates the expression of these genes in a miR-302-dependent manner.

Moreover, AhR agonists including FICZ (6-formylindolo[3,2-*b*]carbazole), DIM (3,3'-diindolylmethane), 3-methylcholanthrene, indirubin, and indirubin-3-monoxime, but not GW610 (2-(3,4-dimethoxyphenyl)-5-fluoro-benzothiazole), promoted

cell reprogramming to different extents (Fig. 10A). Neither FICZ nor DIM could rescue miR-302 antagomir-blocked cell reprogramming in the presence of tranilast (Fig. 10B). However, supplementing miR-302 expression with retroviruses, on the other hand, efficiently rescued the blockage of tranilast-promoted cell reprogramming by AhR knockdown or AhR antagonist (Fig. 10, C and D). Taken together, these results indicate that activation of AhR-mediated miR-302 expression is beneficial for somatic cell reprogramming (Fig 10E).

DISCUSSION

Mouse and human miR-302 orthologs show highly conserved nucleotide sequences and genomic organization (14). MiR-302 is highly expressed in ESCs and has been shown to regulate pluripotency-controlled genes and help somatic cell reprogramming. MiR-302 is expressed at a low level in differentiated cells in general; however, it can be elevated upon specific stimuli or pathological conditions (26, 38). But the mechanisms by which miR-302 is regulated are less well understood. Previous studies show that Oct4 and Sox2 co-occupy the promoter region and regulate expression of miR-302; the expression level of miR-302 correlates well with Oct4 transcript in ESCs and early embryonic development (17, 18, 39). A recent study reports that hyaluronan stimulates the CD44v3 (a hyaluronan receptor) interaction with Oct4-Sox2-Nanog leading to both a complex formation and the nuclear translocation of three transcription factors to promote miR-302 expression, which causes self-renewal, clonal formation, and cisplatin resistance in cancer stem cells from head and neck squamous cell carcinoma (40). Another report demonstrates that the BMP4-Smad pathway represses the transcription of miR-302 and derepresses the expression of the type II BMP receptor in vSMCs (26). During the preparation of this paper, Zhang and Wu (41) reported that sodium butyrate promotes the generation of human iPS cells through induction of the miR302/367 cluster. They also found that expression of miR-302 was not up-regulated by the TGF- β inhibitor SB431542, which also supports our finding that tranilast up-regulates miR-302 expression through activation of AhR instead of inhibition of the TGF- β pathway. Activation of AhR by tranilast leads to the binding of AhR to the miR-302 promoter and thereby promotes miR-302 expression and enhances cell reprogramming. Thus, our study not only elucidates a novel regulatory mechanism of miR-302 expression but also provides a strategy for promoting somatic cell reprogramming by AhR ligands.

AhR belongs to the basic helix-loop-helix transcription factor family (42). Recently reports show that tryptophan metabolites, indole-containing structures, 7-ketocholesterol, and prostaglandins, function as ligands of AhR (43). AhR mediates cellular responses to environmental toxins (44). Besides, AhR has been shown to regulate cellular responses to oxidative stress and inflammation by mediating the transcription level of genes involved in the signaling of Nrf2 (45), p53 (46), RB1 (44), and NF- κ B (47). AhR also has an important function in many developmental pathways, including hematopoiesis (48), lymphoid systems (49), T-cells (50, 51), neurons (52), and hepatocytes (53, 54). In this study, we found that AhR is gradually activated during cell reprogramming and is highly activated in iPS cells

and mES cells compared with MEFs. There are three AhREs in the miR-302 promoter, and activation of AhR by tranilast can promote miR-302 expression and cell reprogramming. Moreover, we found that some other AhR agonists could also enhance iPS generation by promoting miR-302 expression, suggesting an important role for AhR in cell reprogramming. Nevertheless, whether AhR-mediated miR-302 expression is also involved in other physiologic functions of AhR should be explored.

Tranilast is an anti-allergy, over-the-counter drug used against asthma, autoimmune diseases, and atopic and fibrotic pathologies. Tranilast inhibits the growth and metastasis of mammary carcinoma (55). The antiproliferative potential of tranilast depends principally on the capacity of tranilast to interfere with TGF- β signaling through inhibiting TGF- β release (56). Although there is a Smad binding site on the miR-302 promoter, our study shows that induction of miR-302 by tranilast probably does not occur via the TGF- β /Smad pathway. Furthermore, tranilast and RepSox, a TGF- β inhibitor, synergistically promote iPS generation, suggesting that tranilast does not function as an inhibitor of the TGF- β pathway in cell reprogramming process. The cell reprogramming process can be divided into three phases, namely initiation, maturation, and stabilization. Some reports have shown that histone deacetylase inhibitor and TGF- β inhibitors function at the initiation stage of reprogramming, whereas MEK inhibitors function at the maturation and stabilization phase (29, 57, 58). Our study shows that tranilast functions at the initial stage of reprogramming, suggesting that activation of AhR-mediated miR-302 expression might facilitate the bypass of certain reprogramming barriers.

Acknowledgments—We thank Duanqing Pei (Guangzhou Institutes of Biomedicine and Health) for the pMXs-miR-302 plasmid. We also thank all members of our laboratory for sharing reagents and advice.

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Table S1. EC50 of AhR agonists

AhR agonists	EC50
N-(3',4'-Dimethoxycinnamoyl)Anthranilic Acid (Tranilast)	50 μ M
6-formylindolo[3,2-b]carbazole (FICZ)	34-830 pM
3,3'-diindolylmethane (DIM)	10 μ M
3-methylcholanthrene (3-MC)	86 nM
2-(3,4-dimethoxyphenyl)-5-fluoro-benzothiazole (GW610)	1 μ M
Indirubin	100 nM
Indirubin-3-monoxime (I3M)	720 nM

Table S2. Concentration of chemicals used in reporter assay

	Name	Concentration
Epigenetic modifiers	TSA	20 nM
	5'-AZAC	2 μ M
	VPA	1 mM
	NaB	1 mM
	Parnate	2 μ M
GSK-3 β inhibitors	GSK-3 β inhibitor II	3 μ M
	GSK-3 β inhibitor XII	3 μ M
	SB415286	3 μ M
	SB 216763	3 μ M
	GSK-3 β inhibitor IX	3 μ M
	CHIR99021	3 μ M
	LiCl	10 mM
TGF- β inhibitors	Repsox	1 μ M
	SD208	1 μ M
	A83-01	0.5 μ M
	LY2157299	10 μ M
	Tranilast	100 μ M
Other iPS promoting chemicals	Rapamycin	1 nM
	PQ401	1 μ M
	LY294002	0.3 μ M
	PD0325901	0.5 μ M
	Vc	50 μ g/ml

Tabe S3. Primers for Cloning and real time quatitative PCR

Primers for cloning of miR-302 promoter reporters	
pGL3-349-Forward	5'-CGACGCGTTCAAACGGGCAGATAGGAG
pGL3-656-Forward	5'-CGACGCGTGTAAAATCACTTTTGAGTCACTTC
pGL3-872-Forward	5'-CGACGCGTACAGTGATAAGACGACCCA
pGL3-1132-Forward	5'-CGACGCGTAGGAAGCCACTCTATCACT
pGL3-2049-Forward	5'-CGACGCGTTGGGTGGAACACTCTTGAC
pGL3-3344-Forward	5'-CGACGCGTGAAACTCACCTTACCACA
pGL3-3974-Forward	5'-CGACGCGTGGGCTCTGCTAAGGGTAAG
pGL3-4364-Forward	5'-CGACGCGTCGAGGTTCCAAC TGTGCTT
pGL3-4746-Forward	5'-CGACGCGTCTTTTCCAGTCTGAAGCAG
pGL3-Reverse	5'-TACAGATCTGTGACCCGGTTACAGACAT
Primers for cloning of miR-302 promoter reporter mutations	
AhRE-1-Forward	5'-GTAAATGGCCCCTCACTCAGCCTAT TGGAGGAGGTTAGTAC
AhRE-1-Reverse	5'-GTACTAACCTCCTCCAATAGGCTGA GTGAGGGGCCATTTAC
AhRE-2-Forward	5'-CAGCAGCAGCGACTTCTCCTATTAG TTTAAGGATCTCAG
AhRE-2-Reverse	5'-CTGAGATCCTTAAACTAATAGGAGA AGTCGCTGCTGCTG
AhRE-3-Forward	5'-GTCTTAACAGGCAAGGAAAATATAG GAGTTTCCTGGTTGCTTC
AhRE-3-Reverse	5'-GAAGCAACCAGGAAACTCCTATATT TTCCTTGCCCTGTTAAGAC
Primers for real time quatitative PCR	
AhR Forward	5'-AGCCGGTGCAGAAAACAGTAA
AhR Reverse	5'-AGGCGGTCTAACTCTGTGTTC
CYP1A1 Forward	5'-GACCCTTACAAGTATTTGGTCGT
CYP1A1 Reverse	5'-GGTATCCAGAGCCAGTAACCT
pri-miR-302 cluster Forward	5'-CTGTGGGTTTGCTCTTCTGTTTT
pri-miR-302 cluster Reverse	5'-GAGACAGAAAGCATTCCCATGTT
pri-miR-17-92-cluster Forward	5'-TTGGAAC TTCTGGCTATTGGCTCCTC
pri-miR-17-92-cluster Reverse	5'-CCAAGGTGAGGTTCACTTTATTCCTGC
miR-17 stem loop primer Forward	5'-CGGCGGCAAAGTGCTTACAGT
miR-92a stem loop primer Forward	5'-CGGCGGTATTGCACTTGTCC
stem loop primer Reverse	5'-GTGCAGGGTCCGAGGT
endogenous Oct4 Forward	5'-TCTTTCCACCAGGCCCCCGGCTC
endogenous Oct4 Reverse	5'-TGCGGGCGGACATGGGGAGATCC
endogenous Sox2 Forward	5'-TAGAGCTAGACTCCGGGCGATGA
endogenous Sox2 Reverse	5'-TTGCCTTAAACAAGACCACGAAA
Nanog Forward	5'-AGGGTCTGCTACTGAGATGCTCTG
Nanog Reverse	5'-CAACCACTGGTTTTTCTGCCACCG
Rex1 Forward	5'-CGTGTAACATACACCATCCG
Rex1 Reverse	5'-GAAATCCTCTTCCAGAATGG
Exo-Oct4 Forward	5'-GGGTGGACCATCCTCTAGAC
Exo-Oct4 Reverse	5'-CCAGGTTCGAGAATCCAC
Exo-Sox2 Forward	5'-GGGTGGACCATCCTCTAGAC
Exo-Sox2 Reverse	5'-GGGCTGTTCTTCTGGTTG
Exo-Klf4 Forward	5'-GGGTGGACCATCCTCTAGAC
Exo-Klf4 Reverse	5'-GCTGGACGCAGTGTCTTCTC
MBD2 Forward	5'-AGAACAAGGGTAAACCAGACCT
MBD2 Reverse	5'-ACTTCACCTTATTGCTCGGGT
NR2F2 Forward	5'-TCAACTGCCACTCGTACCTG
NR2F2 Reverse	5'-CCATGATGTTGTTAGGCTGCAT
CDKN1A Forward	5'-CCTGGTGATGTCCGACCTG
CDKN1A Reverse	5'-CCATGAGCGCATCGCAATC
RBL2 Forward	5'-AACTTCCCCATGATTAGCGATG
RBL2 Reverse	5'-GGTTAGAACACTGAAGGGCATTT
MECP2 Forward	5'-ATGGTAGCTGGGATGTTAGGG
MECP2 Reverse	5'-TGAGCTTTCTGATGTTTCTGCTT
AOF2 Forward	5'-GTGGTGTTATGCTTTGACCGT
AOF2 Reverse	5'-GCTGCCAAAAATCCCTTTGAGA
RHOC Forward	5'-ATGGCTGCGATCCGAAAGAAG
RHOC Reverse	5'-GCACGTAGACCTCTGGAAACT
TGFβR2 Forward	5'-CCGCTGCATATCGTCCTGTG
TGFβR2 Reverse	5'-AGTGGATGGATGGTCCTATTACA
Fgf5 Forward	5'-GAAAAGACAGGCCGAGAGTG
Fgf5 Reverse	5'-GAAGTGGGTGGAGACGTGTT
GATA4 Forward	5'-ACGGAAGCCCAAGAACCTGA
GATA4 Reverse	5'-AGCTGCTGTGCCCATAGTGA
Tbx5 Forward	5'-GGACCCAGTCCCTTGAATGG
Tbx5 Reverse	5'-TCCAGGCTGAGGAGTTCTAGGC
Primers for ChIP PCR	
AhRE1 ChIP Forward	5'-GGGGCTCTGCTAAGGGTA
AhRE1 ChIP Reverse	5'-GACTCAACTTCTCCAATCCTAT
AhRE2 ChIP Forward	5'-GGTAACCTTCCCAC TCT
AhRE2 ChIP Reverse	5'-AGGCTTTATTCTTTGGTTTAG
AhRE3 ChIP Forward	5'-TGTTAAGCCATTTGTTAAAGC
AhRE3 ChIP Reverse	5'-GGTTGCCTGATTTAGTGATT
Oct4 ChIP Forward	5'-AAGATTTCTCAAATTGCTTCC
Oct4 ChIP Reverse	5'-CACAGAGCCAGGAGATGC
Exo-AhRE2 ChIP Forward	5'-TGGGTGGAACACTCTTGAC
Exo-AhRE2 ChIP Reverse	5'-AGGCTTTATTCTTTGGTTTAG

Table S4. Primers for cloning of AhR shRNA

Scrambled shRNA-Forward	5'-CCGGCCTAAGGTTAAGTCGCCCTCGCTC GAGCGAGGGCGACTTAACCTAGGTTTTTG
Scrambled shRNA-Reverse	5'-AATTCAAAAACCTAAGGTTAAGTCGCCC TCGCTCGAGCGAGGGCGACTTAACCTTAGG
AhR shRNA-1-Forward	5'-CCGGCATCGACATAACGGACGAAATCTCG AGATTTTCGTCCGTTATGTCGATGTTTTTG
AhR shRNA-1-Reverse	5'-AATTCAAAAACATCGACATAACGGACGA AATCTCGAGATTTTCGTCCGTTATGTCGATG
AhR shRNA-2-Forward	5'-CCGGGTCAAGCCTGTTAGCTATATTCTCG AGAATATAGCTAACAGGCTTGACTTTTTTG
AhR shRNA-2-Reverse	5'-AATTCAAAAAGTCAAGCCTGTTAGCTATA TTCTCGAGAATATAGCTAACAGGCTTGAC

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Indirubin	100 nM
Indirubin-3-monoxime (I3M)	720 nM

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	VPA	1 mM
	NaB	1 mM
	Parnate	2 μ M
GSK-3 β inhibitors	GSK-3 β inhibitor II	3 μ M
	GSK-3 β inhibitor XII	3 μ M
	SB415286	3 μ M
	SB 216763	3 μ M
	GSK-3 β inhibitor IX	3 μ M
	CHIR99021	3 μ M
	LiCl	10 mM
TGF- β inhibitors	Repsox	1 μ M
	SD208	1 μ M
	A83-01	0.5 μ M
	LY2157299	10 μ M
	Tranilast	100 μ M
Other iPS promoting chemicals	Rapamycin	1 nM
	PQ401	1 μ M
	LY294002	0.3 μ M
	PD0325901	0.5 μ M
	Vc	50 μ g/ml

Tabe S3. Primers for Cloning and real time quatitative PCR

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pGL3-872-Forward	5'-CGACGCGTACAGTGATAAGACGACCCA
pGL3-1132-Forward	5'-CGACGCGTAGGAAGCCACTCTATCACT
pGL3-2049-Forward	5'-CGACGCGTTGGGTGGAACACTCTTGAC
pGL3-3344-Forward	5'-CGACGCGTGAAACTCACCTTACCACA
pGL3-3974-Forward	5'-CGACGCGTGGGCTCTGCTAAGGGTAAG
pGL3-4364-Forward	5'-CGACGCGTCGAGGTTCCAAC TGTGCTT
pGL3-4746-Forward	5'-CGACGCGTCTTTTCCAGTCTGAAGCAG
pGL3-Reverse	5'-TACAGATCTGTGACCCGGTTACAGACAT
Primers for cloning of miR-302 promoter reporter mutations	
AhRE-1-Forward	5'-GTAAATGGCCCCTCACTCAGCCTAT TGGAGGAGGTTAGTAC
AhRE-1-Reverse	5'-GTACTAACCTCCTCCAATAGGCTGA GTGAGGGGCCATTTAC
AhRE-2-Forward	5'-CAGCAGCAGCGACTTCTCCTATTAG TTTAAGGATCTCAG
AhRE-2-Reverse	5'-CTGAGATCCTTAAACTAATAGGAGA AGTCGCTGCTGCTG
AhRE-3-Forward	5'-GTCTTAACAGGCAAGGAAAATATAG GAGTTTCCTGGTTGCTTC
AhRE-3-Reverse	5'-GAAGCAACCAGGAAACTCCTATATT TTCCTTGCCCTGTTAAGAC
Primers for real time quatitative PCR	
AhR Forward	5'-AGCCGGTGCAGAAAACAGTAA
AhR Reverse	5'-AGGCGGTCTAACTCTGTGTTC
CYP1A1 Forward	5'-GACCCTTACAAGTATTTGGTCGT
CYP1A1 Reverse	5'-GGTATCCAGAGCCAGTAACCT
pri-miR-302 cluster Forward	5'-CTGTGGGTTTGCTCTTCTGTTTT
pri-miR-302 cluster Reverse	5'-GAGACAGAAAGCATTCCCATGTT
pri-miR-17-92-cluster Forward	5'-TTGGAAC TTCTGGCTATTGGCTCCTC
pri-miR-17-92-cluster Reverse	5'-CCAAGGTGAGGTTCACTTTATTCCTGC
miR-17 stem loop primer Forward	5'-CGGCGGCAAAGTGCTTACAGT
miR-92a stem loop primer Forward	5'-CGGCGGTATTGCACTTGTCC
stem loop primer Reverse	5'-GTGCAGGGTCCGAGGT
endogenous Oct4 Forward	5'-TCTTTCCACCAGGCCCCCGGCTC
endogenous Oct4 Reverse	5'-TGCGGGCGGACATGGGGAGATCC
endogenous Sox2 Forward	5'-TAGAGCTAGACTCCGGGCGATGA
endogenous Sox2 Reverse	5'-TTGCCTTAAACAAGACCACGAAA
Nanog Forward	5'-AGGGTCTGCTACTGAGATGCTCTG
Nanog Reverse	5'-CAACCACTGGTTTTTCTGCCACCG
Rex1 Forward	5'-CGTGTAACATACACCATCCG
Rex1 Reverse	5'-GAAATCCTCTTCCAGAATGG
Exo-Oct4 Forward	5'-GGGTGGACCATCCTCTAGAC
Exo-Oct4 Reverse	5'-CCAGGTTCGAGAATCCAC
Exo-Sox2 Forward	5'-GGGTGGACCATCCTCTAGAC
Exo-Sox2 Reverse	5'-GGGCTGTTCTTCTGGTTG
Exo-Klf4 Forward	5'-GGGTGGACCATCCTCTAGAC
Exo-Klf4 Reverse	5'-GCTGGACGCAGTGTCTTCTC
MBD2 Forward	5'-AGAACAAGGGTAAACCAGACCT
MBD2 Reverse	5'-ACTTCACCTTATTGCTCGGGT
NR2F2 Forward	5'-TCAACTGCCACTCGTACCTG
NR2F2 Reverse	5'-CCATGATGTTGTTAGGCTGCAT
CDKN1A Forward	5'-CCTGGTGATGTCCGACCTG
CDKN1A Reverse	5'-CCATGAGCGCATCGCAATC
RBL2 Forward	5'-AACTTCCCCATGATTAGCGATG
RBL2 Reverse	5'-GGTTAGAACACTGAAGGGCATTT
MECP2 Forward	5'-ATGGTAGCTGGGATGTTAGGG
MECP2 Reverse	5'-TGAGCTTTCTGATGTTTCTGCTT
AOF2 Forward	5'-GTGGTGTTATGCTTTGACCGT
AOF2 Reverse	5'-GCTGCCAAAAATCCCTTTGAGA
RHOC Forward	5'-ATGGCTGCGATCCGAAAGAAG
RHOC Reverse	5'-GCACGTAGACCTCTGGAAACT
TGFβR2 Forward	5'-CCGCTGCATATCGTCCTGTG
TGFβR2 Reverse	5'-AGTGGATGGATGGTCCTATTACA
Fgf5 Forward	5'-GAAAAGACAGGCCGAGAGTG
Fgf5 Reverse	5'-GAAGTGGGTGGAGACGTGTT
GATA4 Forward	5'-ACGGAAGCCCAAGAACCTGA
GATA4 Reverse	5'-AGCTGCTGTGCCCATAGTGA
Tbx5 Forward	5'-GGACCCAGTCCCTTGAATGG
Tbx5 Reverse	5'-TCCAGGCTGAGGAGTTCTAGGC
Primers for ChIP PCR	
AhRE1 ChIP Forward	5'-GGGGCTCTGCTAAGGGTA
AhRE1 ChIP Reverse	5'-GACTCAACTTCTCCAATCCTAT
AhRE2 ChIP Forward	5'-GGTAACCTTCCCACTCCT
AhRE2 ChIP Reverse	5'-AGGCTTTATTCTTTGGTTTAG
AhRE3 ChIP Forward	5'-TGTTAAGCCATTTGTTAAAGC
AhRE3 ChIP Reverse	5'-GGTTGCCTGATTTAGTGATT
Oct4 ChIP Forward	5'-AAGATTTCTCAAATTGCTTCC
Oct4 ChIP Reverse	5'-CACAGAGCCAGGAGATGC
Exo-AhRE2 ChIP Forward	5'-TGGGTGGAACACTCTTGAC
Exo-AhRE2 ChIP Reverse	5'-AGGCTTTATTCTTTGGTTTAG

Table S4. Primers for cloning of AhR shRNA

Scrambled shRNA-Forward	5'-CCGGCCTAAGGTTAAGTCGCCCTCGCTC GAGCGAGGGCGACTTAACCTAGGTTTTTG
Scrambled shRNA-Reverse	5'-AATTCAAAAACCTAAGGTTAAGTCGCCC TCGCTCGAGCGAGGGCGACTTAACCTTAGG
AhR shRNA-1-Forward	5'-CCGGCATCGACATAACGGACGAAATCTCG AGATTTTCGTCCGTTATGTCGATGTTTTTG
AhR shRNA-1-Reverse	5'-AATTCAAAAACATCGACATAACGGACGA AATCTCGAGATTTTCGTCCGTTATGTCGATG
AhR shRNA-2-Forward	5'-CCGGGTCAAGCCTGTTAGCTATATTCTCG AGAATATAGCTAACAGGCTTGACTTTTTTG
AhR shRNA-2-Reverse	5'-AATTCAAAAAGTCAAGCCTGTTAGCTATA TTCTCGAGAATATAGCTAACAGGCTTGAC

Effects of long-term exposure to PM₁₀ and NO₂ on asthma and wheeze in a prospective birth cohort

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► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/jech-2013-202681>).

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Received 27 March 2013

Revised 29 July 2013

Accepted 30 July 2013

ABSTRACT

Background Epidemiological studies on the effect of urban air pollution on childhood asthma have shown conflicting results and so far no consistent association has emerged. However, a common limitation in previous studies has been exposure misclassification leading to uncertainties in risk estimates. The aim of this study was to analyse the effects of long-term exposure to particulate matter (PM₁₀) and nitrogen dioxide (NO₂) on the prevalence of asthma and wheeze within a population-based birth cohort—the Manchester Asthma and Allergy Study (MAAS).

Methods The prevalence of asthma and current wheeze within the cohort (N=1185) was determined through parental questionnaires at ages 3, 5, 8 and 11 years. The typical monthly PM₁₀ and NO₂ exposure of each child was estimated through a novel microenvironmental exposure model from birth to age 11. The association between exposure and asthma or wheeze was analysed using generalised estimating equations and multiple logistic regression.

Results The range of asthma prevalence was 15.2–23.3%, with the lowest prevalence at age 3 and the highest at age 5. The prevalence of current wheeze decreased from ages 3 to 8 (23.7–18%). The mean NO₂ exposure decreased from the 1st year of life (21.7 µg/m³) to the 11th year of life (16.0 µg/m³). The mean PM₁₀ exposure showed a smaller decrease (12.8–10.7 µg/m³). The statistical analysis showed no significant association between the exposures and either outcome.

Conclusions No evidence of a significant association between long-term exposure to PM₁₀ and NO₂ and the prevalence of either asthma or wheeze was found.

INTRODUCTION

Asthma is the most common chronic disease during childhood with multiple environmental and genetic risk factors.¹ The UK has the highest prevalence of childhood asthma within Europe² and physician diagnosed asthma in children has increased twofold to threefold from 1970 to 2000.³ The amount of road traffic in the UK has increased fivefold from 1950 to 2008,⁴ resulting in an increase in air pollutants such as nitrogen dioxide (NO₂) and particulate matter (PM) until the 1990s, when the introduction of catalytic converters led to a decrease in air pollution in the UK. This parallel rise in asthma prevalence and road traffic makes traffic-related pollution a potential risk factor for the development of asthma.

Epidemiological studies of air pollution and asthma have been heterogeneous in design, health outcomes and exposure assessment used and have

provided conflicting results.⁵ Within asthma sufferers, increasing exposure to NO₂ and PM is associated with more severe disease.⁶ However, most studies found no association between these pollutants and asthma prevalence or incidence during childhood,^{7–11} although a few did report associations, often in post hoc analysis of subgroups. Therefore, whether air pollution exposure is associated with childhood asthma prevalence remains unclear.

Recent reviews have highlighted the need for improved exposure assessment methods in epidemiological studies.^{5, 12} Although ideally one would monitor personal exposure throughout life, this is clearly not feasible and alternative methods of assigning an index of exposure have been used, for example, central outdoor monitors, land use regression (LUR) models or temporary indoor static monitors. Each methodology has its strengths and weaknesses, which can lead to exposure misclassification, adding uncertainty to the findings of epidemiological studies: using only outdoor estimates may overestimate exposure of children living in industrialised countries as they spend the majority of their time indoors.¹³ Indoor NO₂ and PM₁₀ concentrations tend to be lower than outdoor concentrations in UK homes.¹⁴ However, previous studies have shown large increases in indoor NO₂ and PM₁₀ concentrations, if indoor sources such as gas cookers and cigarette smoke are present.^{15–17} At least 60% of homes in the UK contain gas cooking appliances¹⁴ and the prevalence of adult smokers in the UK was 20% in 2010;¹⁸ therefore, indoor concentrations may make an important contribution to the overall exposure of individuals. We have developed and validated a novel exposure assessment method, the microenvironmental exposure model (MEEM),^{19–21} which models indoor and outdoor exposures longitudinally. A performance evaluation of this model against personal monitoring data showed a lower mean prediction error compared to methods such as LUR or central monitors.²¹

The aims of this study were to estimate time-weighted long-term exposure to PM₁₀ and NO₂ of children participating in the Manchester Asthma and Allergy Study (MAAS)²² and to analyse effects of exposure on asthma and current wheeze prevalence at ages 3, 5, 8 and 11 years. We hypothesised that children exposed to higher PM₁₀ or NO₂ concentrations may be more likely to have asthma than children exposed to low concentrations.

METHODS

Study population

MAAS is a prospective birth cohort based in Greater Manchester, located in the Northwest of England²²

To cite: Mölter A, Agius R, de Vocht F, et al. *J Epidemiol Community Health* Published Online First: [please include Day Month Year] doi:10.1136/jech-2013-202681

(see online supplementary figure A1, appendix 1). Participants were recruited while attending antenatal clinics at two local hospitals between 1995 and 1997. Children were reviewed at ages 3, 5, 8 and 11 years. At each review, parents completed a nurse-administered validated questionnaire. Approval to conduct MAAS has been granted by the local Research Ethics Committee (SOU/00/258; SOU/00/259) and the study is registered as ISRCTN72673620. Parents of all children participating in the study have provided written informed consent.

Clinical outcomes

Asthma: defined as at least two positive answers to the following three questions: (1) doctor diagnosis of asthma ever; (2) child having wheezed during the previous 12 months and (3) child having received asthma medication during the previous 12 months.

Current wheeze: a positive answer to “Has your child ever had wheezing or whistling in the chest in the last 12 months?”.

Modelled exposure to PM₁₀ and NO₂

Children's exposure was estimated using a novel model 'MEEM' described in a previous publication.²¹ The model assumes that children spend the majority of their time in three types of micro-environments (MEs): the child's home (kitchen, living room, bedroom), the child's school and the journey between home and school (see online supplementary table A1, appendix 1). Each of these MEs can be in an indoor or an outdoor ME and different methods were used to model air pollution in each ME: PM₁₀ and NO₂ concentrations in all outdoor MEs were estimated through land use regression models described in detail in previous publications.^{19–20} Concentrations in the kitchen, living room and child's bedroom were estimated through the INDAIR model, which is a validated mass balance model, specifically designed for residential buildings within the UK.¹⁷ Concentrations in the journey indoor and school MEs were estimated using published indoor to outdoor ratios^{23–24} (see online supplementary table A1, appendix 1).

A timeline of each child's home and school addresses, as well as the mode of transport between the two, was collected through parental questionnaires at the age 11 review. The route of the journey from home to school was estimated using a shortest path analysis in ArcMap 9.2 (ESRI Inc). The time a child typically spent in each ME was derived from a parental questionnaire at the age 11 review.

This questionnaire also collected essential input information for the INDAIR model, including room sizes, air exchange rates, presence of gas cooking appliances, typical cooking duration and the number of cigarettes smoked in the home. However, this information was only applicable for the most recent home of each child and it was not feasible to collect such detailed input information for the entire lifetime of each child. Therefore, MEEM was used to estimate exposure for the most recent exposure time period, that is, the summer and winter prior to the age 11 review. Separate exposure estimates were calculated for summer and winter, because we assumed that the air exchange rates in children's homes would differ in summer and winter. We hypothesized that during the winter, the windows would be mainly closed, while during the summer they might be open for extended periods of time. The parental questionnaire described above contained separate questions for winter and summer regarding the windows being open or closed.

To estimate exposure for the whole lifetime of the children, MEEM was slightly simplified by replacing the INDAIR model with an indoor to outdoor ratio (see online supplementary table

A1, appendix 1) and by assuming that the children attended school from 9:00 to 15:00 on weekdays. For clarity, this simplified version of MEEM is referred to as the 'Lifetime model'; however, it should be noted that the two models are almost identical and that the concentration estimates only differ in the home indoor environment.

For analytical purposes, the exposure estimates from the 'Lifetime model' were averaged into the following aetiologically relevant time windows: first year of life (age 0–1), birth to review (age 0–3, 0–5, 0–8, 0–11), 1 year prior to review (ages 2–3, 4–5, 7–8, 10–11).

Statistical analysis

The association between PM₁₀ and NO₂ exposure and asthma and current wheeze prevalence at each review was analysed using multiple logistic regression. To analyse the association between the above exposures and health outcomes over time, generalised estimating equations were used to adjust for the within-subject correlation of repeated measures. Gender and age were included in all analyses; at age 11, the Tanner stage was included to adjust for pubertal development. A list of potential confounding variables (identified in MAAS and the published literature) is shown in online supplementary appendix 1. Initially, we carried out univariate analyses of the potential confounding variables, the clinical outcomes and the exposures to identify variables of further interest. This was followed by a stepwise process to assess the variability explained by each potential confounder within the exposure-outcome models. We decided a priori to include gender and age in all models. Of the remaining variables, only those with a significant effect ($p < 0.05$) were included in the final models. All statistical analyses were carried out in SPSS V.16.0.

RESULTS

Characteristics of the study population

The number of children participating in each review, the corresponding asthma and current wheeze prevalence and the number of exposure estimates available at each time window are shown in figure 1. The children included in this analysis did not differ from those who were excluded, apart from being more likely to have older siblings (55% vs 52.3%) and less likely to have a dog (13.2% vs 15.4% at age 5; 16.8% vs 19.3% at age 8 years; table 1).

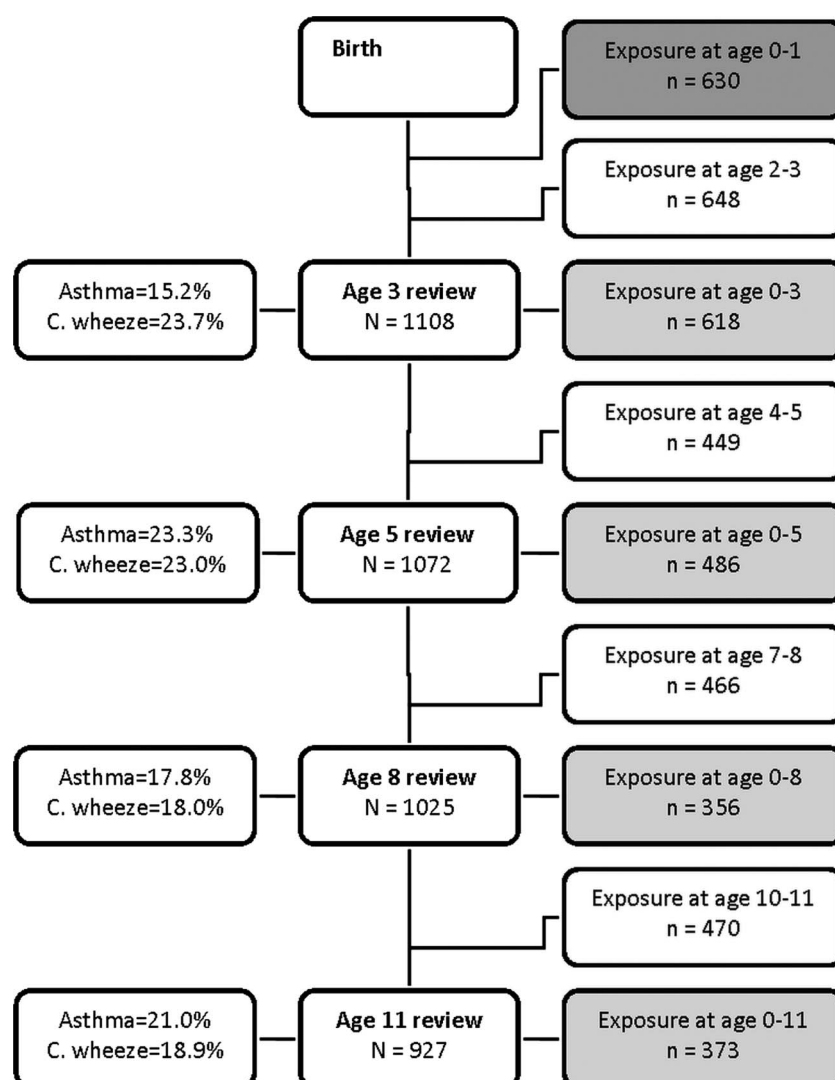
Exposure to pollutants

The mean PM₁₀ and NO₂ concentrations for the different exposure windows are shown in table 2. Annual mean exposure estimates decreased from the 1st year of life to the 11th year of life ($\Delta\text{PM}_{10} = -2.1 \mu\text{g}/\text{m}^3$; $\Delta\text{NO}_2 = -5.7 \mu\text{g}/\text{m}^3$), reflecting a general decrease in the annual mean NO₂ and PM₁₀ concentrations in the study area.²⁵ The SD of most exposure estimates were relatively small, indicating relatively low variability within the lifetime exposure data. However, the mean and SD of the summer and winter exposures estimates were higher, indicating greater variability due to peak exposures in the home indoor ME estimated by INDAIR. The PM₁₀ and NO₂ concentrations were moderately to strongly correlated across all exposure windows (Pearson's $r = 0.59$ – 0.89).

Pollution and asthma or current wheeze

The final models were adjusted for gender, age, body mass index, paternal income at birth, sensitisation, family history of asthma, hospitalisation during the first 2 years of life and smoking within the child's home during the first year of life. At age 11, the mean Tanner stage was used as an additional

Figure 1 Overview of the Manchester Asthma and Allergy Study cohort showing participation rates at each review, the prevalence of asthma and current wheeze and the number of exposure estimates available at different time windows.



covariate to identify pubescent children. Asthma prevalence was not associated with exposure to PM₁₀ and NO₂ during the first year of life (figure 2A), during the lifetime (figure 2B) or during the year prior to the review (figure 2C), in either the cross-sectional or longitudinal models. Furthermore, there was no association between a child having asthma at age 11 and their PM₁₀ and NO₂ exposure during the winter and summer prior to the age 11 review (figure 3).

The results of the analyses of current wheeze prevalence and PM₁₀ and NO₂ exposure are shown in online supplementary figures A2 and A3 (appendix 2). These figures show similar trends as the figures for asthma and none of the analyses with current wheeze indicated a significant association with air pollution exposure. In addition, we have also included results of analyses of asthma incidence and PM₁₀ and NO₂ exposure in online supplementary appendix 2 (figures A4 and A5). However, these were not materially different from the above results for asthma prevalence.

DISCUSSION

This is the first study to model indoor and outdoor air pollution exposure, providing time-weighted estimates of PM₁₀ and NO₂ exposure for multiple time windows throughout primary school age, and to analyse the effects of exposure on childhood asthma and wheeze prevalence from birth to age 11. Using this

comprehensive exposure model, we found no association between PM₁₀ or NO₂ and asthma or wheeze within a population-based birth cohort located in the Northwest of England.

Previous cohort studies on air pollution and childhood asthma have reported conflicting findings. As in our study, several cohort studies on the prevalence of asthma and wheeze in children also found no association with outdoor NO₂ exposure.^{9 10 26 27} However, while some studies found an association between outdoor NO₂ and asthma or current wheeze prevalence in schoolchildren,^{28 29} others only found this in long-term resident girls³⁰ or when relative humidity³¹ was included in the model. Similar to our study, a birth cohort study in Oslo¹⁰ also found no association between modelled outdoor NO₂ exposure during the first year of life and asthma prevalence at age 9–11 years. Furthermore, a birth cohort study in Stockholm, the Barn Allergy Milieu Stockholm Epidemiology (BAMSE), found no significant association between modelled NO_x exposure during the first year of life or lifetime and asthma prevalence at age 1, 2, 4, 8 or 12 years.³² However, in contrast to our findings, a birth cohort study in the Netherlands, the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study,³³ found a significant association between exposure at the birth address and asthma prevalence at age 5 years and longitudinal asthma (age 1–8 years). Fewer studies have been carried out on

Table 1 Overview and descriptive statistics of potential confounding variables

Variable	All children in cohort		Children with exposure data age 0–1		p Value*
	n (N)	Per cent	n (N)	Per cent	
<i>Female gender</i>	543 (1185)	45.8	296 (630)	47.0	0.39
Native ethnicity	1059 (1115)	95.0	578 (605)	95.5	0.87
Older siblings	561 (1073)	52.3	337 (613)	55.0	0.042
<i>Family history of asthma</i>	441 (1185)	37.2	235 (630)	37.3	0.95
Mother atopict	683 (1147)	59.5	357 (618)	57.8	0.18
Father atopict	717 (1138)	63.0	390 (617)	63.2	0.88
<i>Child atopict</i> [†]					
Age 3	225 (983)	22.9	133 (558)	23.8	0.42
Age 5	294 (963)	30.5	171 (563)	30.4	0.90
Age 8	314 (927)	33.9	177 (552)	32.1	0.16
Age 11	281 (784)	35.8	177 (514)	34.4	0.26
Maternal smoking during pregnancy	119 (1025)	11.6	70 (598)	11.7	0.91
<i>Any smoker living in the home</i>					
During first year of life	438 (1024)	42.8	267 (598)	44.6	0.15
Age 3	337 (1080)	31.2	189 (605)	31.2	0.98
Age 5	373 (1094)	34.1	218 (619)	35.2	0.37
Age 8	366 (999)	36.6	226 (586)	38.6	0.13
Age 11	232 (896)	25.9	153 (558)	27.4	0.18
<i>Hospitalisation during the first two years of life for lower respiratory tract infection</i>	109 (1185)	9.2	64 (630)	10.2	0.22
Day care attendance during the first two years of life	741 (1087)	68.2	407 (603)	67.5	0.60
Gas cooker in the home					
Age 1	801 (1028)	77.9	479 (600)	79.8	0.08
Age 8	819 (1029)	79.6	479 (601)	79.7	0.92
age 11	727 (930)	78.2	450 (573)	78.5	0.74
Visible signs of dampness or mould in the home					
Age 1	177 (1030)	17.2	110 (601)	18.3	0.26
Age 8	149 (1030)	14.5	94 (601)	15.6	0.21
Age 11	143 (930)	15.4	87 (573)	15.2	0.84
Dog in the home					
During first year of life	174 (1028)	16.9	95 (600)	15.8	0.27
Age 3	150 (1070)	14.0	75 (602)	12.5	0.10
Age 5	168 (1090)	15.4	82 (620)	13.2	0.022
Age 8	198 (1028)	19.3	101 (600)	16.8	0.019
Age 11	239 (930)	25.7	137 (573)	23.9	0.11
Cat in the home					
During first year of life	205 (1027)	20.0	118 (599)	19.7	0.80
Age 3	208 (1068)	19.5	110 (602)	18.3	0.26
Age 5	216 (1093)	19.8	116 (621)	18.7	0.30
Age 8	217 (1028)	21.1	120 (600)	19.0	0.30
Age 11	228 (930)	24.5	136 (573)	23.7	0.48
<i>Paternal income at birth</i>					
<£10 k	171 (1078)	15.9	88 (588)	15.0	0.009
£10k–£20 k	406 (1078)	37.7	237 (588)	40.3	
£20k–£30 k	297 (1078)	27.6	171 (588)	29.1	
>£30 k	204 (1078)	18.9	92 (588)	15.6	
	Mean (±SD)		Mean (±SD)		p‡
Birth weight (g)	3462 (510)		3480 (518)		0.50
Gestational age (weeks)	39.9 (1.6)		39.8 (1.6)		0.89
Maternal age at birth (years)	30.4 (4.8)		30.8 (4.6)		0.087
Duration of breastfeeding (weeks)	15.5 (20.0)		16.6 (20.6)		0.26
<i>Exact age at review (years)</i>					
Age 3	3.0 (0.1)		3.0 (0.1)		0.56
Age 5	5.0 (0.1)		5.0 (0.1)		0.25
Age 8	8.0 (0.2)		8.0 (0.1)		0.27
Age 11	11.4 (0.5)		11.4 (0.5)		0.79

Continued

Table 1 Continued

Variable	All children in cohort		Children with exposure data age 0–1		p Value*
	n (N)	Per cent	n (N)	Per cent	
	Mean (±SD)		Mean (±SD)		p‡
Height (cm)					
At age 3	95.6 (3.7)		95.5 (3.7)		0.81
At age 5	110.0 (4.5)		109.8 (4.6)		0.50
At age 8	128.1 (5.6)		127.9 (5.7)		0.58
At age 11	148.2 (7.7)		148.2 (7.8)		0.98
Weight (kg)					
Age 3	15.3 (1.9)		15.2 (2.0)		0.80
Age 5	19.9 (2.9)		19.8 (3.1)		0.76
Age 8	28.2 (5.6)		28.1 (5.7)		0.87
Age 11	42.4 (10.4)		42.5 (10.4)		0.86
<i>Body mass index</i>					
Age 3	16.7 (1.4)		16.7 (1.4)		0.81
Age 5	16.3 (1.6)		16.3 (1.7)		0.99
Age 8	17.1 (2.4)		17.1 (2.5)		0.99
Age 11	19.1 (3.4)		19.2 (3.5)		0.80
<i>Mean Tanner stage</i>	2.06 (0.88)		2.05 (0.89)		0.84

Italic variables were included in the adjusted model.

*Based on Pearson's χ -square comparison.

†Based on positive skin prick test to common food and inhalant allergens.

‡Based on t test.

the association between outdoor PM concentrations and asthma or current wheeze in children. In keeping with our results, most of these studies found no association with asthma or wheeze.^{8 11 27 30 34–36} Of the European birth cohort studies, the BAMSE study also found no significant association between PM₁₀ exposure during the first year of life or lifetime and asthma prevalence at age 1, 2, 4, 8 or 12 years,³² further supporting our results. However, the PIAMA study found an association between outdoor particulate matter exposure and asthma and current wheeze prevalence;^{26 33} but it should be noted that they analysed the effects of smaller particles (PM_{2.5}), which may explain some of the discrepancy compared to our results. Nevertheless, a previous cohort study in the UK of 1-year-old to 5-year-old children found a significant association between

modelled outdoor PM₁₀ exposure and current wheeze prevalence.³⁷ Statistically significant associations were only present in adjusted regression models, possibly indicating a strong influence of a covariate within the models. Outside of Europe, a Canadian case-control study of young children (age 0–4 years old)³⁸ found an association between outdoor PM₁₀ exposure and asthma prevalence. This study used health records, and therefore information on potential confounding variables such as smoking or parental asthma was limited.

Of the studies focusing on indoor air pollution exposure, a study in Japan³⁹ found an association between indoor NO₂ exposure and the prevalence of asthma in children, but several other published studies found no significant association between measured indoor NO₂ concentrations and childhood asthma or wheeze prevalence.^{40–43} It is known that children living in industrialised countries spend more than 80% of their time indoors.¹³ Therefore, indoor air pollution exposure may be a better proxy of personal exposure than outdoor exposure and associations found in previous studies based on outdoor exposure may have been affected by exposure misclassification. Although the aim of our study was to analyse time-weighted exposures, we did carry out a small number of 'spot checks' with modelled residential outdoor exposure estimates (see online supplementary table A2, appendix 2). These 'spot checks' did not indicate an association between outdoor exposure and asthma prevalence either. Therefore, it remains unclear to what extent previous findings based solely on outdoor exposure estimates may have been affected by exposure misclassification.

A strength of this study was the novel exposure model used. Although we cannot conclude that exposure misclassification was completely absent from this study, our exposure assessment method eliminated common sources of exposure misclassification by including home and school addresses and by modelling indoor and outdoor exposures. A further advantage of the exposure model was that it provided exposure estimates for

Table 2 Distribution of PM₁₀ and NO₂ exposures (in $\mu\text{g}/\text{m}^3$)

Exposure window	PM ₁₀		NO ₂		N
	Mean	SD	Mean	SD	
Summer before age 11 review*	15.1	3.03	20.3	5.13	392
Winter before age 11 review*	20.0	2.95	31.9	4.55	416
Age 0–1†	12.8	0.58	21.7	1.44	630
Age 0–3†	12.0	0.48	20.8	1.30	618
Age 0–5†	11.6	0.40	20.1	1.25	486
Age 0–8†	11.6	0.42	19.4	1.08	356
Age 0–11†	11.4	0.42	18.7	1.05	373
Age 2–3†	11.2	0.39	20.0	1.33	648
Age 4–5†	11.2	0.63	18.9	1.54	449
Age 7–8†	11.0	0.55	17.5	1.26	466
Age 10–11†	10.7	0.52	16.0	1.21	470

*Three months averages provided by microenvironmental exposure model.

†Twelve to 132 months averages provided by Lifetime model.

PM₁₀, particulate matter; NO₂, nitrogen dioxide.

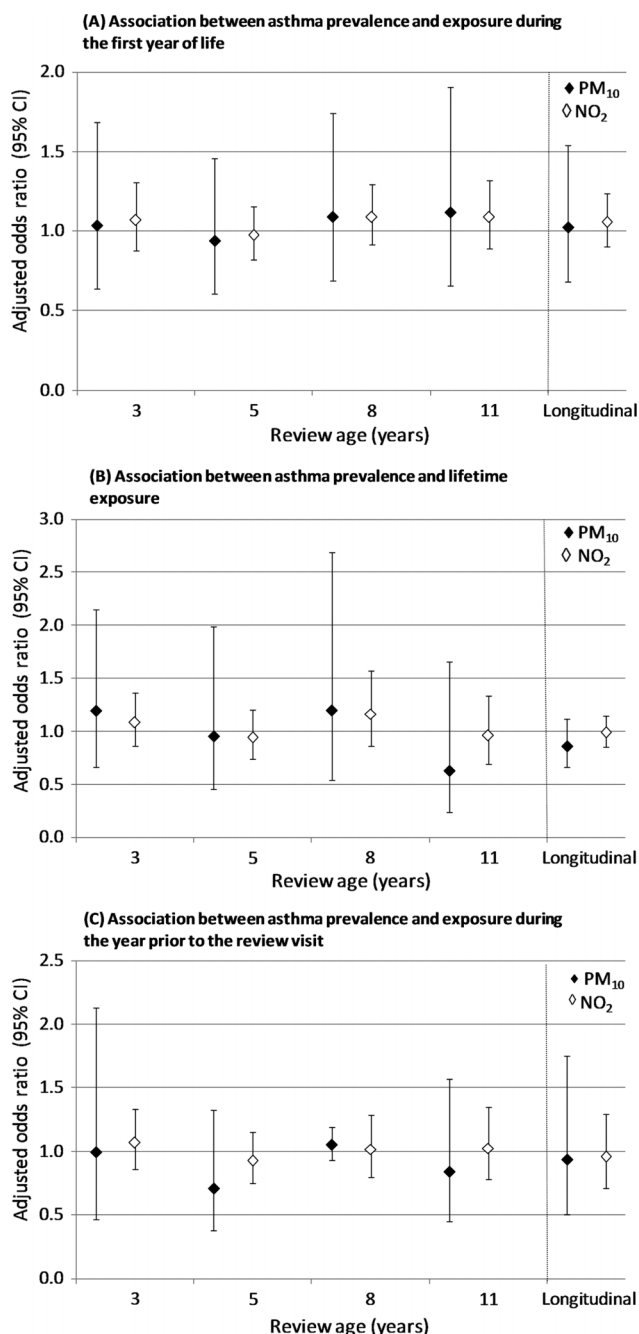


Figure 2 Adjusted cross-sectional and longitudinal associations between asthma prevalence and long-term particulate matter and nitrogen dioxide exposure during different time windows ('Lifetime model'). ORs were calculated per 1 $\mu\text{g}/\text{m}^3$ increase in exposure.

different exposure time windows. There is currently no scientific consensus on which exposure window is most likely to have an effect on asthma in children.³³ While some studies suggest that exposure during early life is most important,³⁸ others found that lifetime exposure had an effect.³³ However, none of the exposure windows used in this study showed a significant association with asthma or wheeze and no trend was apparent.

A limitation of this study was the number of children with a full set of exposure estimates available for the duration of the study (eg, 373 children for the 0–11 years exposure window) compared to the total size of the MAAS cohort (eg, 927 children at age 11, figure 1). This limited availability was due to the

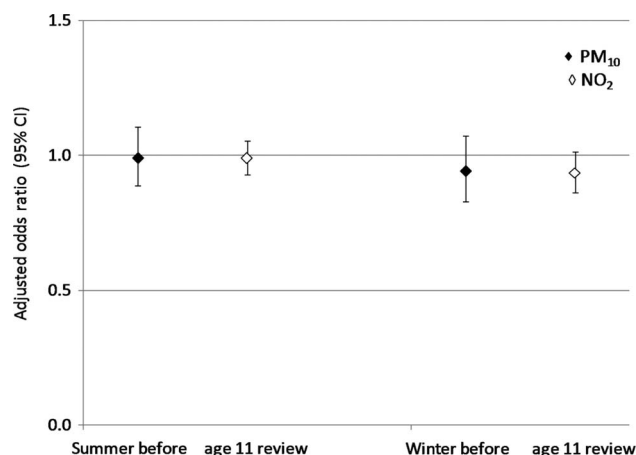


Figure 3 Adjusted association between asthma at age 11 and particulate matter and nitrogen dioxide exposure during the summer and winter before the age 11 review (microenvironmental exposure model). ORs were calculated per 1 $\mu\text{g}/\text{m}^3$ increase in exposure.

following two reasons: (1) the children's address history and school history were collected retrospectively at the age 11 clinical follow-up. However, some of the address/school histories provided were incomplete, resulting in some missing data. (2) Children's home and/or school addresses were outside Greater Manchester and therefore the land use regression model^{19–20} could not be used. Approximately 20% of the children had lived outside this geographic area for part of the follow-up period and hence had to be excluded from the analysis during these time periods. Nevertheless, to the author's knowledge, this is the largest study until now to use a detailed microenvironmental model to estimate PM₁₀ and NO₂ exposure in children over an 11 year time period.

A limitation in previous studies may have been the sole use of the question 'has a doctor ever diagnosed asthma in your child?' to identify the asthma phenotype. In children under the age of 3, it can be difficult to distinguish asthma from other respiratory illnesses.²⁷ Therefore, this question may misclassify some children. In contrast, our study used three variables to identify asthmatic children, which is thought to reduce misclassification and to improve the reliability of our findings.⁴⁴

The aim of the current study was to investigate the association between the modelled exposure to two pollutants (NO₂ and PM₁₀) and asthma prevalence at a population level. Real-life exposures to pollutants are more complex, as many sources emit a range of pollutants and mixtures will vary over space and time, particularly between the outdoor and the indoor environment. While it was beyond the remit of the current study to model all exposures with a high degree of resolution, we acknowledge that other unmeasured pollutants may have an effect on asthma prevalence. In addition, we chose prevalence rather than incidence as our outcome measure; we emphasise that for incident asthma the results are not materially different (see online supplementary appendix 2, figure A4 and A5). As MAAS is a population-based birth cohort, asthma exacerbations were relatively infrequent events precluding any assessment of the association between exposure and exacerbations. We contrast our findings with those seen in asthma populations where increasing exposure is frequently associated with an increase in risk of exacerbations.⁴⁵

In conclusion, using a comprehensive exposure assessment method, modelling indoor and outdoor exposures to NO₂ and

PM₁₀ longitudinally with time weighting, we found no association between pollutant exposure and prevalence of asthma or wheeze. However, these results should not be extrapolated to geographical areas, where the range of exposures is much higher, and they do not imply that in those with pre-existing asthma, exposures at UK levels are safe. Replication of this comprehensive exposure assessment method should now be performed in larger populations.

What is already known on this subject

- Previous studies on air pollution exposure and childhood asthma have shown conflicting results and no consistent association has emerged. Exposure misclassification is thought to be a common limitation and may have contributed to inconsistencies in previous findings.

What this study adds

- This study aimed to reduce exposure misclassification by modelling indoor and outdoor air pollution exposure in multiple microenvironments and through the use of time-weighted estimates of particulate matter and nitrogen dioxide exposure. The comprehensive exposure model provided longitudinal exposure for multiple time windows throughout primary school age, which enabled repeated cross-sectional and longitudinal analyses of air pollution and childhood asthma prevalence.

Acknowledgements The authors would like to thank the families who participated in MAAS and all members of the MAAS study team for their tireless effort, in particular Joseph Nathan and Mandy Mycock who carried out the data quality checks. The exposure model used in this study was partly based on the Greater Manchester air dispersion modelling study carried out by the former Atmospheric Research and Information Centre on behalf of the local authorities of Greater Manchester. Therefore, they would also like to thank the Manchester Area Pollution Advisory Council for permitting us to access this data. Furthermore, they are very grateful to Prof M Ashmore, Dr S Dimitroulopoulou and Dr A Terry for permitting us access to the INDAIR model¹⁷ and for their helpful advice on the use of this model.

Contributors RA, AS and AC contributed to the study conception and design and obtained funding; AM, SL, RA and FdV developed the exposure model; AM and WG contributed to the acquisition of data; AM and AS analysed the data; AM and AS drafted the manuscript and all authors contributed to the revision of the report and approved the version submitted.

Competing interests None.

Patient consent Obtained.

Ethics approval Local Research Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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APPENDIX 1 – ONLINE SUPPLEMENT

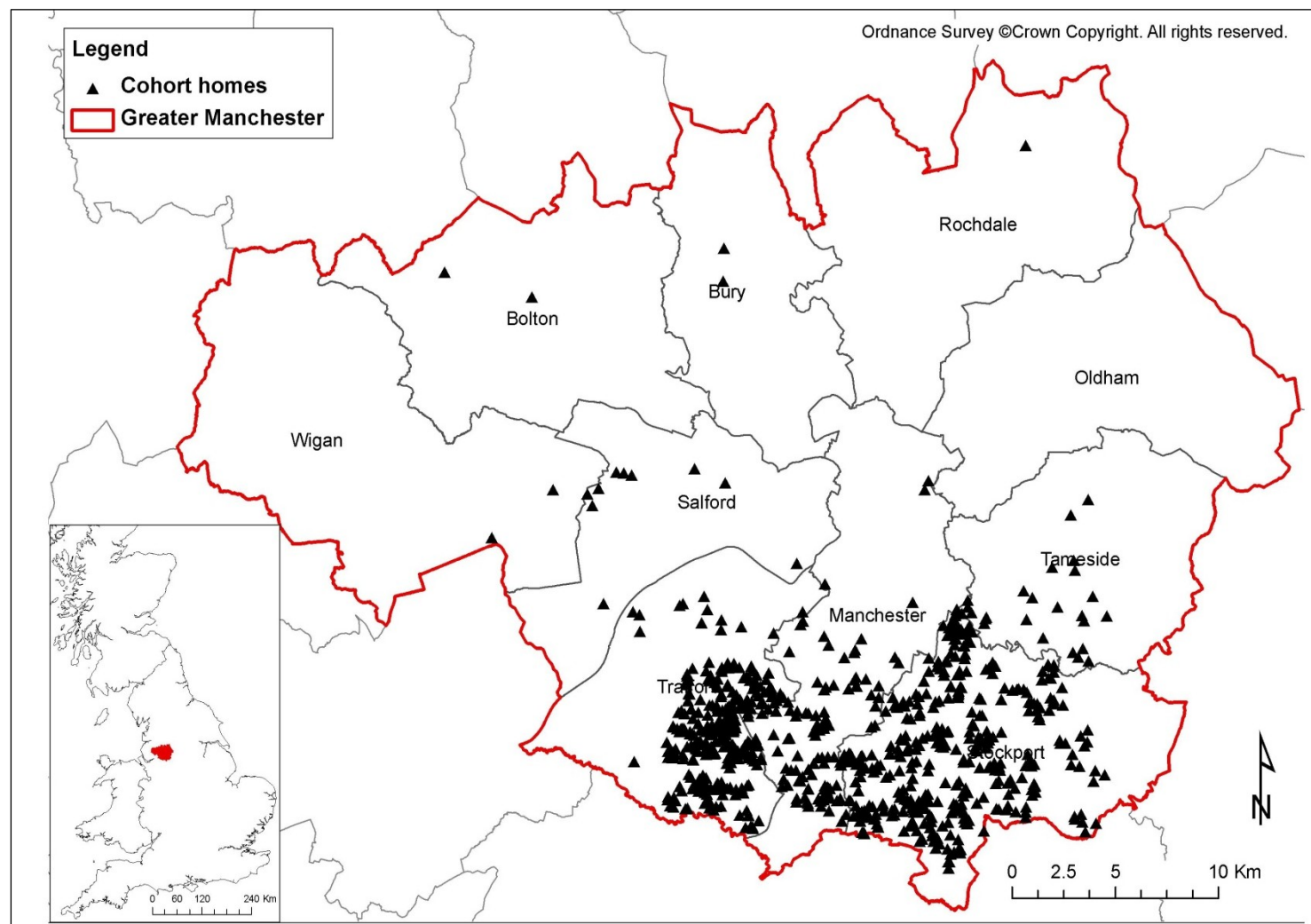


Figure A1: Geographic distribution of cohort homes within the Greater Manchester area

Table A1: List of microenvironments with their estimation methods for PM₁₀ and NO₂ concentrations

Microenvironment		Estimation method
Home	Outdoor	LUR model ^{1;2}
	Kitchen	INDAIR model ^{3;4} (I/O ratio = 0.5 in 'Lifetime model')
	Living room	
	Bedroom	
Journey	Walking or cycling	LUR model ^{1;2}
	Car or Bus	I/O ratio = 2 ⁵
School	Outdoor	LUR model ^{1;2}
	Indoor	I/O ratio = 0.5 ⁶

LUR=Land use regression; I/O=Indoor to outdoor

Due to the data availability for the land use regression model only exposure at addresses within the Greater Manchester area could be modelled. This means exposure could not be modelled for time periods during which children lived or went to school outside of Greater Manchester.

Confounding variables and covariates

Potential confounding variables and covariates were identified through previous research in MAAS and through the published literature ⁷⁻¹⁰. The following variables were identified: gender, age, ethnicity, older siblings, family history of asthma, parental atopy, sensitisation, maternal smoking during pregnancy, any smoker living with the home, hospitalisation during the first two years of life for lower respiratory tract infection, day care attendance during the first two years of life, presence of a gas cooker in the home, Visible signs of dampness or mould in the home, presence of a dog or a cat in the home, socioeconomic status, birth weight, gestational age, maternal age at birth, duration of breastfeeding, body height, body weight, body mass index, and Tanner stage (age 11 only).

Atopic sensitisation was determined through skin prick tests at each review, using a set of common food and inhalant allergens. At age 3, 5, 8 and 11 years children were tested for mite, cat, dog, mould, grass pollen, tree pollen, milk and egg allergens. In addition, at age 8 and 11 years children were also tested for peanut allergens. Atopy was defined as a mean

weal diameter 3mm greater than the negative control to at least one of the allergens tested. Parental atopy was determined through skin prick tests during the recruitment stage.

Body height (to the nearest 0.1cm) and body weight (to the nearest 0.01kg) were measured during hospital visits at each review. All other variables shown above were obtained through parental questionnaires.

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APPENDIX 2 - ONLINE SUPPLEMENT

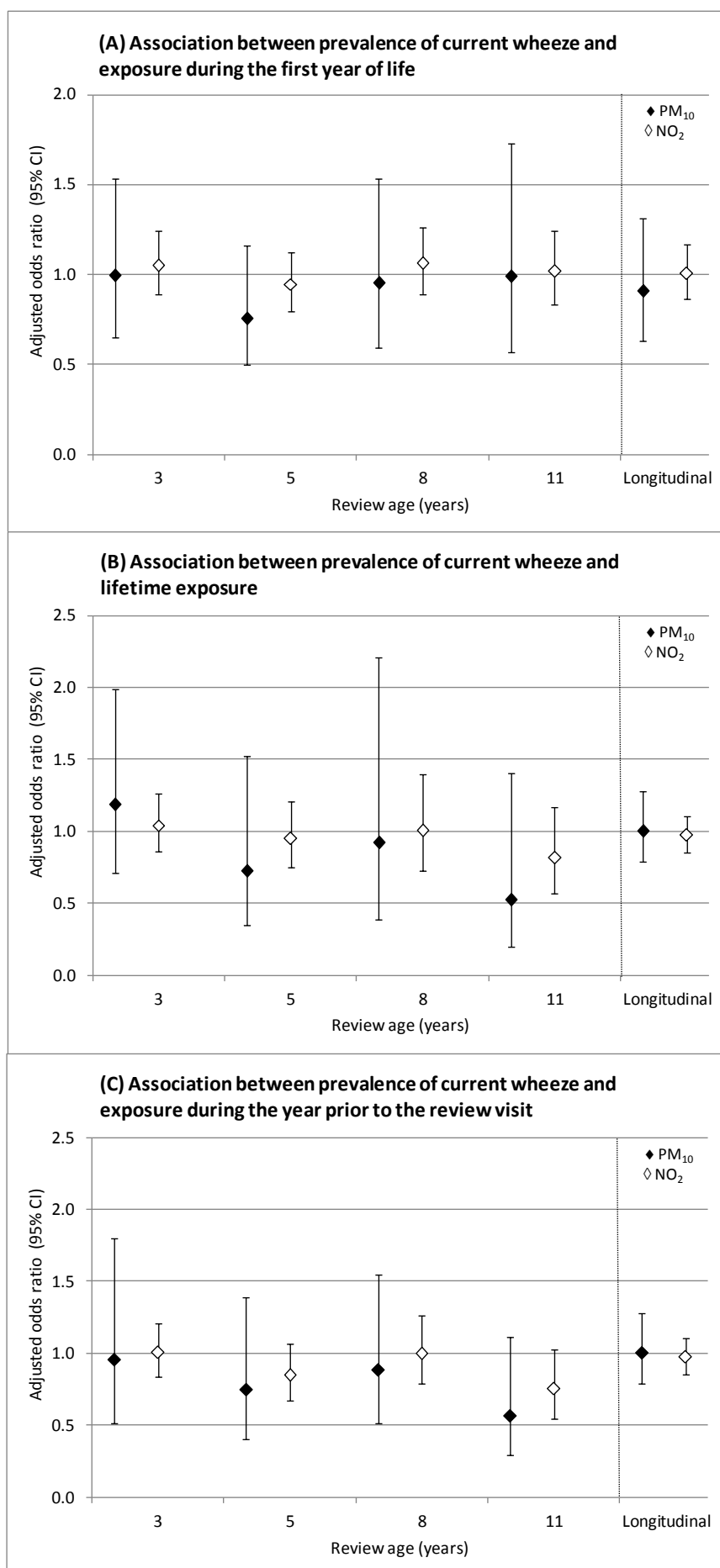


Figure A2: Adjusted cross-sectional and longitudinal associations between current wheeze prevalence and long term PM₁₀ and NO₂ exposure during different time windows (Lifetime model). Odds ratios were calculated per 1µg/m³ increase in exposure.

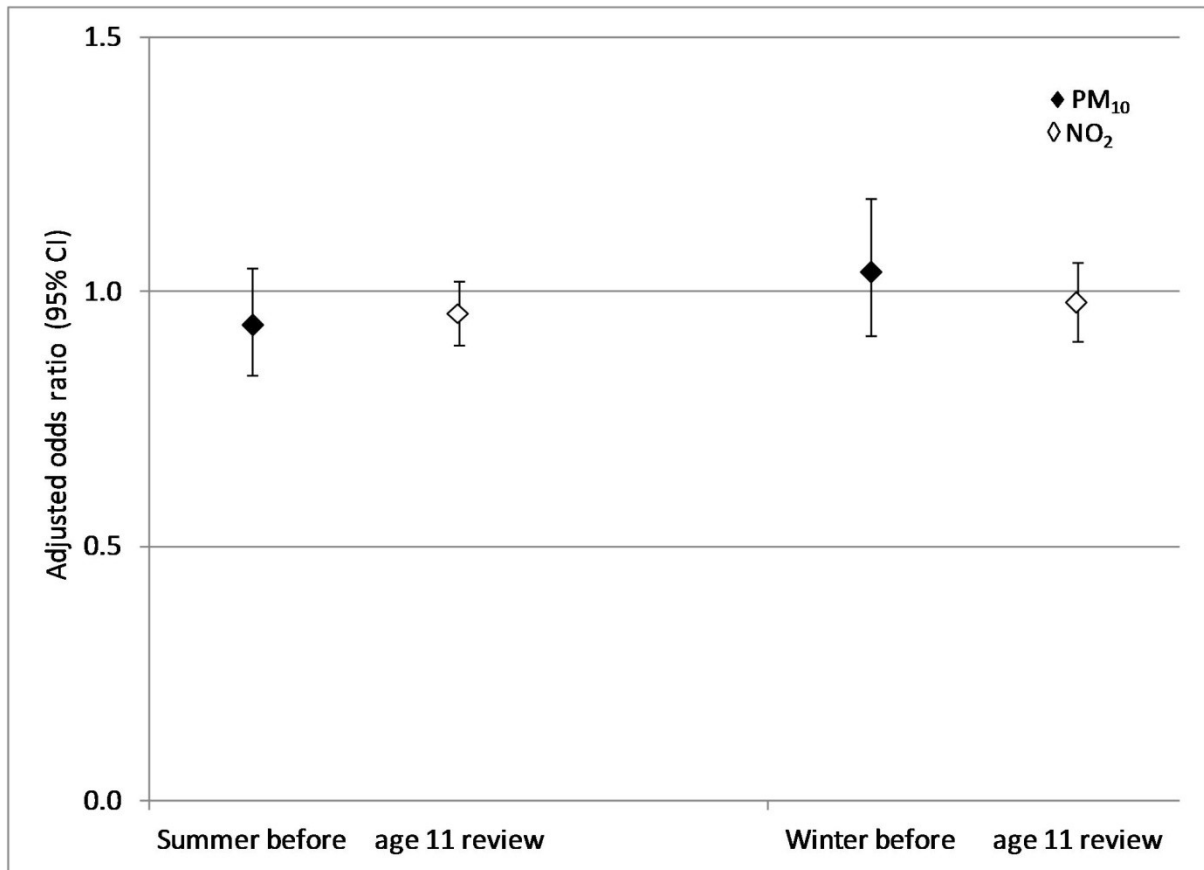


Figure A3: Adjusted association between current wheeze at age 11 and PM₁₀ and NO₂ exposure during the summer and winter before the age 11 review (MEEM). Odds ratios were calculated per 1 μ g/m³ increase in exposure.

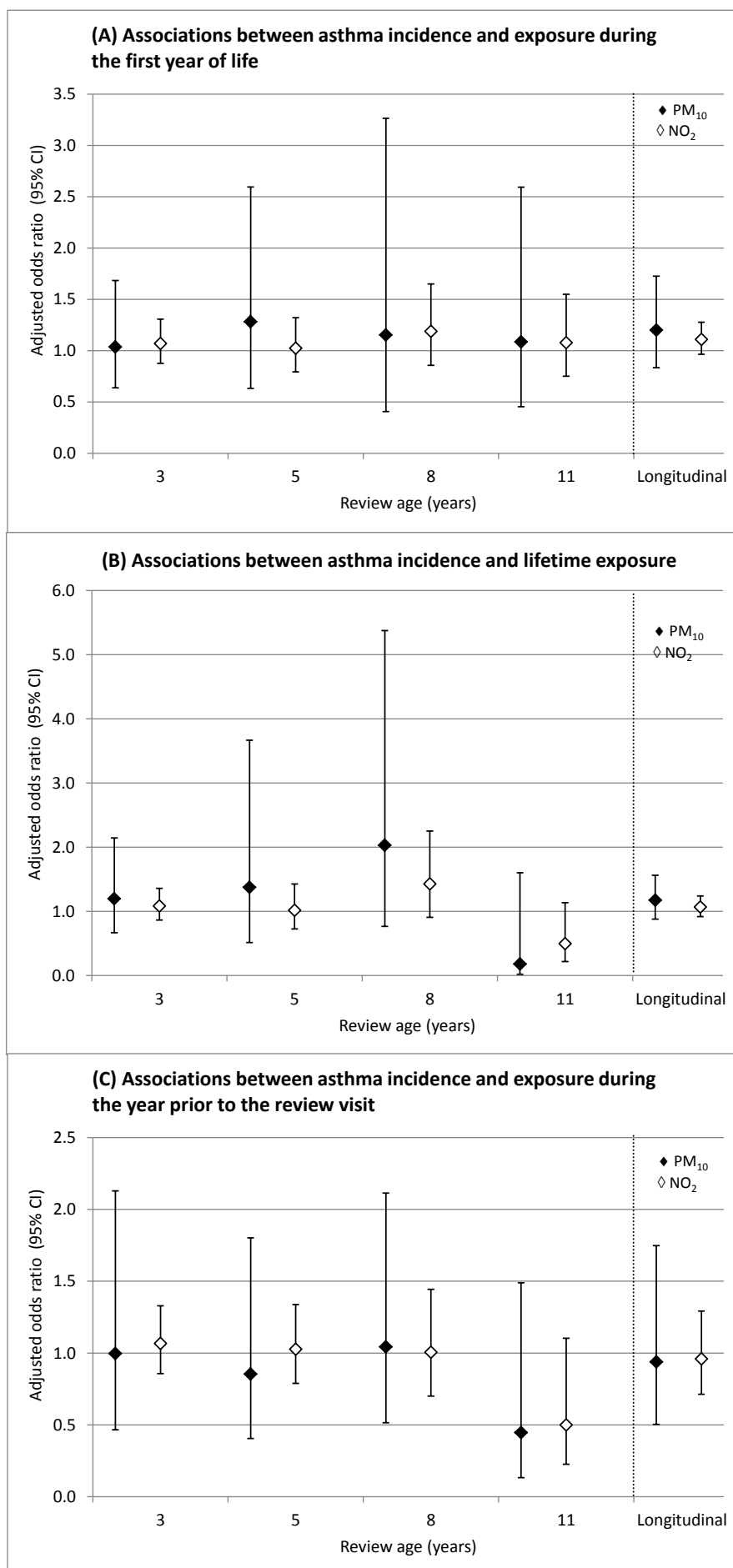


Figure A4: Adjusted cross-sectional and longitudinal associations between asthma incidence and long term PM₁₀ and NO₂ exposure during different time windows (Lifetime model). Odds ratios were calculated per 1 µg/m³ increase in exposure.

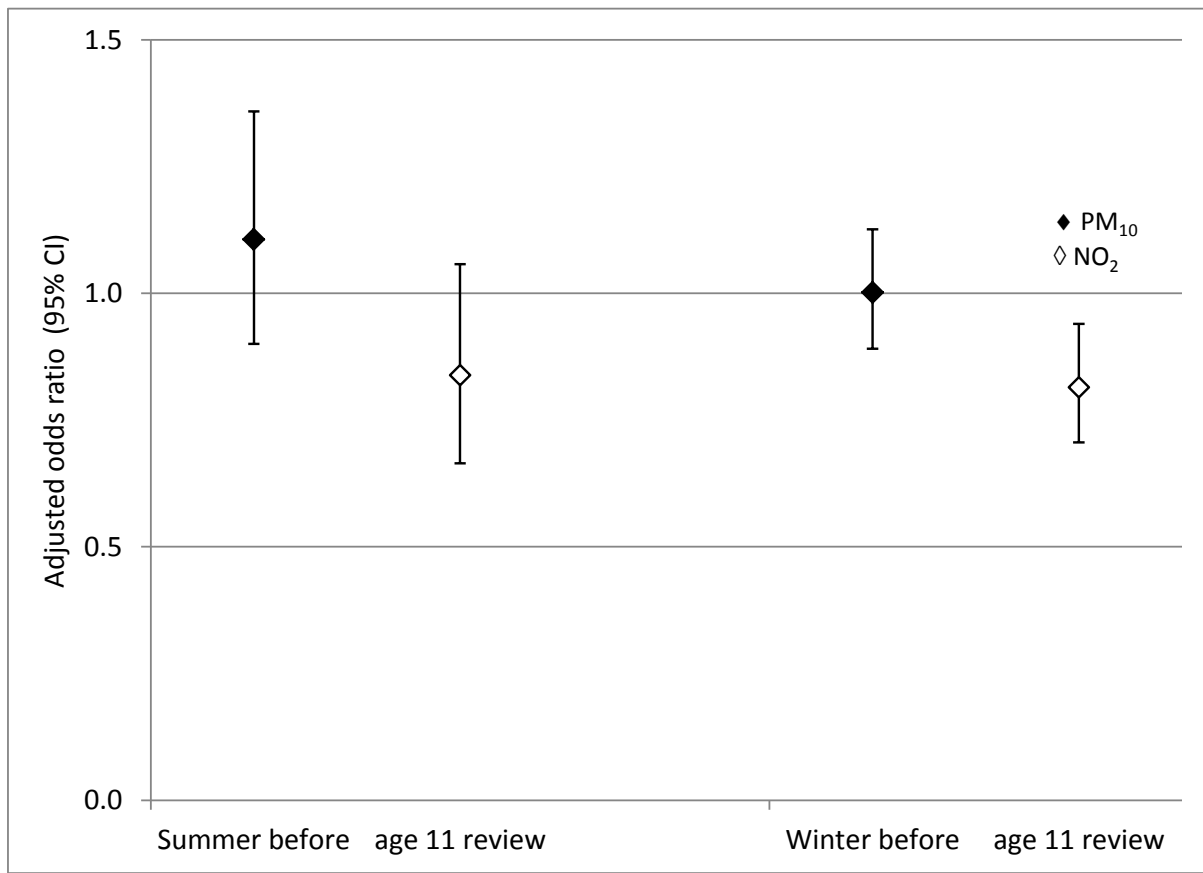


Figure A5: Adjusted association between asthma incidence at age 11 and PM₁₀ and NO₂ exposure during the summer and winter before the age 11 review (MEEM). Odds ratios were calculated per 1µg/m³ increase in exposure.

Table A2: ‘Spot checks’ of associations between asthma prevalence and modelled outdoor PM₁₀ and NO₂ exposure at the child’s home. Odds ratios were calculated per 1µg/m³ increase in exposure. Models were adjusted for gender, age, body mass index, paternal income at birth, sensitisation, family history of asthma, hospitalisation during the first two years of life, smoking within the child’s home during the first year of life and mean Tanner stage (longitudinal analysis only)

Outcome	Exposure window	PM ₁₀			NO ₂		
		adj OR	95% CI	p	adj OR	95% CI	p
Asthma at age 8	First year of life	1.03	0.62-1.69	0.92	1.14	0.90-1.45	0.29
Asthma at age 8	Lifetime	1.31	0.41-1.23	0.65	1.15	0.81-1.62	0.46
Longitudinal	Lifetime (Longitudinal)	0.87	0.55-1.38	0.55	1.05	0.87-1.25	0.63